ABSTRACT

SHAPIRO, SUSAN JANE GRAYDEN. Molecular Characterization of Canine Urothelial Carcinoma: from Chromosome to Clinic. (Under the direction of Dr. Matthew Breen.)

Urothelial carcinoma (UC) is the most common urinary cancer among humans and dogs, comprising ~2% of annual cancer diagnoses. Of these, approximately 20% of human tumors are invasive and carry a devastating prognosis; however, at least 90% of canine tumors are invasive and closely resemble invasive human disease. By studying the molecular pathogenesis of canine UC, including genomic and molecular alterations, we may elucidate diagnostic and therapeutic targets relevant to both dogs and humans.

Particularly in canine UC patients, reliable non-invasive diagnostic assays are lacking, often resulting in inaccurate or delayed diagnosis. In human tumors, including UC, chromosome copy number aberrations are tumor specific, diagnostically significant, and readily accessed. By oligonucleotide array comparative genomic hybridization (oaCGH) of 31 canine UCs, we identified a genomic signature of UC: gains of chromosome 13 (CFA13) and/or CFA36 and/or losses of CFA19 were identified in 100% of canine patients. We then employed single locus fluorescence in situ hybridization (FISH) to develop a targeted +13/-19/+36 diagnostic assay using free catch urine specimens (n=24). Again, 100% of patients possessed a combination of these aberrations, resulting in a highly specific and sensitive diagnostic assay. Comparison of canine aberrations with 285 human tumors showed evolutionary conservation of genomic aberrations in UC, most notably the gain of human chromosome 8 (HSA8) and syntenic CFA13.

To facilitate in vitro molecular studies, canine UC cell lines have been established. However, little work has been done to evaluate their resemblance to the primary tumor. We evaluated the genomic and expression profiles of five UC cell lines using oaCGH, FISH, gene expression microarrays, and quantitative reverse transcription PCR (qRT-PCR). We found that the cell lines not only preserve the genomic aberrations of primary tumors, but
also provide a valuable *in vitro* model for the study of molecules dysregulated in canine UC. Furthermore, cell lines emphasized the aberrant expression profile of genes involved in metabolism (downregulated) and DNA replication (upregulated).

We then utilized the cell line model to investigate a single gene involved in DNA replication; the gene *PTTG1* was overexpressed (~58-fold) in UC cell lines relative to normal urothelium and has been implicated in proliferation, invasion, and metastasis in human carcinomas. *PTTG1* was highly overexpressed, at the mRNA and/or protein level, in 100% of primary canine UCs and cell lines. We further found that siRNA-mediated knockdown of *PTTG1* led to significant reductions in cell line proliferation and invasion. Expression alterations indicative of the epithelial-mesenchymal transition (EMT) in cell lines were also curtailed after *PTTG1* knockdown. Vorinostat, an FDA-approved therapeutic, significantly reduced levels of *PTTG1* and proliferation in cell lines, suggesting vorinostat may be a readily translatable means of *PTTG1* reduction with the potential to impede tumor growth and invasion.

Lastly, we investigated two additional druggable targets: microRNA-10b (miR-10b) and the BRAF<sup>V600E</sup> mutation. miR-10b is located in the amplified region of CFA36 and was, therefore, hypothesized to be overexpressed, leading to epigenetic silencing of tumor suppressor genes. We found that miR-10b is underexpressed in UC, suggesting miR-10b amplification does not play a role in tumorigenesis. We also investigated the prevalence of the canine equivalent of the human BRAF<sup>V600E</sup> mutation (V450E) in tumors and cell lines by Sanger sequencing. We found that ~70% of specimens possessed the mutation, but siRNA-mediated *BRAF* knockdown failed to significantly reduce cell line proliferation or invasion. Thus, although miR-10b and BRAF<sup>V450E</sup> represent differential markers of normal and neoplastic urothelium, they do not appear to be involved in the invasive nature of canine UC. Overall, our findings provide deeper understanding of the pathogenesis of canine UC, genomically and molecularly, and espouse the value of the canine model in human UC.
Molecular Characterization of Canine Urothelial Carcinoma: from Chromosome to Clinic

by

Susan Jane Grayden Shapiro

A dissertation submitted to the Graduate Faculty of North Carolina State University in partial fulfillment of the requirements for the degree of Doctor of Philosophy

Comparative Biomedical Sciences

Raleigh, North Carolina

2015

APPROVED BY:

Dr. Matthew Breen
Committee Chair

Dr. John Cullen

Dr. Anthony Blikslager

Dr. Steven Suter
DEDICATION

To the person who has always made sure my dreams were within my reach—my Mom.
**BIOGRAPHY**

Mrs. Shapiro grew up in the rural town of Lebanon, Virginia. The majority of her young life was spent on her beautiful family farm nestled in the Blue Ridge Mountains. There, she often took it upon herself to not only take care of the lives of her pets and farm animals, but every creature she encountered. She knew from the approximate age of nine that she wanted to be a veterinarian, and her menagerie of animals, mainly dogs and horses, allowed her to begin to hone her skills at an early age. In the 3rd grade, Mrs. Shapiro further discovered an extreme love for science—learning about what drives all life, from plant to person. Her passion for the mechanisms of life and experimentation followed her throughout high school and college and remains today.

In 2006, Mrs. Shapiro graduated from high school and attended the University of Virginia in Charlottesville, Virginia, in pursuit of a bachelor of science in biology, with hopes of one day becoming a veterinarian. To address her love for science, Mrs. Shapiro found a veterinarian at the UVA School of Medicine, Dr. Sanford Feldman, who allowed her to become involved in research. As director of the Center for Comparative Medicine, Dr. Feldman showed her the value of animals in advancing human health. Dr. Feldman mentored Mrs. Shapiro through four years of undergraduate pre-veterinary education, taught her numerous research techniques she still utilizes today, and helped her acquire her first scientific publication. Mrs. Shapiro was overwhelming excited about being able to contribute to a constantly evolving and growing understanding of life through research.

In 1995, Mrs. Shapiro’s mother was diagnosed with breast cancer, an event that most certainly changed not only her mother’s life, but Mrs. Shapiro’s as well. The 8-year-old knew little of what cancer was, aside from the sadness it brought to her mom and her family. Over the coming years, however, everything that dealt with that ominous word “cancer”
captivated Mrs. Shapiro. In July 2005 at a summer governor’s school at the Medical College of Virginia, Mrs. Shapiro was given the opportunity to investigate the appearance of “cancer clusters” in Virginia related to carcinogen exposures. In the summer of 2009, Mrs. Shapiro studied the role of genetics in chemotherapeutic sensitivity at Purdue University College of Veterinary Medicine. Everything about cancer, particularly the interaction between genes and the environment in its development, fascinated Mrs. Shapiro and, combined with her love for science and animals, drove her to pursue a combined DVM/Ph.D. Mrs. Shapiro quickly recognized not only the intellectual satisfaction of research, but the impact she could have on the field of veterinary medicine and the lives of many through research.

One of the greatest days of Mrs. Shapiro’s young life came when Dr. Sam Jones called her one snowy January day to let her know she was being offered admission into North Carolina State University’s DVM/Ph.D. Program. She was knew exactly how she wanted to dedicate the years of her Ph.D.—to investigate canine bladder cancer, a disease her beloved Shetland sheepdog Gibita was predisposed to simply because of her breed-associated genetics. Mrs. Shapiro joined the lab of Dr. Matthew Breen who allowed her to pursue this topic so close to her heart.

When she’s not busy playing with dogs or studying cancer, Mrs. Shapiro enjoys dancing, singing, and doing anything outdoors. Her future aspirations following completion of veterinary school include an oncology residency and an academic career as a veterinary oncologist.
ACKNOWLEDGEMENTS

First I would like to express my sincere and utmost gratitude to Dr. Matthew Breen for providing my research “home” over the past five years. Thank you for providing the means to allow me to pursue work on something so dear to my heart and taking it on as a passion of your own. From my DVM/PhD interview, through our first chat on the phone about my research project interests, to today, you have always motivated me, kept my spirits high, and had faith in my abilities as a researcher, clinician, and person. With your guidance, I have gotten everything out of my PhD that I could have hoped. Thank you for showing me what it takes to be a successful scientist and giving me confidence that I, too, can be one someday. In addition, I extend immense appreciation to my other committee members—Dr. Anthony Blikslager, Dr. John Cullen, and Dr. Steve Suter—for their unrelenting guidance throughout my graduate career. You are all an inspiration!

My mom Kim and my siblings Anne and Shor: Thank you for constantly supporting my love of animals and desire to be a vet throughout my life. Thank you for not only motivating me to be a hard worker and making sure my grades were good enough, but also convincing me that I could do anything to which I set my heart and mind. Even more importantly, you all taught me how to love, which has pervaded every aspect of my life.

To my husband Zach. Thank you so much for your unwavering support, intellectual challenges, and encouragement to “take a study break and do something fun”. You never let me get down on myself and know exactly what to say to boost my confidence. You have not only been my major support system over the last five years, but you enriched the whole experience by reminding me how important and fascinating everything I do is. Thank you for the unconditional love, patience, and respect you show me.
My previous research mentors, most notably Dr. Sanford Feldman at the UVA Hospital. You opened my eyes to research and the important role veterinarians play in advancing the health of both humans and animals. You have inspired me to be better from the first day I stepped into your lab, and I am forever grateful for the opportunities you gave me and doors you have unknowingly opened for me since.

Megan Schreeg, I would never have thought we would somehow end up in the exact same program, defending our PhDs the same summer, and making our dreams a reality. Words cannot describe how thankful I am to have you as a friend and colleague!

To the members of the Breen lab, past and present. Thank you for instilling in me your wisdom and being a fabulous support system over the past five years! Remember to always get excited when a urine comes to the lab!

In addition to my lab, I must express sincere gratitude to Dr. Debbie Knapp and her lab at Purdue University. Thank you for not only educating me about canine bladder cancer back in 2009, but for helping make my PhD project possible ever since.

Finally, all my sweet gone, but not forgotten, animals--Snowflake, Queen Gibagi, and Gibagi II. The past five years of hard work, (at least) two years of hard work to come, and following pages are the result of your constant presence in my heart. Thank you for inspiring me to pursue a rewarding career in which I can not only save lives, but help preserve the kind of bond between owner and pet that you showed me. Gibita, my sweet sheltie, you inspired me to pursue learning about canine bladder cancer. I know you cannot understand English, but thank you for reminding me every day the significance of what I do.
# TABLE OF CONTENTS

LIST OF TABLES .............................................................................................................................. xiii

LIST OF FIGURES ............................................................................................................................. xv

CHAPTER 1: LITERATURE REVIEW ................................................................................................. 1

Human Urothelial Carcinoma ....................................................................................................... 1

  Current diagnostics ..................................................................................................................... 5

  Tumor staging and grading ......................................................................................................... 7

  mRNA and protein expression ................................................................................................... 8

  Genomic aberrations .................................................................................................................. 10

  Epigenetic alterations ............................................................................................................... 17

Models of Urothelial Carcinoma .................................................................................................. 20

  The cell line model ................................................................................................................... 20

  Animal models .......................................................................................................................... 21

Canine Urothelial Carcinoma ...................................................................................................... 24

  Tumor staging and grading ........................................................................................................ 29

  Molecular alterations ............................................................................................................... 30

Dissertation Rationale and Outline .............................................................................................. 32

References ...................................................................................................................................... 34

CHAPTER 2: CANINE UROTHELIAL CARCINOMA: GENOMICALLY ABERRANT AND

COMPARATIVELY RELEVANT ......................................................................................................... 52

Abstract ........................................................................................................................................ 52

Introduction .................................................................................................................................... 53

Methods and Materials ................................................................................................................. 54

  Canine UC case collection ........................................................................................................ 54

vii
Pathologic evaluation of canine UC........................................................................54
Canine oaCGH........................................................................................................54
Selection of canine diagnostic regions..................................................................55
Fluorescence in situ hybridization of canine FFPE biopsies using BAC and custom
SureFISH probes........................................................................................................56
FISH of urine sediment.............................................................................................56
Human oaCGH data...................................................................................................57
Humanization of canine oaCGH data for comparative analysis...............................57
Generation of call data and probe median values for comparative assessment........57
Isolation of regions of overlapping copy number aberration...................................57
Statistical validation of the gene list..........................................................................58
Comparative pathologic staging analysis...................................................................58

Results.......................................................................................................................58
Canine UC biopsies show recurrent DNA copy number aberrations......................58
FISH analysis of FFPE tumor biopsies and urine sediment allows direct visualization of
urothelial cell copy number status..........................................................................59
Copy number changes in canine UC—an aid to diagnosis.......................................61
Canine and human urothelial tumors share copy number aberrations.....................62
Comparative pathologic staging.................................................................................65

Discussion.................................................................................................................66
Canine UCs exhibit diagnostically relevant copy number aberrations.....................66
Canine UC is of comparative value to human medicine............................................68

Conclusion.................................................................................................................70

Acknowledgements..................................................................................................70
CHAPTER 3: A CULTURED APPROACH TO CANINE UROTHELIAL CARCINOMA
RESEARCH: CHARACTERIZATION OF FIVE CELL LINES ........................................... 74

Introduction ....................................................................................................................... 75

Methods and Materials ..................................................................................................... 77

- Canine UC cell lines and urothelial controls ................................................................. 77
- Culture preparation ....................................................................................................... 78
- oaCGH ........................................................................................................................... 79
- Primary tumor oaCGH data ......................................................................................... 80
- Fluorescence in situ hybridization ................................................................................. 80
- Gene expression analysis ............................................................................................ 81

Results ............................................................................................................................... 83

- Canine UC cell lines display recurrent chromosome copy number and structural aberrations. ......................................................................................................................... 83
- Gene expression profiles of cell lines differ from those of normal urothelium......... 85

Discussion .......................................................................................................................... 86

Conclusions ......................................................................................................................... 94

Acknowledgements ........................................................................................................... 95

References ........................................................................................................................ 95

CHAPTER 4: INVASION OF THE CELL LINES: THE ROLE OF CANINE PTTG1 IN UC
INVASION .......................................................................................................................... 112

Abstract ............................................................................................................................ 112

Introduction ....................................................................................................................... 113

Methods and Materials ................................................................................................... 115
Tissue specimens ........................................................................................................115
UC cell lines ...........................................................................................................117
qRT-PCR of PTTG1 .................................................................................................117
Immunohistochemistry .........................................................................................119
Protein isolation and western blotting ...............................................................120
siRNA-mediated knockdown of PTTG1 ................................................................121
Invasion analysis ....................................................................................................123
qRT-PCR analysis of epithelial and mesenchymal cell markers ......................125
Vorinostat treatment of PTTG1-overexpressing UC cell lines .........................125

Results .................................................................................................................126

PTTG1/Securin is highly overexpressed in primary canine tumors and cell lines and
suggestive of the epithelial-mesenchymal transition ........................................126
PTTG1 knockdown results in decreased cell proliferation and invasion in canine UC cell
lines ........................................................................................................................129
PTTG1 knockdown leads to reduced expression of mesenchymal markers ....130
Vorinostat treatment reduced levels of PTTG1 and limited cell proliferation ....131

Discussion ..............................................................................................................132

Acknowledgements ..............................................................................................140
References ...............................................................................................................140

CHAPTER 5: THERAPEUTIC TARGET PRACTICE: INVESTIGATION OF MIR-10B AND
BRAF^{V600E} IN CANINE UC ..................................................................................155

Abstract ...............................................................................................................155

Introduction ..........................................................................................................156

Methods for miRNA analysis ..............................................................................159
Case collection...........................................................................................................................................159
miRNA isolation...............................................................................................................................................160
miRNA TaqMan® Assays................................................................................................................................160
Reference miRNA analysis...............................................................................................................................162
mRNA isolation and qRT-PCR of miR-10b target HOXD10 ..............................................................................162
oaCGH/fold change correlation analysis ......................................................................................................163
Methods for BRAF analysis ............................................................................................................................163
Case collection ..................................................................................................................................................163
DNA isolation....................................................................................................................................................164
Primer design, PCR, and targeted Sanger Sequencing......................................................................................164
Sequencing analysis..........................................................................................................................................165
Cell culture ......................................................................................................................................................166
DsiRNA-mediated BRAF knockdown ..............................................................................................................166
RNA isolation, cDNA conversion, and qRT-PCR analysis .............................................................................166
Invasion analysis ...............................................................................................................................................168
Results .............................................................................................................................................................169
A combination of reference miRNA provides the most stable reference for quantitative miRNA analysis ...............................................................................................................................................169
miR-10b is significantly downregulated in canine UC ........................................................................................170
BRAF V600E (V450E) mutations are prevalent in canine bladder and prostate carcinomas and UC cell lines ...............................................................................................................................................171
Knockdown of BRAF minimally reduces cell proliferation and unpredictably and minimally affects invasion ...............................................................................................................................................172
Discussion .....................................................................................................................................................173
miR-10b in canine UC ................................................................. 173
BRAF^{V600E} in canine UC.......................................................... 178
Conclusions.................................................................................. 181
Acknowledgements...................................................................... 182
References.................................................................................... 182

CHAPTER 6: CONCLUSIONS AND FUTURE DIRECTIONS.................. 197
References.................................................................................... 204

APPENDIX...................................................................................... 207
Appendix A: Chapter 3 Supplementary Table ............................... 208
LIST OF TABLES

Chapter 1
Table 1.1. *Currently available non-invasive diagnostics for the detection of human urothelial carcinoma with associated clinical value.* ................................................................. 44
Table 1.2. *American Joint Committee on Cancer’s accepted scheme for the staging of human urothelial carcinomas according to the extent of primary tumor invasion and regional and distant metastases.* ................................................................. 45
Table 1.3. *Frequent genomic aberrations observed in human urothelial carcinoma and their associated prevalence among patients with UC at various tumor stages/grades.* ............... 46
Table 1.4. *Comparison of demographical, clinical, molecular, and prognostic features of canine and human urothelial carcinoma.* ................................................................. 47
Table 1.5. *World Health Organization (WHO) accepted staging guidelines for canine UC (1980) according to the extent of invasion and regional and distant metastasis.* ............... 48

Chapter 2
Table 2.1. *Canine patient signalment and staging.* ................................................................. 55
Table 2.2. *Single-locus probes used to represent the regions of CFA 13, 19, and 36 with the highest recurrence of copy number aberration in canine UC.* ................................................................. 57
Table 2.3. *Frequency of categorical (neutral, loss, or gain) copy number status of the peak unidirectional aberrations on CFA 13, 19, and 31 in cases and controls.* ......................... 62
Table 2.4. *Summary statistics of the power of each of the three selected regions of CFA 13, 19, and 36 as predictors of the presence of canine UC.* ................................................................. 63
Table 2.5. *Human and canine UC share syntenic regions of copy number aberration.* ....... 65

Chapter 3
Table 3.1. *Characteristics of five canine UC cell lines.* ................................................................. 100
Table 3.2. Regions chosen for FISH validation of oaCGH. .................................................. 101
Table 3.3. Copy number aberrant genes analyzed by qRT-PCR. ........................................ 102
Table 3.4. Comparisons analysis of primary tumors and cell lines ..................................... 103
Table 3.5. Regions implicated in cell line GISTIC analysis represent regions relevant to neoplasia .......................................................................................................................... 104
Table 3.6. Compiled FISH and oaCGH Log2 data. Log2 ratio values obtained from oaCGH analysis of cell lines and calculated by raw FISH data are shown. .......................... 105
Table 3.7. Gene expression microarray results. .................................................................... 106

Chapter 4

Table 4.1. Signalment, tumor stage, and the presence of overt metastases of patients and cell lines included in PTTG1 analyses along with PTTG1/securin expression data .......... 145
Table 4.2. Data acquired from Matrigel Invasion analyses, including total cell number, average number of invaded cells, average invasive indices, and associated p-values. ..... 146

Chapter 5

Table 5.1. Patients analyzed in the miR-10b study and their respective miR-10b fold changes (qRT-PCR), CFA36 Log2 ratios (oaCGH), and HOXD10 fold changes (qRT-PCR). .......................... 188
Table 5.2. The effects of BRAF knockdown on neoplastic cell invasion .............................. 189

Appendix A

Supplementary Table 1. Regions implicated in GISTC analysis of primary tumors .......... 208
LIST OF FIGURES

Chapter 1

Figure 1.1. Tumor staging is based on the extent of primary tumor invasion and metastasis. .................................................................49

Figure 1.2. Recurrent genomic aberrations in urothelial carcinoma and associated neoplastic alterations. .................................................................50

Figure 1.3. Major cell signaling pathways involved in the development of urothelial carcinoma. ........................................................................51

Chapter 2

Figure 2.1. Representative oaCGH profiles of four canine UC cases (TCC10, 15, 28, and 50) from the cohort used in the present study ........................................59

Figure 2.2. Representative chromothriptic-like events detected in canine UC. .................................................................60

Figure 2.3. Frequency of chromothriptic-like events across the canine genome. .................................................................60

Figure 2.4. Penetrance data of primary canine UC tumors reveal highly recurrent genomic alterations. ................................................................61

Figure 2.5. BAC and SureFISH methods of copy number detection yield comparable results and elucidate the extent of copy number alteration in canine UC. .................................................................62

Figure 2.6. Visual comparison of "humanized" canine and human UC oaCGH data. .................................................................63

Figure 2.7. Key regions of shared CNA shared between human and canine UC. .................................................................66

Figure 2.8. Comparison of penetrance of CNAs in human and canine (recoded to human) UC when evaluated by extent of muscle invasion. ........................................................................67

Figure 2.9. (SOM Figure 1). Gene ontology analysis highlighted gene function categories frequently affected by shared copy number aberration in human and canine UC. ........................73
Chapter 3

Figure 3.1. Genomic aberrations of canine UC cell lines recapitulate those seen in primary tumors. ................................................................. 107

Figure 3.2. The cell lines demonstrate evidence of chromothriptic-like events in tumor evolution. ................................................................. 108

Figure 3.3. Structural aberrations exist in oaCGH-flagged regions. ................................................................. 109

Figure 3.4. Clustered heat map of cell line differential expression. ................................................................. 110

Figure 3.5. Gene expression arrays, qRT-PCR, and oaCGH results support one another and emphasize the role of genomic copy number on mRNA levels. ................................................................. 111

Chapter 4

Figure 4.1. PTTG1 is highly overexpressed in canine UC cell lines and tumor biopsies. ... 147

Figure 4.2. Securin expression in canine testicular tissue recapitulates that seen in humans. .................................................................................................................. 148

Figure 4.3. IHC and Western blot validate protein expression of securin in canine UC tumors and highlight its potential role in the epithelial-mesenchymal transition. ................................................................. 149

Figure 4.4. DsiRNA knockdown of PTTG1 in canine UC cell lines successfully reduced cellular levels of PTTG1 to more physiologically-normal levels. ................................................................. 150

Figure 4.5. PTTG1-knockdown cells demonstrated decreased invasion relative to PTTG1-overexpressing neoplastic cells. .................................................................................................................. 151

Figure 4.6. PTTG1 knockdown results in reduced proliferation and invasion of canine UC cell lines. .................................................................................................................. 152

Figure 4.7. Expression markers of EMT are altered in UC cell lines and partially recovered by PTTG1 knockdown. .................................................................................................................. 153

Figure 4.8. Vorinostat treatment reduces cell proliferation in canine UC cell lines. ........ 154
Chapter 5

Figure 5.1. miR-148b/-874/-181b provides the most stable miRNA reference gene value. 190

Figure 5.2. miR-10b levels are significantly decreased in neoplastic urothelium compared to control. ............................................................................................................................................................................. 191

Figure 5.3. miR-10b genomic levels do not appear to influence mature miR-10b levels in canine UC. .................................................................................................................................................................................. 192

Figure 5.4. The canine equivalent of the human BRAF<sup>V600E</sup> mutation is evident in canine UC and prostatic carcinoma. .................................................................................................................................................................. 193

Figure 5.5. DsiRNA knockdown of BRAF resulted in significantly reduced levels of cellular BRAF in BRAF<sup>V450E</sup> and wild type patients.................................................................................................................................................................................. 194

Figure 5.6. BRAF knockdown marginally reduced cell number and invasiveness in UC cell lines. .................................................................................................................................................................................................................................................. 195

Figure 5.7. The miR-10b locus is located in a region of CFA36 amplified in >80% of primary canine UCs and cell lines. ........................................................................................................................................................................................................... 196
CHAPTER 1: LITERATURE REVIEW

Human Urothelial Carcinoma

Cancer of the urinary bladder represents the most common cancer of the urinary tract in men and women and fourth most common cancer in men overall. In 2014, a projected 74,690 men and women were diagnosed with the disease, claiming 15,580 lives nationally and 150,000 globally (Cancer Genome Atlas Research, 2014). Although several histological subtypes of bladder cancer exist, malignancies of the epithelium (urothelium) represent >90% of cases. Due to the mucosal location of the majority of bladder tumors, combined with approximately six-fold higher incidence rates in industrialized countries than undeveloped counties, environmental interactions with urothelial cells at a molecular level are of profound relevance (Fu, Kohaar, Moore, & Lenz, 2014; Schulz, 2006).

Three major risk factor categories have been established in the development of urothelial carcinoma (UC): genetics, chronic urothelial irritation, and chemical and environmental exposures. Individuals with an affected primary relative have a 50-100% increased risk compared to those without a family history of UC, emphasizing a potential genetic component to the disease (Mitra & Cote, 2009). Conditions involving prolonged urothelial irritation, including exposure to the cyclophosphamide metabolite acrolein and chronic urinary tract infections, have been linked to UC development with nine-fold increased risk (Kaufman, Shipley, & Feldman, 2009). Nevertheless, the vast majority of cases are directly related to carcinogen exposure, most commonly those inhaled in cigarette smoke (Cheng et al., 2011).

Comprised of transitional cells, the urothelium consists of a mere 5-7 cell layers when the bladder is relaxed and as few as one when the bladder expands to accommodate urine. This ability of the urinary bladder to expand, although essential for function, inevitably
exposes nearly every urothelial cell to corrosive compounds contained within the urine, making urinary carcinogen exposure paramount. Industrial chemicals, including aromatic amines, aniline dyes, and coal, are also associated with increased risk of UC. Consequent to the latency periods associated with carcinogen exposure, UC generally affects the elderly, with the average age of diagnosis being 65 years (Oliveria, Arantes-Rodrigues, & Vasconcelos-No´brega, 2014). Due to more aggressive cigarette habits, more frequent industrial jobs, and potentially genetics, UC is diagnosed two to five times more frequently in men than women. In undeveloped countries, however, incidence rates among men and women are nearly identical, suggesting that a gender-associated risk is more environmental than hormonal (Knapp et al., 2000).

Two major subtypes of UC exist: superficial and invasive. Superficial tumors generally arise from localized areas of urothelial hyperplasia and project into the bladder lumen without invading the basement membrane. The majority (~80%) of UCs are located superficially, with 37% located in the lateral bladder wall and subject to curative surgical removal and a resultant favorable prognosis (Stephenson, Holmes, Noble, & Gerald, 1990). Nevertheless, a significant proportion (~20%) of UCs are highly invasive, evolving from flat carcinoma in situ (CIS) lesions and invading through the epithelial basement membrane and often the underlying muscle and blood vessels, leading to dissemination of neoplastic cells throughout circulation (Netto, 2012). In fact, the development of micrometastases is considered an early event in invasive UC (Gupta & Mahipal, 2013; X. Wang, Oldani, Zhao, Huang, & Qian, 2014); 50% of patients with invasive UC develop distant metastases, and 90% of these patients will die within two years of diagnosis (Cekanova & Rathore, 2014). Even in instances of early diagnosis, 95% of patients with invasive UC fail to respond to
treatment, attaching a highly guarded prognosis to invasive UC diagnosis—an approximate 12-month survival time (Cheng et al., 2011; Kaufman et al., 2009; Wu, 2005).

Current treatments of invasive UC include cystectomy and/or chemotherapy and radiation, with radical cystectomy preferred (Gupta & Mahipal, 2013). Cystectomies necessary for invasive UC are aggressive, often including prostatectomy in men and excision of the uterus and ventral vaginal wall in women (Kaufman et al., 2009). To treat presumed metastases, adjunct chemotherapy is typically administered, despite a known lack of efficacy (Kaufman et al., 2009). The chemotherapeutic protocol of choice MVAC (combination methotrexate, vinblastine, Adriamycin, and cisplatin) is extremely toxic, particularly in compromised elderly individuals who make up the majority of UC patients, and provides a 6-year disease-free rate of only 3.7% (Cekanova & Rathore, 2014; Milowsky & Kim, 2014). Newer protocols that include gemcitabine, cisplatin, and paclitaxel have improved response while decreasing toxicity, but over half of all patients are unable to receive cisplatin chemotherapeutics due to poor performance status, renal function, or co-morbidities (Galsky et al., 2011). Still yet, no efficacious and well-tolerated chemotherapeutics have been established for metastatic UC (Bellmunt et al., 2014; Calderaro et al., 2014).

Although initially favorable, superficial tumors, also carry a guarded prognosis. The majority (>80%) recur within two years of initial treatment and are often more invasive and aggressive (Cheng et al., 2011; Juanpere et al., 2012). The mechanism of recurrence is yet to be elucidated, but is thought to be due, at least in part, to the “field effect” of urothelial carcinogenesis. The field effect posits that neoplastic alterations in the urothelium are not limited to the overt tumor but rather distributed among all cells in contact with carcinogenic compounds of the urine, predisposing every cell to neoplasia (Jones et al., 2005; McConkey
et al., 2010). Due to the associated concerns for recurrence and metastasis, thorough screening must be done on a near monthly basis, making UC the most expensive solid tumor to manage and the fifth most expensive cancer overall ($3.7 billion annually) (Gogalic, Sauer, Doppler, & Preininger, 2015; Mitra & Cote, 2009; Netto, 2012). A cystoscopy is required every three months for two years, every six months for the next two years, and annually thereafter (Kaufman et al., 2009). Current alternative diagnostics fail to reliably identify recurrences of UC, making the development of non-invasive, yet sensitive, biomarkers for cost-saving testing paramount. In addition, UC shows a highly diverse compilation of biological and functional characteristics, hindering appropriate treatment selection and prognosis determination.

An ideal biomarker would detect all tumors with high sensitivity, especially high-grade, invasive tumors, and minimize false positive results while being economically feasible (Lerner, et al., 2006). It should further be quantifiable, allowing stratification of patients displaying varying levels and prognostic correlation of these levels to prognosis. Using information obtained at diagnosis, the biomarker would help direct clinicians’ selection of therapeutic protocols and deduce a probable clinical outcome (Cheng et al., 2014). Biomarker research, although somewhat extensive, has failed to identify a hugely successful marker of diagnosis and recurrence. Three molecular changes are posited to be at the crux of UC development and hold promising biomarkers: 1) Chromosomal changes initially activate the carcinogenic event, highlighting the importance of thorough molecular characterization in UC, 2) Alterations in cell cycle regulatory proteins facilitate uncontrolled tumor proliferation, and 3) Loss of epithelial adhesions allows cells to adopt a migratory phenotype and form metastases (Abat et al., 2014). Studies have focused mainly on
cytogenomic markers and differential mRNA/protein expression, and, although neoplastic associations have been found, high sensitivity and specificity for UC detection is lacking.

**Current diagnostics**

As with many solid tumors, excisional biopsy remains the gold standard of UC diagnosis. Excisional biopsy is typically performed via cystoscopy-guided transurethral resection of bladder tumor (TURB-T), enabling tumor removal, diagnosis, and appropriate histopathologic staging, and grading (Cheng et al., 2011). This method, however, is not without limitations; staging accuracy is highly dependent on complete resection of the tumor, and over 50% of TURBTs fail to include muscle layers (Lenis & Chamie, 2015). Muscle invasion is the most critical histopathologic indicator of prognosis, highlighting the need for appropriate thickness biopsy (Lenis & Chamie, 2015). Moreover, in cases in which minimally invasive (T1) tumors are not entirely resected, 50% are identified as muscle invasive (>T2) upon repeat resection, speaking to the rapid progression of the tumor or limited capacity for histopathologic staging in the face of suboptimal biopsy. Due to the invasive nature of biopsy, risk for inaccurate diagnosis, and rapid evolution of the tumor, less invasive, less subjective, and more rapid diagnostic techniques are necessary.

Numerous non-invasive assays have been developed for the detection of UC in urine and are reviewed in Table 1. Urine cytology, although specific, provides very low sensitivity for low grade tumors, presumably due to the low exfoliation of neoplastic cells. In an attempt to increase sensitivity while retaining a less invasive technique, the Urovysion™ cytogenetic assay was recently developed (Abbott Molecular, Des Plaines, IL). Gains of chromosomes 3, 7, 17, and loss of 9p21 (the CDKN2A locus) are detected by fluorescence in situ hybridization of exfoliated urothelial cells in the urine (Bonberg et al., 2013). Sensitivities and specificities range from 70-86% and 66-93%, respectively, showing a relatively high
sensitivity for detection of recurrent tumors, but low sensitivity for low grade tumors, limiting
the assay’s utility for routine diagnostics (Martyn-Hemphill, Mak, Khan, Challacombe, &
Bishop, 2013). Typically, patients with a low risk of recurrence display 9p21 monosomy
and/or gain of HSA3, while high risk patients show gain of 7 and/or 17 (Matsuyama et al.,
2014). The 9p21 locus is, in particular, associated with recurrence. Although these
aberrations are significantly associated with recurrence, they carry no further prognostic
value. Nevertheless, Urovysion’s objective assessment of neoplastic cells is desirable for
any diagnostic test and provides an excellent foundation for a promising diagnostic test.

Several antigens have been targeted for UC diagnosis in urine, including
components of the mitotic spindle (NMP22), complement proteins (BTA), and epithelial
markers (mucins). Nuclear matrix protein 22 (NMP22) is a component of the mitotic spindle
which can be detected even in low levels in healthy human urine (Onal, Han, Yilmaz,
Köybasioglu, & Altuğ, 2014). NMP22 levels are elevated in neoplastic urothelium, making
them of diagnostic relevance. The diagnostic assay for NMP22 (NMP22® BladderChek®,
Alere, Waltham, MA) relies on a sandwich ELISA with two antibodies and two epitopes and,
although a fairly sensitive and noninvasive means of UC detection, it carries a high false
positive rate (Balci et al., 2015; Goodison, Rosser, & Urquidi, 2013). The bladder tumor
antigen test (BTA®, Polymedco, Cortlandt Manor, NY) relies on the detection of
complement factor H detection by two monoclonal antibodies and has increased sensitivity
with increased tumor grade (Guo et al., 2014; Raitanen et al., 2001); however, the assay
has poor specificity in inflammatory conditions with symptoms similar to UC, limiting its
clinical utility. Lastly, ImmunoCyt™ (Scimedx, Denville, NJ) detects two mucin-like proteins
and a carcinoembryonic antigen via fluorescent antibodies. Although sensitive for low grade
tumors, the assay has low specificity and requires large numbers of exfoliated cells (Yang et
al., 2014). Despite numerous attempts to improve UC diagnostics, a “perfect” biomarker for non-invasive detection of UC is yet to be illuminated.

*Tumor staging and grading*

Accurate grading and staging of bladder tumors is at the crux of treatment selection and prognosis and is *the* most important determinant of progression (Clemo, DeNicola, Carlton, Morrison, & Walker, 1994; van Rhijn, van der Poel, & van der Kwast, 2009). Staging encompasses the extent of tumor invasion and metastasis, both locally and throughout the body, while grading refers to the cellular and nuclear atypia within the primary tumor.

Histopathologic staging is achieved via the tumor-node-metastasis (TNM) staging protocol, as established by the American Joint Committee on Cancer (American Joint Committee on Cancer, 2002). In order to fully stage tumors using the TNM scheme, a tumor extent (T), regional nodal metastasis status (N), and distant metastasis status (M) are determined for the individual patient (Table 2). When the tumor has not invaded the urothelial basement membrane, it is classified as either Ta or Tis based on morphology: papillary tumors as Ta and flat tumors (*in situ*) as Tis. Tumors invading the basement membrane into the lamina propria are denoted as T1, while those invading through the muscularis are T2 and further divided by extent of muscle invasion (Figure 1). More aggressive tumors displaying invasion of perivesical tissues or neighboring organ structures (prostate, uterus, abdominal wall, vagina, pelvic wall) are classified by T3 and T4, respectively. Regional lymph node metastasis is defined by N0-N3, increasing with increasing size of the associated metastatic lesion and number of involved lymph nodes. Distant metastasis (M) is simplistically shown as M1 when distance metastases are evident, most commonly in the lung, and M0 otherwise (Kurian, Lee, & Born, 2011).
Bladder tumors are placed into one of three grading categories based on cytologic features, tumor heterogeneity, and nuclear characteristics (Patrick, Fitzgerald, Sesterhenn, Davis, & Kiupel, 2006). Grade I tumors are regarded as the least malignant and are identified by orderly cellularity with some abnormal cytologic features. Grade II tumors are more advanced and show tissue disorganization with some polarity. Mitotic figures are frequently seen in all layers of the urothelium, and invasion is likely. Grade III tumors show complete tissue disorganization without polarity. Cells are highly pleomorphic with clumped chromatin and numerous mitotic figures, speaking to the high proliferative capabilities of the tumor (Patrick et al., 2006). Grade II and III tumors display a high propensity for deep tumor invasion, exemplifying their aggressive nature (Valli, Norris, Jacobs, & Laing, 1995).

Tumor stage and grade carry significant prognostic value, making accurate biopsy paramount. Low grade Ta tumors have a 95% 15-year survival rate and rarely progress, even despite a high recurrence rate (Dip, Reis, Abe, & Viana, 2014). High grade Ta tumors, however, have a drastically reduced 15-year survival rate at 61%. Minimally invasive T1 tumors show a 44% survival rate. High grade papillary tumors often progress (>30%) to invasive disease, emphasizing the importance of accurate grading in the approach to thorough diagnosis, patient education, and treatment (Weilandt et al., 2014).

**mRNA and protein expression**

Numerous studies have investigated the molecular profile of urothelial tumors. mRNA and protein analyses have flagged cell cycle regulatory pathways (93% of UC cases), p53/Rb pathways (73%), PI3K pathways (72%), and chromatin remodeling pathways (89%). Most commonly, over-activation of PI3K leads to activation of RAS-associated pathways, specifically the AKT pathway (Calderaro et al., 2014; Cancer Genome Atlas Research, 2014; Mitra & Cote, 2009). Following phosphorylation by PI3K, PIP₃ recruits
PDK1 and AKT to the cellular membrane in close proximity, enabling phosphorylation and activation of AKT by PDK1 (Calderaro et al., 2014). PTEN normally dephosphorylates PIP$_3$, aborting pathway activation, and is thus associated with tumorigenesis in many cancers. In bladder cancer, however, gene silencing experiments have shown that PI3K/AKT activation is unrelated to losses of PTEN (Calderaro et al., 2014). Nevertheless, levels of phosphorylated AKT (pAKT) are increased in 88% of UC patients, and upregulation of the pAKT downstream target, p56, confirms increased activity of pAKT. pAKT also activates numerous proteins involved in cell cycle regulation, including MDM2, p21, and p27, making AKT a key regulator of the cell cycle, cell proliferation, and oncogenesis (Mitra & Cote, 2009). pAKT levels are similar in all UC grades and stages, suggesting deregulation of this pathway is generally involved in UC development and valuable for elucidating a potential broad-spectrum therapeutic target for UC (Calderaro et al., 2014).

Invasive tumors are specifically characterized by down regulation of epithelial barrier genes, including E-cadherin, and upregulation of proliferation and invasion genes, including MMPs, VEGF, and COX2, which together allow the disassociation of the epithelial barrier and transformation to a more motile, mesenchymal-type cell (Cheng et al., 2011). Notably, cyclooxygenase-2 (COX2) is an inflammation-associated gene which has been linked to the development of numerous carcinomas, including urothelial. It is also a well-recognized angiogenesis and invasion factor, promoting the survival and dissemination of neoplastic cells from the primary tumor. Although not normally expressed in the urinary bladder, high levels of COX2 are seen in UC and correlate with tumor stage and grade, making levels prognostically valuable (K Rathore & M Cekanova, 2014). Consequently, non-steroidal anti-inflammatory drugs (NSAIDs), which inhibit the cyclooxygenase enzymes, have established and profound efficacy for the treatment and prevention of bladder tumors (Bhattacharya, Li,
DNA microarray analysis has been used to identify additional genes differentially expressed in UC patients. Results have enabled separation of normal and tumor urothelium based on differential expression, emphasizing the altered landscape of gene expression in UC (Mengual et al., 2009). *CEACAM6*, a promoter of the epithelial-mesenchymal transition, is among the most highly overexpressed genes in tumors relative to normal urothelium. Tumors further cluster into high grade and low grade groups based on differential gene expression only, with over 500 genes differentially expressed between high and low grade tumors. Most notably, the metabolic gene carboxylesterase-1 (*CES1*) is highly under expressed in neoplastic urothelium compared to normal, highlighting a potential role of altered xenobiotic and carcinogen metabolism in UC pathogenesis.

*Genomic aberrations*

Previous studies have supported cancer as a “chromosomal disease” (Duesberg, 2005). Chromosome instability is a crucial event in carcinogenesis, leading to genomic deletions, amplifications, and rearrangements and subsequent alterations in gene dosage (Ma et al., 2012). In UC, genomic instability is associated with poor prognosis in both superficial lesions, in which instability correlates to tumor progression to invasion, and invasive tumors, which are highly genomically unstable (Fu et al., 2014; Netto, 2012). Numerous causes of genomic instability are recognized in UC and other cancers, including centrosome dysfunction, dysregulation of the cell cycle, breakage-fusion-bridge cycles, and exogenous carcinogenic compounds.

Aneuploidy, or an abnormal number of chromosomes per cell, results from a lack of proper spindle attachment during mitosis or non-disjunction. On a genome level, this is
reflected as relative chromosome copy number losses or gains. Non-muscle invasive bladder cancer has a nearly diploid karyotype; muscle invasive tumors, however, are highly aneuploid and display numerous chromosomal alterations, including the chromosome shattering phenomenon known as chromothripsis (Knowles & Hurst, 2015). Dysregulation of the mitotic spindle-organizing proteins is recognized as a major cause of aneuploidy in cancer, as well as centrosome dysfunction (Florl & Schulz, 2008). Normal centrosome duplication is crucial for proper chromosome segregation in anaphase and is controlled by the cyclin-dependent kinases (CDKs) throughout the cell cycle. CDKN2A, known as p16, is critical for the appropriate localization of the centrosomes in mitosis and is reduced in both genomic copy number and gene expression in UC (Abat et al., 2014; McDermott et al., 2006). Reduced levels of p16 lead to severe centrosome dysfunction and widespread chromosome gains and losses which may play a role in UC development and progression (Florl & Schulz, 2008).

Centrosome amplification represents an alternative manifestation of aberrant centrosomes. Increased centrosome numbers correlate to a decreased disease-free interval and is the strongest known clinical predictor for human UC recurrence in non-muscle invasive tumors (Chan, 2011). Centrosome amplification in UC is associated with gain of chromosome 20q, p53 inactivation, AURKA overexpression, and cyclin D/E overexpression, making these markers prognostically relevant (Chan, 2011).

Cell cycle dysregulation is frequently caused by activation of cellular proto-oncogenes, leading to constitutive entrance of cells into mitosis and tumor expansion along with genomic instability. Although healthy cells have mechanisms to protect DNA from damage and promptly repair it when it occurs, neoplastic cells readily evade DNA damage response (DDR) mechanisms. The DDR is central to coordinating cell cycle checkpoints with
DNA repair and cell death. In particular, the Timeless and Tipin genes are involved in maintenance of genome integrity during replicative stress. Downregulation of these genes, among others, leads to chromosome fragility, while overexpression leads to maintenance of an aberrant genome and cell survival (Schepeler et al., 2013). Bladder tumors with high levels of genomic instability, increased proliferation, and invasion show increased Timeless, allowing the persistence of aneuploidy. The ensuing cycles of increasing replicative stress lead to selection for more replicatively promiscuous DDR defective tumor clones, ultimately leading to DNA instability and aberration accumulation (Schepeler et al., 2013).

Breakage-fusion-bridge (BFB) cycles represent yet another major mechanism of chromosomal instability and rearrangements in cancer and UC. When a chromosome breaks or telomeres shorten, chromosome ends become “sticky” due to the electrostatic forces of exposed nucleotides (Florl & Schulz, 2008). Sticky ends of broken chromosomes may then associate, forming a derivative chromosome with aberrantly placed genomic material. As cells divide more rapidly or are continuously exposed to break-inducing agents, the BFB cycles continue, often leading to duplication or loss of genetic material involved in these rearrangements. In UC, research suggests defective telomeres are the most common reason for aneuploidy via BFB cycles (Florl & Schulz, 2008).

In addition to endogenous causes of aneuploidy, environmental carcinogens, such as those highlighted in UC risk, function as aneuploidogens. Previous studies have shown that treatment of cultured cells with potent carcinogens leads to unstable chromosomes, higher breakage rates, and higher prevalence of chromosomal copy number aberrations in 100% of cases (Duesberg, 2005). Other carcinogens, including aromatic hydrocarbons, destroy microtubule polymers, promoting inappropriate mitotic spindle formation, centrosome dysfunction, and abnormal chromosome segregation, suggesting the interaction
of carcinogens with chromosomes may instigate tumorigenesis (Das, Choudhury, Chakrabarty, Bhattacharya, & Chakrabarti, 2012).

As a result of the high occurrence of aneuploidy in cancer, numerous studies have identified and profiled specific and recurrent cytogenomic aberrations observed in UC (Table 3). In general, losses predominate the profile of low grade, superficial UC, while gains predominate those of high grade, invasive tumors. Losses can have severe biological consequences when the genomic region contains a tumor suppressor gene, while gains of oncogenes result in malignant transformation. Overall, the most common aberrations among UCs of all stages and grades include loss of 2q, 5q, 8p, 9p, 10q, and 18q and gain of 1q, 5p, 8q, and 17q (Jana, Galsky, Hahn, Milowsky, & Sonpavde, 2012).

Loss of chromosome 9 (HSA 9) is the single-most common chromosomal aberration in UC (>50% of low and high grade tumors), including pre-neoplastic lesions of the bladder (Bonberg et al., 2013; Nascimento e Pontes, da Silveira, Trindade Filho, Rogatto, & Viana de Camargo, 2013). Considered an early karyotypic event in UC, the prevalence of the loss of HSA9 decreases as tumor stage and grade increases. Loss of 9p21 is also associated with a decreased disease-free interval (Cheng et al., 2011). The oncogenic role of loss of HSA9 is presumably due to loss of the CDKN2A locus at 9p21, leading to unregulated S-phase entry, neoplastic transformation, and uncontrolled cell proliferation. In addition, HSA9 contains the deleted in bladder cancer protein-1 gene (DBC1), a suspect tumor suppressor gene which is deleted or methylated in 50% of bladder tumors (Knowles & Hurst, 2015; Schulz, 2006). Healthy urothelium acquiring loss of 9q leads to hyperplastic lesions, which evolve to low grade, papillary (pTa) tumors with subsequent mutation of FGFR3 (present in >80% of patients) (Dip et al., 2014). When loss of 9q occurs in TP53- and/or Rb-mutated
urothelium, dysplastic lesions evolve to high grade UC (CIS) with a high propensity for invasion (Netto, 2012) (Figure 2).

As tumor stage and grade increase, so does the total number of chromosomal aberrations observed in the tumor karyotype (Mitra & Cote, 2009). Although HSA9 loss becomes less frequent with advanced stage, the gain of another chromosome—HSA8—greatly increases in frequency. Gain of 8q is exclusive to high stage, high grade bladder tumors and recurrent low grade tumors (Högland, Säll, Heim, & al., 2001; Kang, Li Y Fau - Yu, Yu Y Fau - Guo, & Guo, 2014; Nascimento e Pontes et al., 2013). HSA8q contains the genomic locus of c-Myc, with resultant overexpression associated with high grade UC via promotion of cell cycle progression (Kang, Li Y Fau - Yu, et al., 2014). High grade tumors also show redundancy in gains of chromosome 3p, 10q, 13q, 17p, and 18q. Interestingly, smokers tend to develop invasive lesions possessing these aberrations, suggesting the carcinogenic compounds in cigarette smoke specifically target these chromosomes and their associated pathways (Mitra & Cote, 2009).

In addition to chromosome copy number aberrations, recurrent sequence mutations are associated with UC development. Activating mutations of FGFR3 are present in >80% of non-invasive, low grade UCs and <30% of invasive, high grade tumors (Calderaro et al., 2014; Cancer Genome Atlas Research, 2014; Juanpere et al., 2012; Kang, Li, Yu, & Guo, 2014). Activation of FGFR3, by either genomic mutation or ligand interaction, initiates a tyrosine kinase pathway able to activate both MAPK and PI3K signaling pathways (Figure 3). As a result, FGFR3 activation is involved in in embryogenesis, development, angiogenesis, wound healing, tissue homeostasis, tumorigenesis, cell proliferation, migration, and apoptosis (Dip et al., 2014). Most commonly, a single point mutation in FGFR3 exon 7 leads to ligand-independent constitutive activation of the receptor and
downstream pathway (Dip et al., 2014). However, FGFR3 mutations are also seen in hyperplastic lesions of the urothelium, rendering mutation detection a non-specific test of malignancy (van Rhijn et al., 2002; van Oers et al., 2006; Cheng et al., 2011). For these reasons, mutated FGFR3 suggests a low grade tumor and, therefore, favorable prognosis, even despite a high potential for tumor recurrence.

Genomic alterations observed in advanced UC center around the loss of tumor suppressor genes, as opposed to the activation of proto-oncogenes prevalent in low grade tumors. Carcinoma in situ (CIS) lesions and high grade tumors are characterized by loss-of-function mutations in the TP53 and/or Rb pathways (Juanpere et al., 2012; Mitra & Cote, 2009; Schulz, 2006). Similarly, at least 50% of high grade, muscle invasive UCs possess mutations in p53 (HSA17, codons 2-11), genomically linking CIS lesions as precursors to high grade tumors (Calderaro et al., 2014). Loss of p53 activity can be used as therapeutic guide, rendering DNA more sensitive to DNA damaging compounds such as cisplatin (Lin & Howell, 2006). In addition to p53 mutations, inactivating mutations of the Rb gene lead to an inability to inhibit the E2F transcription factor, thereby allowing constant promotion of the cell cycle (Milowsky & Kim, 2014).

As mentioned previously, the PI3K-AKT pathway appears to be central to the development of high grade, muscle-invasive UC. In addition to gene expression and protein alterations, over 40% of high-grade UCs possess a mutated gene in the PI3K-AKT pathway (Juanpere et al., 2012). Several pathway players, including RAS and AKT1, are altered via point mutations, leading to constitutive pathway activation and subsequent uncontrolled cell division and apoptosis evasion. AKT activation further promotes centrosome amplification and chromosome instability, perpetuating a continuous cycle of chromosome gain/loss and tumor evolution (Ma et al., 2012). Mutations in HRAS (codon 61), a central proto-oncogene
involved in multiple cell signaling pathways, can similarly affect the MAPK pathway, providing a "double-edge sword" for cellular integrity via two central proliferation pathways (Sjödahl et al., 2011). Interestingly, mutations in FGFR3 mimicking phosphorylation, as seen in low-grade tumors, can lead to AKT activation, suggesting the mechanisms to low- and high-grade tumors may be more similar than different.

Previous genome wide association studies (GWAS) have elucidated single nucleotide polymorphisms (SNPs) associated with UC. In particular, gains of the SNP identified by rs8102137 (HSA19q12) are associated with high grade tumors (Fu et al., 2014). The associated gene cyclin E, which is necessary for cell cycle progression, is 6 Kb downstream of the SNP and is similarly increased in both copy number and expression in UC. When comparing the expression levels of cyclin E in over 6,000 tumors and healthy bladders, increased expression was significantly associated with increased genomic instability; rapid progression through the cell cycle leads to the rapid accumulation of genomic alterations. This central idea is not only recapitulated with numerous potential oncogenic drivers in UC, but in all neoplasia. Curtailing uncontrolled proliferation may prevent genomic damage and neoplastic transformation.

Due to the rapid acquisition of secondary chromosome rearrangements during cell transformation, the genomic “drivers” of neoplasia are difficult to ascertain (Höglund et al., 2001). Nevertheless, the identification of marker associated with the progression of UC is advantageous for numerous clinical applications, including diagnostic markers, treatment guides, and prognostic indicators. The identification of genomic markers may lead to the identification of a central pathway and effector molecular, leading to a clinically relevant biomarker and potential therapeutic target (Cheng et al., 2014).
Epigenetic alterations

Altered epigenetic regulation has been extensively associated with UC. Most commonly, epigenetic alterations lead to the suppression of crucial tumor suppressor genes, leading to uncontrolled tumor cell proliferation (Dip et al., 2014). Three major epigenetic changes have repeatedly been implicated in UC development: microRNA (miRNA) imbalances, DNA methylation, and histone modifications.

MicroRNA represent a class of small, non-coding RNA molecules (18-25 nucleotides) that serve to regulate post-transcriptional expression of up to 30% of mammalian genes (Y. Lu & Cheng, 2014). Via interaction with the RNA-induced silencing complex (RISC), miRNAs bind 3’ untranslated regions (UTRs) in target genes, leading to mRNA degradation or, more frequently, impaired translation (Y. Lu & Cheng, 2014; Zabolotneva et al., 2013). It is believed that chromosomal alterations are a major mechanism leading to dysregulated miRNA expression (Wilting et al., 2013). The genomic loci of miRNA are located within gene introns and transcribed along with host genes (L. Zhang et al., 2006). A single miRNA can target up to 300 genes, many of which are involved in angiogenesis, cell proliferation, apoptosis evasion, the epithelial-mesenchymal transition, and tissue invasion (A. Zaravinos et al., 2012). Due to the various and profound effects each miRNA can have on numerous target mRNAs and, therefore, proteins, copy number aberrations in regions of crucial miRNA can have major effects on the cell transcriptome and homeostasis.

Depending on their targets, miRNA can act as tumor suppressors or oncogenes by targeting oncogenes or tumor suppressors, respectively. Over half of all miRNA loci are associated with CpG islands and, therefore, silencing of associated miRNA is believed to be methylation-mediated. For this reason, it is generally accepted that neoplastic
transformation, which is characterized by global hypermethylation in UC, leads to decreased levels of mature miRNAs (A. Zaravinos et al., 2012).

Over 50% of miRNAs are located near cancer associated genomic regions (CAGRs) or chromosomal fragile sites (Y. Lu & Cheng, 2014), with 43% located in exact regions of chromosome loss of amplification (Calin et al., 2004). Fragile sites represent genomic regions of preferential sister chromatid exchanges and, therefore, increased incidence of translocation, deletion, amplification, or integration of tumor-associated viruses (Calin et al., 2004). In one study, the incidence of miRNAs in fragile sites was over nine times greater than in non-fragile regions. Consequently, miRNAs are often in regions of aberrant chromosome copy number, particularly in epithelial cancers, often leading to altered miRNA transcript levels (L. Zhang et al., 2006).

One prominent oncogenic miRNA (oncoMiR) is miR-10b. Located within the HOXD gene cluster and prominent chromosome fragile site at HSA2q31, miR-10b has been implicated in numerous cancers, including breast, ovarian, esophageal, gastric, renal, colorectal, pancreatic, and bladder carcinomas (Y. Lu & Cheng, 2014). miR-10b appears generally decreased in tumors, but metastatic cells and established metastases have significantly increased levels. As a result, increased miR-10b is associated with a poor prognosis (Parrella et al., 2014). Due to the ability to therapeutically silence overexpressed miRNA and supplement those underexpressed, miRNA have become a popular and valuable molecular tool with massive physiologic consequences (Pereira, Rodrigues, Borralho, & Rodrigues, 2013).

DNA methylation is an important regulator of transcriptional control in ~50% of all known genes, and hypermethylation has been implicated as the major cause of tumor suppressor gene (TSG) silencing in cancer genomes. Methylation is characterized by the
attachment of a methyl group to the 5’ carbon of cytosine and most commonly occurs in cytosine-rich CpG islands in the promoter of target genes or exon 1, thereby silencing gene transcription. Aberrant methylation is an attractive biomarker target due to the stability of DNA in bodily fluids and even after tissue fixation and processing (Reinert, 2012). Previous studies of UC have shown the extent of DNA methylation increases with increasing tumor stage and grade, suggesting an association of methylation with tumor aggression (Wolff et al., 2010). Specifically, >20% of bladder tumors demonstrate increased methylation of potential tumor suppressors PAX6, RASSF1A, APC, and cadherin (CDH1, CDH13) (Maruyama et al., 2001). On the contrary, hypo-methylation is often predominant in non-invasive tumor types, including the non-neoplastic urothelium of UC patients, advocating a role of methylation on the “field effect” observed in UC and suggesting a mechanism for a high tumor recurrence rate (Wolff et al., 2010). Further evidence for the value of methylation in UC comes from therapeutic success; 5-aza-2’-deoxycytidine (DAC, 5-aza), a demethylating agent, increases tumor sensitivity to cisplatin and radiation in vitro and antitumor activity in canine models (Hahn et al., 2012; Jiang et al., 2014; Ramachandran, Gordian, & Singal, 2011).

An additional and integral part of gene regulation includes histone modification. Normally facilitated by acetylation/deacetylation, methylation, and phosphorylation, histone modifications control transcriptional activity of genomic regions (Vallot et al., 2011). Histone modification is thought to be responsible for the silencing of numerous tumor suppressor genes, as well as activation of oncogene transcription, in human cancers. For example, HOXA and HOXD genes on chromosome 3 and 2, respectively, are abnormally silenced in epithelial cancers due to reduced acetylation (Vallot et al., 2011). Numerous chemotherapeutics, including FDA-approved Vorinostat (suberanilohydroxamic acid),
effectively inhibit the removal of acetyl groups from histones, maintaining gene expression. Past studies have shown treatment of bladder cancer cell lines with Vorinostat stops tumor progression and promotes cell differentiation and apoptosis, providing a viable therapeutic option for UC (D. Wang et al., 2013).

Despite a vast amount of genomic and molecular research into UC, an effective targeted chemotherapy has not been established for UC patients. Particularly in patients with invasive and metastatic disease, therapeutic advancements are the sine qua non of UC research and yet are severely lacking. Consequently, patients diagnosed with invasive UC are treated aggressively, ineffectively, and suffer a decreased quality of life. For these reasons and more, the molecular stratification of tumors and development of personalized medicine approaches in UC is crucial to the advancement of UC clinical care (Gupta & Mahipal, 2013).

**Models of Urothelial Carcinoma**

*The cell line model*

*In vitro* models are necessary to study molecular mechanisms of pathogenesis and for evaluation of drug safety and efficacy; *in vitro* models allow for controlled experimental conditions, reducing the number and effect of confounding factors and increasing experimental reproducibility (Cekanova & Rathore, 2014). Their manipulability facilitates discovery and investigation of precise factors, specifically how they respond to and are affected by environmental influences. Due to the ability to manipulate and thoroughly analyze *in vitro* models, in addition to sparing animals lives, they are invaluable for intricate molecular studies.
The cell line model is amenable to the study of molecular mechanisms involved in UC. The prevalence of abnormal molecular signatures in human tumors suggests involved pathways, including MAPK and PI3K/AKT, should be evaluated further to acquire improved understanding of disease and targeted therapeutic options. By allowing detailed analysis of the cell cycle and numerous other molecular mechanisms, cultured systems facilitate understanding the molecular changes involved in neoplastic urothelial cells over time (Florl & Schulz, 2008). The clinical predictive value of a cell line determines its utility in in vivo trials, in which intact physiologic processes are present. When utilizing an in vitro model, it is crucial to ascertain and acknowledge the value, or limitations, of tested drug dosages and potential role of biological systems. In fact, the loss of a natural biological background, including tumor heterogeneity, perfusion, stromal interactions, and immune cells, is the major limitation of in vitro models. For this reason, it is paramount to assess exactly how the loss of these factors affects the cells studied by comparing the disease as seen in vivo to than in vitro (Cekanova & Rathore, 2014). Numerous UC cell lines have been established for in vitro study. The more translational experiments conducted in these models, the more will be learned about them and how well they mirror the disease in vivo, augmenting their clinical predictive value.

Animal models

Cancer is the second most common cause of death in humans, highlighting the importance of continued research into the underlying mechanisms of development and subsequent diagnostic, prognostic, and therapeutic advancements (Cekanova & Rathore, 2014; Oliveria et al., 2014). In order for these studies to maintain biological and in vivo relevance while upholding ethical standards, animal models are routinely used. Animal cancer models enable researchers to not only identify potentially efficacious treatments by
molecular and clinical studies, but they also help determine the most sensitive stages and optimal therapeutic doses. Which model serves to provide relevant information about the disease, however, is a constantly evolving opinion. The ideal model organism for any disease is readily available, inexpensive to obtain and maintain, biologically similar with an intact immune response, and responds to treatments similarly to humans. In the case of UC, there exists a critical need for improved treatments of invasive and metastatic disease.

Although many laboratory rat and mice strains possess an intact immune system, they fail to develop bladder tumors de novo. Injection of N-butyl-N-(4-hydroxybutyl)nitrosamine induces bladder tumors in mice, but these tumors do not metastasize, leading to skepticism about the clinical utility of the murine model in oncology, and, in particular, metastatic cancers such as invasive UC (Cekanova & Rathore, 2014; Oliveria et al., 2014). Aside from biological differences in tumor development and behavior, mice can tolerate a much higher dose of chemotherapeutics than humans, limiting their utility in drug efficacy and safety studies. In fact, fewer than 8% of rodent oncology trials result in translation to human clinical trials (Cekanova & Rathore, 2014). Considering the critical need for developing diagnostics and therapeutics for metastatic human UC, this poses a major problem in using laboratory rodents in the investigation of UC.

The need for a model of spontaneously-derived cancers in an appropriate biological context has led to the acknowledgement that pets, including dogs, develop spontaneous cancer and provide a highly relevant model beneficial to all species (Knapp et al., 2000). Over six million dogs are diagnosed with a naturally occurring cancer each year, making tumors for study readily available (Cekanova & Rathore, 2014). Additionally, similarities to humans, along with unique genetic characteristics, make dogs an optimal model for oncology research, particularly in drug development and metastasis (Knapp et al., 2000;
Shearin & Ostrander, 2010). Strong behavioral and phenotypic selection due to pure breeding has led to decreased genetic heterogeneity and inadvertent selection of deleterious genetic traits. Although unfortunate for our pets, these characteristics enable more straightforward genetic analyses than those of a heterogeneous, outbred human population (Shearin & Ostrander, 2010). By using a more homogenous population with reduced genetic noise, we may elucidate genes at the crux of tumor development, so called “driver mutations”. In addition to genetic advantages, both species have an extremely complex and largely identical immune system, allowing evaluation of tumor behavior within a biologically relevant context. Lastly, humans and their pets share the same environment—drinking water, air, living spaces, and often physical habits. For these reasons, the frequency of neoplasia and treatment responses are similar between humans and dogs, providing a highly useful and accurate model for therapeutic advancements. In fact, many studies of invasive human UC have been based on findings in dogs (Patrick et al., 2006).

The average age of cancer diagnosis in dogs is 8.4 years, which is similar to the 50-year average in humans, suggesting tumor development in dogs, just as in humans, is influenced by age and environment (Cekanova & Rathore, 2014). In the case of serious environmental carcinogens, as is suspected in UC, dogs can serve as a sentinel for their human cohabitants. The latency between carcinogen exposure and neoplastic transformation in dogs is as little a one year, whereas carcinogenesis may take decades in their owners. This phenomenon has been reported numerous times in homes with asbestos in which dogs are diagnosed with mesothelioma, leading to increased awareness and early detection among owners (Knapp et al., 2013).

The dog is a particularly good model for the study of UC. Aside from humans, dogs are the only domestic mammals with a significant incidence of UC. Furthermore, canine UC
closely resembles that of its human counterpart, including clinical signs, histopathology, molecular features, and biological behavior, including metastases, therapeutic response, and prognosis (Table 3) (Bellmunt et al., 2014). Naturally occurring canine UC allows us to study the entire clinical course of disease—from development to diagnosis, through treatment selection and response, to progression, and death—rapidly and in a way that benefits both dogs and people (Dhawan, Ramos-Vara, Stewart, Zheng, & Knapp, 2009).

With selection of the appropriate animal model, researchers can better address questions central to the treatment of both canine and human UC—how are metastases prevented, and how can we effectively treat existing metastases? (Knapp et al., 2000)

**Canine Urothelial Carcinoma**

Although uncommon relative to other tumor types, UC comprises 1.5-2% of all canine cancer diagnoses. Forty-five percent of household pet dogs over the age of 10 develop cancer, meaning that despite a small incidence rate, over 20,000 dogs are diagnosed with UC each year (Knapp et al., 2014). Just as in humans, UC is a geriatric disease, with the mean age of diagnosis being 11 dog years (60 equivalent human years) (Knapp et al., 2000). Unlike humans, however, the majority of canine tumors are located in the bladder trigone, the area of the bladder in which the ureters and urethra join to the bladder body. The anatomical location of canine bladder tumors makes them of serious physiological concern due to their close proximity to the ureters and urethra, whose patency is required for life, and precludes surgical resection of the tumor. Thus, a diagnosis of UC evokes far more concern among owners than other solid tumors (Hamilton, Sarcornrattana, Illiopoulou, Xie, & Kitchell, 2012).
Just as in humans, numerous environmental risk factors are associated with UC development. Exposure to flea and tick dips, mosquito control, industrial areas, and cyclophosphamide treatment are highly suspected risk factors (Knapp et al., 2014; Mutsaers, Widmer, & Knapp, 2003). Additionally, dogs who are obese, female, neutered, and/or of predisposed breeds have an increased risk for developing UC. The Scottish terrier is diagnosed approximately 20 times more frequently than the average dog. Shetland sheepdogs, beagles, West Highland white terriers, and wire hair fox terriers have three to five times more diagnoses than average (Knapp et al., 2014). Consequently, UC is known as a “small dog disease”, with mean body weight of affected dogs being 15.7 kilograms.

The risk of developing UC associated with flea and tick dips is dose-related and increased with obesity, presumably due to increased lipid stores in which lipophilic compounds can be stored (Knapp et al., 2000). Accordingly, lipophilic “inert” ingredients in insecticides, which make up 95% of the product, are the suspected culprits in carcinogenesis (Knapp et al., 2013; Mutsaers et al., 2003). These inert compounds, including benzene, toluene, xylene, and petroleum, are ideal for storage in body fat and may be constantly present in the body, particularly of obese and female dogs with a higher percentage of body fat. Additionally, active lipophilic herbicide compounds are found at high concentrations in exposed dogs. In a study of 25 dogs living with herbicide-treated lawns, at least one in three test chemicals was detected in the urine of 76% of participants (Knapp et al., 2013). Most commonly, the residual chemical was methylchlorophenoxypropionic acid (MCPP), reported in 60% of dogs. The presence of chemicals in the urine was not correlated with the amount of time spent on the lawn, making these findings crucial even for dogs without treated home lawns who may walk on publicly treated lawns or whose lawns may be contaminated by neighbors’ runoff. Despite companies recommending 24 hours
post-treatment before allowing pets and children onto treated lawns, significant chemical uptake was evident even past 48 hours post-treatment. Due to the enormous amount of information linking UC development in both dogs and humans to environmental carcinogens, aberrant metabolic activation and detoxification pathways are suspected to play a major role in UC pathogenesis.

Further evidence for aberrant metabolic pathways have been demonstrated in urinary metabolite studies (J. Zhang et al., 2012). When comparing chemical metabolite levels in urine, dogs diagnosed with UC had increased levels of urea, choline, methylguanidine, citrate, acetone, and B-hydroxybutyrate compared to healthy dogs. In addition, levels of citrate, acetone, B-hydroxybutyrate, and methylguanidine were significantly increased even in healthy at risk breeds (Scottish terrier, Shetland Sheepdogs, West Highland White Terriers) compared to healthy non-risk dogs and elevated approximately to levels of non-risk dogs diagnosed with UC. Such findings suggest inherent and detectable metabolic differences may play a role in breed-associated UC risk.

Clinical symptoms of UC are similar to those seen in people, including hematuria, dysuria, pollakiuria, and stranguria (Knapp et al., 2014). Unfortunately, these symptoms are common to most urinary tract diseases, including bacterial cystitis—one of the most commonly diagnosed diseases in veterinary medicine (Allen, Waters, Knapp, & Kuczek, 1996). Non-specific symptoms, combined with a dog who generally appears happy and healthy, often lead to misdiagnosis and prolonged inappropriate treatment for bacterial cystitis, delaying therapeutic intervention. Additionally, polyploid cystitis represents a non-neoplastic urothelial mass arising from chronic cystitis and detectable on ultrasound, further complicating definitive diagnosis (Patrick et al., 2006). It is suspected that the resultant delay in diagnosis contributes to the extremely high incidence of invasive UC in dogs compared to
humans (80% and 20%, respectively). At diagnosis, canine bladder tumors are nearly always papillar y (projecting into the bladder lumen), high grade, and invasive (Knapp et al., 2000; Vinall, Kent, & deVere White, 2012). Moreover, metastases, even when not overt, are presumably present. At diagnosis, approximately 16% of patients have regional lymph node metastasis, 14% have distant metastasis (lung, most commonly, in humans and dogs), and 10% show metastasis to both (Knapp et al., 2014). At death, 50-90% of patients show evidence of distant metastases (Nikula, Benjamin, Angleton, & Lee, 1989; Valli et al., 1995). These rates are similar to metastatic rate in invasive human UC, in which 50% of individuals with invasive tumors succumb to metastasis (Milowsky & Kim, 2014). Due to the advanced tumor stage at diagnosis, treatment is rarely curative (Knapp et al., 2014; Vinall et al., 2012). Nevertheless, lagging diagnostic methods preclude knowledge of the true aggression of early bladder tumors and prevent more efficacious therapeutic timing.

Current canine prognostic indicators are limited to tumor location, depth of tumor invasion, and the presence of metastases (Patrick et al., 2006). Proper tumor classification, however, requires tumor biopsy. Despite histopathologic evaluation being the “gold standard” of diagnosis, the majority of veterinary clinics rarely biopsy canine bladder tumors, precluding accurate assessment of tumor histopathology and definitively diagnosis. Currently, the only adjunct UC diagnostic assay available for veterinary use is the veterinary bladder tumor antigen assay (V-BTA™; Polymedco), which detects complement factor H in urine specimens as in the human BTA assay (Henry, Tyler, McEntee, Stokol, & Rogers, 2003; Vinall et al., 2012). However, this assay is not clinically relevant, as fewer than 3% of positive test results actually detect UC. False positive results are spawned by glucosuria, proteinuria, and/or hematuria, all common urinary symptoms in geriatric dogs, as well as a plethora of other urinary ailments. Thus, a similarly convenient assay which is unaffected by
common clinical signs is desirable. Moreover, metastasis evaluation lacks sensitivity, limited to diagnostic imaging and lymph node aspirates, complicating determination of disease extent.

Due to the delicate anatomical location of the majority of canine tumors, common and extensive urethral involvement, and presence of metastases, surgery is typically not a valid option for canine patients. Reports suggest that, even when possible, surgery with clean margins does not preclude local recurrence or the development of distant metastases (Knapp et al., 2000; Mutsaers et al., 2003). Surgical debulking of the tumor, however, has been shown to provide survival benefits, as well as palliative care. Despite a relatively poor response to chemotherapy (<35%), chemotherapy remains the treatment of choice for canine UC (Henry et al., 2003; Fulkerson & Knapp, 2015).

Current median survival time with medical therapy alone is approximately 150 days without biopsy. Survival time improves to 195 days with biopsy and medical therapy and 272 days with more extensive surgical debulking (Mutsaers et al., 2003). The highest survival rate has been associated with combination antracycline (mitoxantrone, doxorubicin) and platinum chemotherapeutic protocols (358 days), but high toxicity limits the utility of these more efficacious protocols (Mutsaers et al., 2003). When combined with radiation and piroxicam, however, mitoxantrone provides a median 240 day survival, providing the most universally well-tolerated and efficacious treatment for canine UC (Fulkerson & Knapp, 2015).

Piroxicam, a non-steroidal anti-inflammatory with non-selective COX inhibition, has been shown to have great anti-tumor effects even at low doses in canine UC; the median survival with Piroxicam therapy alone is 180 days. Over 30% of dogs treated with Piroxicam alone went into partial remission, while 6% of dogs showed a complete remission and were
tumor-free over 2 years after diagnosis (Knapp et al., 2000). The successes of Piroxicam in the canine mode have led to invaluable human studies of COX inhibitors in humans with carcinoma in situ, enabling the discovery a new human therapeutic option. The shared tumor heterogeneity and response between humans and dogs is a perfect example of how dogs can reveal efficacious treatments and realistic expectations for humans.

The advent of early diagnostic techniques for canine UC might help identify tumors prior to invasion, enabling more prompt and efficacious treatment. Great increases in survival rates in human carcinomas, including prostate, colorectal, and breast, have been due to advancements in education and early detection (Vinall et al., 2012). Hopefully with such advancements in veterinary medicine, similar improvements can be achieved.

*Tumor staging and grading*

A classification scheme for tumor staging separate from that of humans has been established by the World Health Organization (WHO) and is the most broadly applied scheme for characterization of canine tumors (Table 5) (Owen, 1980). Although still a TNM (tumor-node-metastasis) scheme as seen in humans, the extent of invasion differs in dogs, as well as the classification of nodal metastases. In humans, the extent of invasion into the bladder wall is divided among T1-T3 stages; in dogs, T2 tumors include all tumors invading into the lamina propria, muscularis, and/or perivesical tissue, while T1 are superficial. T3 tumors invade neighboring organs, with the majority (>90%) of canine tumors being of stage T2 or T3 (Knapp et al., 2014). No T4 stage exists in the canine staging scheme. Nodal metastases are also simplified compared to the human scheme: N1 tumors show regional lymph node involvement, while N2 tumors show metastasis to both regional and juxtaregional lymph nodes. No N3 nodal stage exists in dogs. Distant metastases are staged just as in humans: M1 signifies distant metastases are present. Despite the existence of this
separate staging scheme, previous studies have found the histopathologic characteristics of canine UC similar between humans and dogs and human staging applicable to the staging of canine tumors, allowing direct comparison of canine and human tumors (Patrick et al., 2006; Valli et al., 1995).

The grading scheme for canine UC mirrors that of human, with grades I-III correlating to increasing levels of atypia in neoplastic cells. Unfortunately, the majority of canine tumors are grade II or III (78%), representative of the aggressiveness of canine tumors (Patrick et al., 2006). A significant association exists between survival and tumor grade, but only between grade I and grades II/III (Valli et al., 1995). No differences in survival time between grades II and III are known, limiting the prognostic value of tumor grade. Considering the similarly invasive clinical behaviors and prognostic value of tumor grade, there is support for a two-tiered grading scheme in dogs relying on only “low” (grade I) and “high” grade (grades II, III) (Patrick et al., 2006). However, canine UCs are rarely biopsied and, therefore, rarely staged and graded histopathologically, impeding an understanding of disease progression and potential prognostic value.

Molecular alterations

In dogs, very little is known about the genomic landscape of UC aside from general karyotypic observations. It is known that 79% of canine tumors are aneuploid, with 47% hyperploid, 50% tetraploid, and 3% hypertetraploid, as assessed by flow cytometry (Clemo et al., 1994; Knapp et al., 2000). In dogs, grade III tumors tend to be tetraploid, and, therefore, tetraploidy corresponds to a rapid clinical course and poor prognosis. Aneuploidy is not, however, exclusive to neoplastic tissues; hyperplastic/inflamed urothelium can also be aneuploid, limiting the clinical significance of ploidy in canine UC.
Minimal research has investigated the specific molecular profile of canines UC. Most notably, the \textit{BIRC5} gene, which encodes the Survivin protein, is highly upregulated in both mRNA and protein expression in neoplastic canine urothelial cells (Rankin, Henry, Turnquist, & Turk, 2008a, 2008b). Survivin is normally expressed during fetal development, where it contributes to cell proliferation, angiogenesis, cardiogenesis, and neural tube closure. Survivin further inhibits apoptotic caspases 3 & 7 and promotes continued progression from the G2 to M phase of the cell cycle (Chen et al., 2014). The \textit{BIRC5} promoter has a cell cycle-dependent element (CDE), allowing fluctuations with various stages of the cell cycle (Chen et al., 2014). A cytosine/guanine polymorphism at the CDE repressor binding site is associated with advanced tumor stage and grade in humans but has not been evaluated in dogs. Moreover, increased levels of Survivin suggest a decreased recurrence-free survival period, decreased overall survival, and increase in tumor invasiveness in human patients (Chen et al., 2014). Although highly expressed in neoplastic urothelium of both species, Survivin has also been detected in cystitis at nearly equal frequency in dogs and at low levels in normal urothelium, limiting its diagnostic specificity (Rankin et al., 2008a).

Just as in humans, the FGF signaling cascade has been implicated in canine UC development, specifically basic fibroblast growth factor (bFGF). bFGF is a proangiogenic molecule and a natural ligand for FGFR3, the main culprit in superficial human UC. Canine UC patients have an average of nearly five-fold increased levels of urinary bFGF compared to normal dogs or dogs with urinary tract infections, representing a significant increase in bFGF levels (Allen et al., 1996; Mutsaers et al., 2003). bFGF elevations and presumed over activity of the FGFR3 pathway further draws parallels between the pathogenesis of canine and human UC and emphasizes potential therapeutic targets.
A more recent study investigated the role of yet another growth factor in canine UC--epidermal growth factor receptor (EGFR) (Hanazono et al., 2014). EGFR is a receptor tyrosine kinase able to activate both MAPK and PI3K cell signaling pathways, promoting cell cycle progression and the epithelial-mesenchymal transition (EMT) similar to FGFR3. EGFR is known to be overexpressed in numerous human carcinomas, including urinary bladder, and is associated with high-grade, invasive tumors (Bryan et al., 2015; Lipponen & Eskelinen, 1994). Although EGFR overexpression is also overexpressed in canine UC, no prognostic association has been noted. Moreover, EGFR immunohistochemistry successfully discriminates between UC and polyploid cystitis but shows poor diagnostic sensitivity (72%) and relies on biopsied specimens, limiting its relevance in an infrequently biopsied tumor.

The current knowledge base of the molecular pathogenesis of canine UC is limited. Precision medicine, formerly known as personalized medicine, relies on the identification of tumor-promoting molecules amenable to therapeutic targeting. Until more is learned about the molecular profile of canine UC, extension of precision medicine protocols into canine UC will prove challenging. Furthermore, a deeper understanding of disease pathogenesis, at molecular and clinical levels, facilitates improved ability to appreciate disease predispositions and advocate preventive measures.

**Dissertation Rationale and Outline**

The detection and therapeutic management of UC is a challenge to both human and veterinary medicine. In both species, the poor prognosis associated with advanced, invasive tumors necessitates detailed investigation of tumor pathogenesis. Since differences in genomic aberrations between superficial and invasive tumors in humans are well recognized
and clinically relevant, the potential value of genomic alterations in veterinary oncology is evident. Although much is known about the aberrant genome of human UC, virtually nothing is known about the equivalent canine tumor. In the following pages, we aimed to learn more about the molecular profile of canine UC, including genomic, gene expression, and functional alterations. We maintained four major hypotheses throughout our studies: 1) the genome of canine bladder tumors is aberrant and of diagnostic relevance, 2) dogs provide a valuable genomic model for human UC and assist the identification of potential driver genomic aberrations, 3) canine UC cell lines maintain the genomic characteristics and behavior of the primary tumor and, therefore, provide a valuable model of the canine and human disease in vitro, and 4) the cell lines provide a means with which we can assess the invasive tendencies of canine UC and evaluate therapeutic options for both canine and human UC.

First, we evaluated the genomic profile of canine UC tumors, with detailed analysis of suspect regions. Three canine chromosomes were exceptionally aberrant in copy number, including CFA 13, 19, and 36. Due to the prevalence of these mutations throughout the UC tumor population, we developed a fluorescence in situ hybridization (FISH) assay to detect numerical abnormalities in tumor biopsies and, more clinically, urine sediments. By comparing aberrations across the canine and human genome, we successfully identified suspected pathogenesis-associated genomic regions which were shared between humans and dogs, most notably gains of HSA8/CFA13. Interestingly, HSA8 gains predominate the genomic profile of invasive UC, further emphasizing similarities between invasive human UC and canine UC.

Canine UC cell lines were similarly interrogated, along with gene expression analysis, to determine the clinical predictive value of the cell lines in relation to the dog and
human disease and elucidate global molecular abnormalities. The cell lines were found to be demonstrative of the primary tumor. Hence, we used the cell lines for functional analyses of potential biomarkers identified in cell line expression analysis and potentially relevant to neoplasia, most notably the invasive behavior of canine tumors. Expression analysis of UC cell lines highlighted aberrant metabolic pathways, including lipid and xenobiotic metabolism, theorized to be at the crux of UC development. Additionally, expression analysis elucidated aberrantly expressed genes with potentially diagnostic, prognostic, and therapeutic implications. We evaluated the role of one of the most highly overexpressed among cell lines, proto-oncogene PTTG1, and its involvement in the invasiveness of neoplastic cells. We found PTTG1 overexpression to not only increase tumor cell proliferation and invasiveness, but also to be a valid therapeutic target using currently available chemotherapeutics.

Finally, we evaluated the role of two specific genomic aberrations prevalent in canine UC: the miR-10b locus affected by CFA36 gains and BRAFV600E mutations. Although diagnostically relevant, we found little association of these alterations to the invasiveness of canine UC. Altogether, results greatly augmented our knowledge of canine UC at a molecular level and bridged them to clinically relevant applications with comparative potential.

References


39


Table 1.1. *Currently available non-invasive diagnostics for the detection of human urothelial carcinoma with associated clinical value.*

<table>
<thead>
<tr>
<th>Assay</th>
<th>Principle</th>
<th>Biomarker Target</th>
<th>Sensitivity/Specificity (%)</th>
<th>Advantages</th>
</tr>
</thead>
<tbody>
<tr>
<td>Accu-Dx®</td>
<td>Qualitative immunoassay</td>
<td>Fibrin, FDP</td>
<td>68-70/68-86</td>
<td>Simple</td>
</tr>
<tr>
<td>BladderChek®</td>
<td>Qualitative immunoassay</td>
<td>NMP22</td>
<td>55.7/85.7</td>
<td>Sensitive for high stage tumors</td>
</tr>
<tr>
<td>BTA stat®</td>
<td>Qualitative immunoassay</td>
<td>bCFHrp</td>
<td>9.3-89/50-90</td>
<td>Sensitive for high stage/grade tumors</td>
</tr>
<tr>
<td>BTA TRAK®</td>
<td>ELISA</td>
<td>bCFHrp</td>
<td>52-83/50-90</td>
<td>Sensitive for high stage/grade tumors</td>
</tr>
<tr>
<td>ImmunoCyt™</td>
<td>Immunocytofluorescence</td>
<td>Carcinoembryonic antigen, mucin</td>
<td>70-80/60-70</td>
<td>Cytology increases sensitivity</td>
</tr>
<tr>
<td>NMP22®</td>
<td>Sandwich ELISA</td>
<td>NMP22</td>
<td>48-100/70-91</td>
<td>Sensitive for high stage tumors</td>
</tr>
<tr>
<td>UroVysion™</td>
<td>FISH</td>
<td>Chromosome 3, 7, 9, 17</td>
<td>69-87/89-96</td>
<td>Sensitive for high grade, CIS, &amp; occult tumors</td>
</tr>
</tbody>
</table>


Table 1.2. American Joint Committee on Cancer’s accepted scheme for the staging of human urothelial carcinomas according to the extent of primary tumor invasion and regional and distant metastases.

<table>
<thead>
<tr>
<th>Primary Tumor</th>
<th>Tumor Invasion (T)</th>
<th>Extent</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Ta</td>
<td>Papillary, non-invasive</td>
</tr>
<tr>
<td></td>
<td>T1s</td>
<td>Flat, non-invasive (in situ)</td>
</tr>
<tr>
<td></td>
<td>T1</td>
<td>Lamina propria</td>
</tr>
<tr>
<td></td>
<td>T2</td>
<td>Muscularis</td>
</tr>
<tr>
<td></td>
<td>T2a</td>
<td>Superficial half of muscularis</td>
</tr>
<tr>
<td></td>
<td>T2b</td>
<td>Deep half of muscularis</td>
</tr>
<tr>
<td></td>
<td>T3</td>
<td>Perivesical tissue</td>
</tr>
<tr>
<td></td>
<td>T3a</td>
<td>Microscopic perivesical invasion</td>
</tr>
<tr>
<td></td>
<td>T3b</td>
<td>Macroscopic perivesical invasion</td>
</tr>
<tr>
<td></td>
<td>T4</td>
<td>Prostate, uterus, vagina, pelvic/adominal walls</td>
</tr>
<tr>
<td></td>
<td>T4a</td>
<td>prostate, uterus, vagina</td>
</tr>
<tr>
<td></td>
<td>T4b</td>
<td>pelvic/abdominal wall</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Metastases</th>
<th>Regional Lymph Nodes (N)</th>
<th>Extent</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N0</td>
<td>None</td>
</tr>
<tr>
<td></td>
<td>N1</td>
<td>Single lymph node (≤2 cm)</td>
</tr>
<tr>
<td></td>
<td>N2</td>
<td>Single lymph node (&gt;2 and ≤5 cm) Multiple lymph nodes (≤5 cm)</td>
</tr>
<tr>
<td></td>
<td>N3</td>
<td>&gt;5 cm in any number of lymph nodes</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Distant Metastasis (M)</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>M0</td>
<td>None</td>
</tr>
<tr>
<td>M1</td>
<td>Present</td>
</tr>
</tbody>
</table>
Table 1.3. Frequent genomic aberrations observed in human urothelial carcinoma and their associated prevalence among patients with UC at various tumor stages/grades.

<table>
<thead>
<tr>
<th>Aberrant Genomic Region</th>
<th>Tumor Stage/Grade</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>pTa G1-2</td>
<td>pTa G3</td>
</tr>
<tr>
<td>LOSSES</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1p</td>
<td>50%</td>
<td></td>
</tr>
<tr>
<td>2p</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2q</td>
<td>5%</td>
<td>39%</td>
</tr>
<tr>
<td>3p</td>
<td>6%</td>
<td>5%</td>
</tr>
<tr>
<td>4p</td>
<td>5%</td>
<td>22%</td>
</tr>
<tr>
<td>4q</td>
<td>10%</td>
<td>17%</td>
</tr>
<tr>
<td>5q</td>
<td>20%</td>
<td>33%</td>
</tr>
<tr>
<td>6q</td>
<td>16%</td>
<td>33%</td>
</tr>
<tr>
<td>8p</td>
<td>19%</td>
<td>28%</td>
</tr>
<tr>
<td>9p</td>
<td>45%</td>
<td>45%</td>
</tr>
<tr>
<td>9q</td>
<td>45%</td>
<td>38%</td>
</tr>
<tr>
<td>10q</td>
<td>9%</td>
<td>28%</td>
</tr>
<tr>
<td>11p</td>
<td>16%</td>
<td>17%</td>
</tr>
<tr>
<td>11q</td>
<td>6%</td>
<td>23%</td>
</tr>
<tr>
<td>13q</td>
<td>20%</td>
<td>17%</td>
</tr>
<tr>
<td>14q</td>
<td>9%</td>
<td>70%</td>
</tr>
<tr>
<td>17p</td>
<td>6%</td>
<td>11%</td>
</tr>
<tr>
<td>18q</td>
<td>10%</td>
<td>39%</td>
</tr>
<tr>
<td>Y</td>
<td>40%</td>
<td></td>
</tr>
<tr>
<td>GAINS</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1q</td>
<td>13%</td>
<td>17%</td>
</tr>
<tr>
<td>2p</td>
<td></td>
<td>5%</td>
</tr>
<tr>
<td>3q</td>
<td>1%</td>
<td>5%</td>
</tr>
<tr>
<td>5p</td>
<td>3%</td>
<td>28%</td>
</tr>
<tr>
<td>6p</td>
<td>5%</td>
<td>11%</td>
</tr>
<tr>
<td>7p</td>
<td>19%</td>
<td>5%</td>
</tr>
<tr>
<td>8q</td>
<td>10%</td>
<td>22%</td>
</tr>
<tr>
<td>10p</td>
<td>3%</td>
<td>5%</td>
</tr>
<tr>
<td>11q</td>
<td>25%</td>
<td></td>
</tr>
<tr>
<td>12p</td>
<td>1%</td>
<td>5%</td>
</tr>
<tr>
<td>12q</td>
<td>15%</td>
<td>5%</td>
</tr>
<tr>
<td>17q</td>
<td>30%</td>
<td>33%</td>
</tr>
<tr>
<td>20q</td>
<td>15%</td>
<td>33%</td>
</tr>
</tbody>
</table>
Table 1.4. Comparison of demographical, clinical, molecular, and prognostic features of canine and human urothelial carcinoma.

(Adapted from Urologic Oncology, 5(2), Knapp et al., Naturally-occurring canine transitional cell carcinoma of the urinary bladder: A relevant model of human invasive bladder cancer, 47-59, Copyright 2000, with permission from Elsevier.)

<table>
<thead>
<tr>
<th>Demographics</th>
<th>Canine UC</th>
<th>Human UC</th>
</tr>
</thead>
<tbody>
<tr>
<td>% of all cancers</td>
<td>1.5–2</td>
<td>2</td>
</tr>
<tr>
<td>Age at diagnosis</td>
<td>11 years (=60 human years)</td>
<td>65 years</td>
</tr>
<tr>
<td>Male:female ratio</td>
<td>1:2</td>
<td>2.8:1</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Risk factors</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Environment</td>
<td>Urban-associated</td>
<td></td>
</tr>
<tr>
<td>Carcinogens</td>
<td>Cyclic compounds</td>
<td>Cyclic compounds, cigarette smoking, industrial chemicals</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Clinical Features of UC</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Clinical signs</td>
<td>Hematuria, dysuria, urinary tract infection</td>
<td>Hematuria, urinary tract infection</td>
</tr>
<tr>
<td>Tumor Location</td>
<td>Trigone, most commonly</td>
<td>Lateral wall, most commonly</td>
</tr>
<tr>
<td>% Invasive</td>
<td>&gt;90</td>
<td>20</td>
</tr>
<tr>
<td>% Metastasis at diagnosis</td>
<td>20</td>
<td>10</td>
</tr>
<tr>
<td>Common sites of metastasis</td>
<td>Regional nodes and lung</td>
<td></td>
</tr>
<tr>
<td>Treatment of choice</td>
<td>Mitoxantrone +/- Piroxicam</td>
<td>Surgical resection</td>
</tr>
<tr>
<td>% Response to cisplatin</td>
<td>12–20</td>
<td>17–34</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Molecular Features of UC</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Urine bFGF concentration</td>
<td></td>
<td>Increased</td>
</tr>
<tr>
<td>Survivin Expression</td>
<td>Increased</td>
<td></td>
</tr>
<tr>
<td>EGFR Expression</td>
<td>Increased</td>
<td></td>
</tr>
<tr>
<td>COX2 Expression</td>
<td>Increased</td>
<td></td>
</tr>
<tr>
<td>Mutated p53</td>
<td>Yes</td>
<td></td>
</tr>
<tr>
<td>Recurrent Chromosome Aberrations</td>
<td>Unknown</td>
<td>-1p, +1q, -2p, -3p, -4p, -5q, +5p, -6q, +7, -8p, +8q, -9, -10, -11p, -15, -16, -17p, -18, -22p</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Prognostic factors</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>TNM stage</td>
<td>Increased TNM=decreased survival</td>
<td></td>
</tr>
<tr>
<td>Aneuploidy</td>
<td>No association</td>
<td>Negatively associated</td>
</tr>
</tbody>
</table>
Table 1.5. *World Health Organization (WHO) accepted staging guidelines for canine UC (1980) according to the extent of invasion and regional and distant metastasis.*


<table>
<thead>
<tr>
<th>Primary Tumor</th>
<th>Extent</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tumor Invasion (T)</td>
<td></td>
</tr>
<tr>
<td>Tis</td>
<td>Flat, non-invasive (in situ)</td>
</tr>
<tr>
<td>T1</td>
<td>Papillary, non-invasive</td>
</tr>
<tr>
<td>T2</td>
<td>Bladder Wall (submuscosa, muscularis, and/or perivesical tissue)</td>
</tr>
<tr>
<td>T3</td>
<td>Neighboring organs</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Metastases</th>
<th>Extent</th>
</tr>
</thead>
<tbody>
<tr>
<td>Regional Lymph Nodes (N)</td>
<td></td>
</tr>
<tr>
<td>N0</td>
<td>None</td>
</tr>
<tr>
<td>N1</td>
<td>Regional (internal/external iliac LN)</td>
</tr>
<tr>
<td>N2</td>
<td>Regional + Juxtaregional</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Distant Metastasis (M)</th>
<th>Extent</th>
</tr>
</thead>
<tbody>
<tr>
<td>M0</td>
<td>None</td>
</tr>
<tr>
<td>M1</td>
<td>Present</td>
</tr>
</tbody>
</table>
Figure 1.1. *Tumor staging is based on the extent of primary tumor invasion and metastasis.*

Excisional biopsy of urothelial tumors allows appropriate tumor staging. Tumor staging refers to the extent (or lack) of local invasion of the primary tumor throughout the body. Tis (CIS) and Ta lesions are the most superficial, localized to the urothelium. Stages T1-T4 are progressively invasive, with T4 stage tumors invading adjacent tissues and organs, including the uterus or prostate, among other tissues. Reprinted by permission from Macmillan Publishers Ltd: Nature Reviews Cancer, Knowles & Hurst, Molecular biology of bladder cancer: new insights into pathogenesis and clinical diversity, 15(1): 25-41, copyright 2015.
Loss of chromosome 9q is among the initiating genomic changes in tumor development. Hyperplastic lesions are typically less aggressive and progress to low grade papillary tumors characterized by FGFR3 mutations and the propensity to recur. Dysplastic lesions arise from TP53 and RB1 mutations, leading to high grade carcinomas in situ (CIS) characterized by loss of 9q. Upon acquiring a gain of chromosome 8q, CIS lesions undergo an epithelial-mesenchymal transition, invade the submucosa and muscularis, and likely metastasize (>50% of patients; Klatte et al., 2012). If papillary tumors receive a “second hit” as a TP53 mutation, they acquire invasive ability. Blue denotes prognostically favorable tumor stages, while black denotes those that are prognostically poor. Dashed lines represent possible yet less frequent pathways of tumor evolution.
Figure 1.3. Major cell signaling pathways involved in the development of urothelial carcinoma.

Pathways associated with constitutive activation of RAS are believed to be at the crux of UC development, most notably via activation of AKT. Various causes of constitutively active RAS have been documented in UC, including FGFR3 mutation and constitutive activation and overexpression of FGFR3 ligands, including bFGF. Concurrent activation of the AKT and MAPK pathways leads to a plethora of oncogenic cellular effects, including cell cycle progression, cell growth, and apoptosis inhibition. Red boxes represent inhibitor molecules, while gold represent activating.
CHAPTER 2: CANINE UROTHELIAL CARCINOMA: GENOMICALLY ABERRANT AND COMPARATIVELY RELEVANT

Canine urothelial carcinoma: genomically aberrant and comparatively relevant

S. G. Shapiro • S. Raghunath • C. Williams • A. A. Motsinger-Reif • J. M. Cullen • T. Liu • D. Albertson • M. Ruvolo • A. Bergstrom Lucas • J. Jin • D. W. Knapp • J. D. Schiffman • M. Breen

Received: 19 January 2015 / Revised: 7 February 2015 / Accepted: 10 February 2015
© Springer Science+Business Media Dordrecht 2015

Abstract Urothelial carcinoma (UC), also referred to as transitional cell carcinoma (TCC), is the most common bladder malignancy in both human and canine populations. In human UC, numerous studies have demonstrated the prevalence of chromosomal imbalances. Although the histopathology of the disease is similar in both species, studies evaluating the genomic profile of canine UC are lacking, limiting the discovery of key comparative molecular markers associated with driving UC pathogenesis. In the present study, we evaluated 31 primary canine UC biopsies by oligonucleotide array comparative genomic hybridization (aCGH). Results highlighted the presence of three highly recurrent numerical aberrations: gain of dog chromosome (CFA) 13 and 36 and loss of CFA 19. Regional gains of CFA 13 and 36 were present in 97 % and 84 % of cases,

Electronic supplementary material The online version of this article (doi:10.1007/s10577-015-9471-y) contains supplementary material, which is available to authorized users.

S. G. Shapiro • S. Raghunath • C. Williams • M. Breen
Department of Molecular Biomedical Sciences, College of Veterinary Medicine, North Carolina State University, 1060 William Moore Drive, Raleigh, NC 27607, USA
E-mail: Matthew_Breen@ncsu.edu

S. Raghunath • J. D. Schiffman
Department of Pediatrics and Huntsman Cancer Institute, University of Utah, 2000 Circle of Hope, Salt Lake City, UT 84112, USA

A. A. Motsinger-Reif
Department of Statistics, College of Sciences, North Carolina State University, Raleigh, NC, USA

J. M. Cullen
Department of Population Health and Pathobiology, College of Veterinary Medicine, North Carolina State University, Raleigh, NC, USA

T. Liu • D. Albertson
Anatomic Pathology Division Department of Pathology, University of Utah, 1950 Circle of Hope, RM N3105, Salt Lake City, UT 84112, USA

M. Ruvolo • A. Bergstrom Lucas • J. Jin
Agilent Technologies, 5301 Stevens Creek Blvd., Santa Clara, CA 95051, USA

D. W. Knapp
Department of Veterinary Clinical Sciences, Purdue University, School of Veterinary Medicine, West Lafayette, IN, USA

A. A. Motsinger-Reif • J. M. Cullen • M. Breen
Center for Comparative Medicine and Translational Research, North Carolina State University, Raleigh, NC, USA

M. Breen
Center for Human Health and the Environment, North Carolina State University, Raleigh, NC, USA

M. Breen
Limbacher Comprehensive Cancer Center, University of North Carolina, Chapel Hill, NC, USA

Published online: 18 March 2015
respectively, and losses on CFA 19 were present in 77% of cases. Fluorescence in situ hybridization (FISH), using targeted bacterial artificial chromosome (BAC) clones and custom Agilent SureFISH probes, was performed to detect and quantify these regions in paraffin-embedded biopsy sections and urine-derived urothelial cells. The data indicate that these three aberrations are potentially diagnostic of UC. Comparison of our canine oatCGH data with that of 285 human cases identified a series of shared copy number aberrations. Using an informatics approach to interrogate the frequency of copy number aberrations across both species, we identified those that had the highest joint probability of association with UC. The most significant joint region contained the gene PABPC1, which should be considered further for its role in UC progression. In addition, cross-species filtering of genome-wide copy number data highlighted several genes as high-profile candidates for further analysis, including CDKN2A, S100A8/9, and LRPIB. We propose that these common aberrations are indicative of an evolutionarily conserved mechanism of pathogenesis and harbor genes key to urothelial neoplasia, warranting investigation for diagnostic, prognostic, and therapeutic applications.

**Keywords** Canine · Urothelial carcinoma · Transitional cell carcinoma · Cytogenetics · Chromosome aberration · Array comparative genomic hybridization · Comparative oncology

**Abbreviations**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>AMADID</td>
<td>Agilent MicroArray Design Identifier</td>
</tr>
<tr>
<td>BAC</td>
<td>Bacterial artificial chromosome</td>
</tr>
<tr>
<td>CDKN2A</td>
<td>Cyclin-dependent kinase inhibitor 2A</td>
</tr>
<tr>
<td>CFA</td>
<td><em>Canis familiaris</em> (also used as a prefix to canine chromosome numbers)</td>
</tr>
<tr>
<td>CNA</td>
<td>Copy number aberration</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>ECCS</td>
<td>Evolutionarily conserved chromosome segment</td>
</tr>
<tr>
<td>FASST2</td>
<td>Fast Adaptive States Segmentation Technique 2</td>
</tr>
<tr>
<td>FFPE</td>
<td>Formalin-fixed paraffin embedded</td>
</tr>
<tr>
<td>FISH</td>
<td>Fluorescence in situ hybridization</td>
</tr>
<tr>
<td>GO</td>
<td>Gene ontology</td>
</tr>
<tr>
<td>H&amp;E</td>
<td>Hematoxylin and eosin</td>
</tr>
<tr>
<td>HSA</td>
<td><em>Homo sapiens</em> (also used as a prefix to human chromosome numbers)</td>
</tr>
<tr>
<td>oatCGH</td>
<td>Oligo-array comparative genomic hybridization</td>
</tr>
</tbody>
</table>

**Introduction**

Urothelial carcinoma (UC) of the bladder, also known as transitional cell carcinoma (TCC), is the second most common human urogenital cancer, surpassed only by prostate cancer. In 2014, an estimated 74,690 UC diagnoses were made in the USA alone, comprising 4–5% of all human cancer diagnoses (American Cancer Society 2014). Similarly, UC is the most common urogenital cancer of dogs, representing 1.5–2% of canine cancer diagnoses annually in the USA (Mutsaers et al., 2003; Oliveira et al., 2014). At diagnosis, approximately 20% of canine and 10% of human patients have overt metastasis, with microscopic metastases likely present in many more cases (Knapp et al., 2000; Oliveira et al., 2014). Due to the high metastatic rate and, particularly in canines, delayed diagnosis, the identification of potential early diagnostic indicators, including genomic changes, is highly desirable.

Previous studies have established that canine UC resembles and behaves similarly to invasive human UC, providing a beneficial in vivo model for the disease (Knapp et al., 2000; Dhawan et al., 2009). The canine cancer model is an optimal one for human cancers: people and their pets share an environment, have similar relative life spans, and develop the disease naturally (Shearin and Ostrander, 2010). Additionally, bottlenecks in the domestic dog population due to tightly controlled breeding have created breeds of dogs with high genetic similarity and resultant disease predispositions (Dobson, 2013). Scottish Terriers are 20 times more likely to develop UC than the average dog (Knapp et al., 2014). Other breeds with high incidence of UC include Shetland Sheepdogs, Beagles, West Highland White Terriers, and Wire Hair Fox Terriers, who are three to five times more likely to develop UC than the general dog population. The overwhelming breed predispositions associated with canine UC suggest a strong genetic component to disease development. Interspecies
chromosome synteny allows us to capitalize on the unique genetic homogeneity of purebred dogs to elucidate more specific, evolutionarily conserved, and relevant genomic aberrations in humans, regions that may prove clinically relevant to both dogs and people.

In the current study, we evaluated 31 primary canine UC tumor biopsies via genome-wide oligonucleotide array comparative genomic hybridization (oaCGH). Validation of CGH data was performed using formalin fixed paraffin-embedded (FFPE) UC biopsies (n=5) and urine sediments (n=24) via fluorescence in situ hybridization (FISH). A hematoxylin and eosin (H&E)-stained slide of each tumor biopsy was evaluated and staged in parallel by human and veterinary pathologists. Canine oaCGH data were compared with those derived from 285 UCs, revealing striking similarities of copy number gains and losses shared between species. Additionally, we describe a high-specificity FISH-based method for canine UC diagnosis utilizing low-volume, free-catch urine specimens. These data suggest: (1) the diagnostic relevance of genomic aberrations in veterinary medicine, (2) a non-invasive method of canine UC diagnosis, and (3) the canine model is a valuable model in the study of human UC.

Pathologic evaluation of canine UC

H&E-stained slides previously used for canine UC diagnosis were reviewed by a board-certified veterinary pathologist (JC) and two human genitourinary pathologists (TL, DA). Slides were first scanned at low magnification to locate the neoplastic regions of the biopsy. Magnification was then increased to allow identification of specific histologic criteria in the neoplasms. The urothelial basement membrane was evaluated for evidence of invasion, as shown by a clear breach in basement membrane and invasion of the lamina propria of the bladder (stage T1). If no such invasion was observed, tumors were classified as either Ta (papillary carcinoma) or Tis (in situ “flat tumor”). When the biopsy included deeper layers of the bladder wall, the UC was graded on the basis of the extent of invasion of the bladder. UC that invaded the muscularis propria were graded as T2, and those that extended into the perivesical tissues were graded as T3. Pathologic staging was performed in accordance with the World Health Organization (WHO) and American Joint Committee on Cancer (AJCC) guidelines (American Joint Committee on Cancer 2002). The compiled pathologic data are included in Table 1.

Methods and materials
Canine UC case collection

Cystoscopy or cystotomy specimens were obtained from a series of chemotherapy-naïve, client-owned dogs presenting with suspected UC at the veterinary hospitals at Purdue University (PU) and North Carolina State University (NCSU). Each biopsy specimen was either snap frozen and subsequently stored in liquid nitrogen, or fixed in 10% neutral buffered formalin (NBF) for 24 h prior to embedding in paraffin. Histopathological evaluation confirmed a diagnosis of UC for all 31 specimens (Table 1). Urine samples (n=24) were collected via free catch (1–5 mL) from additional NCSU patients with confirmed UC (diagnosis via urine cytology or histopathology) and stored at 4 °C for a maximum of 24 h until further processing (see below). Control urothelium was collected from recently euthanized dogs at necropsy and confirmed to be non-neoplastic by histopathologic evaluation. All biopsy and urine specimens were obtained with informed client consent and under institutionally approved protocols at PU and NCSU.

Canine oaCGH

DNA was extracted from biopsy specimens using either the DNeasy Kit (frozen samples, Qiagen, Valencia, CA) or QI Amp DNA FFPE Tissue Kit (FFPE samples, Qiagen). DNA samples were verified to be of sufficient quantity and integrity by agarose gel electrophoresis (>1 kb) and spectrophotometry (260:280 and 260:230 both >1.8). Gender-specific DNA reference samples were generated from whole blood of healthy mixed-breed dogs, pooling equimolar quantities of DNA from ten males or ten females. DNA was labeled using the Genomic DNA Enzymatic Labeling Kit (Agilent, Santa Clara, CA), as described previously (Poorman et al. 2014; Thomas et al. 2014). Fluorescently labeled test and reference samples were hybridized to Canine G3 SurePrint 180,000 feature oaCGH arrays (Agilent, AMADID 025522) for 40 h, as described previously (Poorman et al. 2014; Thomas et al. 2014). Arrays were scanned at 3 μm (Agilent, Model G2505C); data were extracted using Feature Extraction v10.10 software (Agilent Technologies, Santa Clara, CA) and assessed for quality using the Quality Metrics report tool in
Table 1. Canine patient signalment and staging

<table>
<thead>
<tr>
<th>Case ID</th>
<th>Sample type (biopsy)</th>
<th>Breed</th>
<th>Sex</th>
<th>Vet stage</th>
<th>Human stage</th>
<th>Muscular invasion</th>
<th>Stage group</th>
</tr>
</thead>
<tbody>
<tr>
<td>TCC7</td>
<td>Frozen</td>
<td>Scottish Terrier</td>
<td>FS</td>
<td>Carcinoma</td>
<td>pTis</td>
<td>N</td>
<td>A</td>
</tr>
<tr>
<td>TCC8</td>
<td>Frozen</td>
<td>Boxer</td>
<td>MC</td>
<td>pT1</td>
<td>pT1</td>
<td>N</td>
<td>A</td>
</tr>
<tr>
<td>TCC9</td>
<td>Frozen</td>
<td>Welsh Corgi</td>
<td>MC</td>
<td>Carcinoma</td>
<td>pT1</td>
<td>N</td>
<td>A</td>
</tr>
<tr>
<td>TCC10</td>
<td>Frozen</td>
<td>Mixed Breed</td>
<td>FS</td>
<td>Carcinoma</td>
<td>pT1</td>
<td>N</td>
<td>A</td>
</tr>
<tr>
<td>TCC11</td>
<td>Frozen</td>
<td>Treeing Walker Coonhound</td>
<td>FS</td>
<td>Carcinoma</td>
<td>pTis</td>
<td>N</td>
<td>A</td>
</tr>
<tr>
<td>TCC12</td>
<td>Frozen</td>
<td>Beagle</td>
<td>MC</td>
<td>pT1</td>
<td>pT1</td>
<td>N</td>
<td>A</td>
</tr>
<tr>
<td>TCC13</td>
<td>Frozen</td>
<td>Miniature Schnauzer</td>
<td>FS</td>
<td>Carcinoma</td>
<td>pT1</td>
<td>N</td>
<td>A</td>
</tr>
<tr>
<td>TCC14</td>
<td>Frozen</td>
<td>Collie</td>
<td>FS</td>
<td>Carcinoma</td>
<td>pT1</td>
<td>N</td>
<td>A</td>
</tr>
<tr>
<td>TCC16</td>
<td>Frozen</td>
<td>Fox Terrier</td>
<td>FS</td>
<td>Carcinoma</td>
<td>pTis</td>
<td>N</td>
<td>A</td>
</tr>
<tr>
<td>TCC17</td>
<td>Frozen</td>
<td>Siberian Husky</td>
<td>MC</td>
<td>pT1</td>
<td>pT1</td>
<td>N</td>
<td>A</td>
</tr>
<tr>
<td>TCC18</td>
<td>Frozen</td>
<td>Dachshund</td>
<td>FS</td>
<td>Carcinoma</td>
<td>pTis</td>
<td>N</td>
<td>A</td>
</tr>
<tr>
<td>TCC19</td>
<td>Frozen</td>
<td>Labrador Retriever</td>
<td>MC</td>
<td>Carcinoma</td>
<td>pT1</td>
<td>N</td>
<td>A</td>
</tr>
<tr>
<td>TCC20</td>
<td>Frozen</td>
<td>Jack Russell Terrier</td>
<td>FS</td>
<td>Carcinoma</td>
<td>pTis</td>
<td>N</td>
<td>A</td>
</tr>
<tr>
<td>TCC26</td>
<td>Frozen</td>
<td>Beagle</td>
<td>FS</td>
<td>pT1</td>
<td>pT1</td>
<td>N</td>
<td>A</td>
</tr>
<tr>
<td>TCC28</td>
<td>Frozen</td>
<td>Beagle</td>
<td>FS</td>
<td>Carcinoma</td>
<td>pT1</td>
<td>N</td>
<td>A</td>
</tr>
<tr>
<td>TCC33</td>
<td>FFPE</td>
<td>Scottish Terrier</td>
<td>FS</td>
<td>pT3</td>
<td>pT2</td>
<td>Y</td>
<td>B</td>
</tr>
<tr>
<td>TCC34</td>
<td>FFPE</td>
<td>West Highland Terrier</td>
<td>FS</td>
<td>pTis</td>
<td>pTis</td>
<td>N</td>
<td>A</td>
</tr>
<tr>
<td>TCC35</td>
<td>FFPE</td>
<td>Dalmation</td>
<td>FS</td>
<td>pT1</td>
<td>pT1</td>
<td>N</td>
<td>A</td>
</tr>
<tr>
<td>TCC36</td>
<td>FFPE</td>
<td>German Shepherd</td>
<td>MC</td>
<td>pT2</td>
<td>pT2</td>
<td>Y</td>
<td>B</td>
</tr>
<tr>
<td>TCC38</td>
<td>FFPE</td>
<td>Dachshund</td>
<td>MC</td>
<td>pT3</td>
<td>pT3</td>
<td>Y</td>
<td>B</td>
</tr>
<tr>
<td>TCC39</td>
<td>FFPE</td>
<td>Keeshond</td>
<td>MC</td>
<td>pT2</td>
<td>pT2</td>
<td>Y</td>
<td>B</td>
</tr>
<tr>
<td>TCC40</td>
<td>FFPE</td>
<td>Doberman Pinscher</td>
<td>FS</td>
<td>pT2</td>
<td>pT2</td>
<td>Y</td>
<td>B</td>
</tr>
<tr>
<td>TCC42</td>
<td>FFPE</td>
<td>Dachshund</td>
<td>FS</td>
<td>pT2</td>
<td>pT2</td>
<td>Y</td>
<td>B</td>
</tr>
<tr>
<td>TCC43</td>
<td>FFPE</td>
<td>Hound</td>
<td>FS</td>
<td>pT1</td>
<td>pT1</td>
<td>N</td>
<td>A</td>
</tr>
<tr>
<td>TCC44</td>
<td>FFPE</td>
<td>Corgi</td>
<td>FS</td>
<td>&gt;pT1</td>
<td>&gt;pT1</td>
<td>N</td>
<td>A</td>
</tr>
<tr>
<td>TCC45</td>
<td>FFPE</td>
<td>Labrador Retriever</td>
<td>FS</td>
<td>pT1</td>
<td>&gt;pT1</td>
<td>N</td>
<td>A</td>
</tr>
<tr>
<td>TCC46</td>
<td>FFPE</td>
<td>Miniature Schnauzer</td>
<td>FS</td>
<td>pT2</td>
<td>pT3</td>
<td>Y</td>
<td>B</td>
</tr>
<tr>
<td>TCC47</td>
<td>FFPE</td>
<td>Unknown</td>
<td>FS</td>
<td>pTis</td>
<td>&gt;pTis</td>
<td>N</td>
<td>A</td>
</tr>
<tr>
<td>TCC48</td>
<td>FFPE</td>
<td>Unknown</td>
<td>FS</td>
<td>Carcinoma</td>
<td>pT1</td>
<td>N</td>
<td>A</td>
</tr>
<tr>
<td>TCC49</td>
<td>FFPE</td>
<td>Shetland Sheepdog</td>
<td>FS</td>
<td>pT1</td>
<td>At least pT1</td>
<td>N</td>
<td>A</td>
</tr>
<tr>
<td>TCC50</td>
<td>FFPE</td>
<td>Basenji</td>
<td>FS</td>
<td>Carcinoma</td>
<td>pTis</td>
<td>N</td>
<td>A</td>
</tr>
</tbody>
</table>

Thirty-one primary UC cases were analyzed in this study. The case identifier, sample type (frozen or FFPE biopsy), breed, and sex are noted for each patient (F female, M male, S spayed, C castrated). Stage, as determined by veterinary and human pathologists, is indicated, along with presence of absence of muscle invasion and the consequent analysis group (A vs. B).

Agilent’s Feature extraction software (v10.5) (Agilent Technologies). Raw data were evaluated to identify and exclude probes displaying non-uniform hybridization or signal saturation and imported into Nexus copy number v7.5 (BioDiscovery, Hawthorne, CA) for analysis. Copy number calls were made using the FASST2 segmentation algorithm with a significance threshold of 5.05⁻⁶. Aberrations were defined as a minimum of three consecutive probes with log2 tumor: reference value of >1.14 (high gain), 1.13 to 0.201 (gain), -0.234 to -1.1 (loss), and ≤-1.1 (big loss).

Selection of canine diagnostic regions

Selection of canine diagnostic regions

Genome-wide oCGH data for the canine cohort were evaluated to identify chromosomal regions that exhibited the highest penetrance of aberrations. To develop a DNA copy number-based assay for the detection of...
neoplastic epithelial transitional cells shed in the urine, three regions with the highest frequency of unidirectional aberration in neoplastic biopsies were identified and compared with qPCR data from a cohort of 100 nonneoplastic DNA specimens. Aberrant regions were classified as gains, losses, or neutral for the purposes of classification. Potential predictive model(s) were built using the classification tree algorithm J48, as implemented in Weka c5.7 (Hall et al. 2009). The J48 algorithm was implemented both with and without fivefold cross-validation, in order to assess the potential predictive performance. In both cases, the same model (in terms of variables selected and tree architecture) resulted, so only the model without cross-validation is presented here (for simplicity). Several measures of model fit were calculated, including the relative risk, odds ratio, sensitivity, specificity, and overall misclassification rate for each of the three regions.

**Fluorescence in situ hybridization of canine FFPE biopsies using BAC and custom SureFISH probes**

To validate qPCR data, regions on CFA 13, 19, and 36 with >75% aberration frequency among patients and one balanced region on CFA 8 (qPCR Log2=0 in 100% of patients) were selected for FISH analysis. Differentially labeled FISH probes for each of these four regions were generated using DNA isolated from canine bacterial artificial chromosome (BAC) clones of the CHORI-82 canine BAC library (http://bacpac.chori.org/library.php?id=253) (Table 2). In addition, six-silico-selected sequences from non-repetitive canine genomic sequences (canfam2) were selected for the three unbalanced regions (segments of on CFA 13, 19, and 36) (Table 2) and submitted for massively parallel de novo synthesis and generation of custom SureFISH probes (Agilent Technologies), labeled with one of the three current color options (red, green, or aqua). Each FISH probe was first assessed to verify a unique cytogenetic location when hybridized to canine metaphase spreads and interphase nuclei from healthy dogs using protocols reported previously for BACs (Poorman et al. 2014) and per the manufacturer’s recommendations for SureFISH probes (Agilent Technologies).

FISH was performed on 5 μm sections of FFPE biopsy specimens from healthy canine urothelium and from pathologically confirmed cases of UC, using protocols reported previously (Poorman et al. 2014; Thomas et al. 2014), with modifications. After deparaffinization with xylene treatment, slides were exposed to Hyaluronidase VIII (45 U/μL in 250 mM TRIS-buffered saline, pH 7.4, Sigma-Aldrich, St. Louis, MO) at 37 °C for 1 h, followed by a combined collagenase treatment (236 U/μL each of collagenases I and II, and 0.4 U/μL of collagenase III (Invitrogen/Life Technologies, Carlsbad) in HBSS with 2.4 mM CaCl2 and pretreatment in 80 °C sodium thiocyanate for 60 min. Slides were rinsed in MQ water, briefly dried, and then immersed in Abbott Protease II solution (Abbott Laboratories, Chicago, IL) for 45 min at 37 °C. Pretreated slides were dehydrated through an ethanol series (70, 90, 100 %), and fluorescent probe (100 ng of each BAC or SureFISH probe) in hybridization solution was added. Probes and DNA were co-denatured at 80 °C for 5 min, followed by overnight hybridization at 37 °C. All hybridization steps and post-hybridization washes and imaging were as described previously (Thomas et al. 2014). For each specimen, probes were visualized by multiplane fluorescence microscopy, using a BioView imaging system built around an Olympus BX61. Probe signals were enumerated in >50 cells, each classified as aberrant or not aberrant, and mean copy number per cell also obtained.

**FISH of urine sediment**

Free-catch urine was mixed with an equal volume of 1× phosphate-buffered saline (PBS) and exfoliated cells concentrated by centrifugation (1500 RPM). Urine sediment was rinsed in 10 mL 1× PBS. Following centrifugation, supernatant was aspirated to approximately 1 mL, the cells resuspended and fixed three times in 3:1 methanol/glacial acetic acid. The concentrated fixed cell suspension was spotted onto a glass slide for FISH analysis. Slides were dehydrated in an ethanol series (70/90/100 %) prior to being heated for 2 min at 65 °C, transferred to a 37 °C slide moist, and treated with pepsi solution for enzyme-induced epitope retrieval (Thermo Fisher Scientific, Cheshire, UK) for 30 s to eliminate signal background due to protein. Slides were rinsed twice in water and then fixed in 3:1 methanol/glacial acetic acid for 5 min, rinsed twice in water, and passed through a second dehydration series. Hybridization, washes, and imaging were as for the FFPE FISH.
Table 2. Single-locus probes used to represent the regions of CFA 13, 19, and 36 with the highest recurrence of copy number aberration in canine UC.

<table>
<thead>
<tr>
<th>Chromosome</th>
<th>BAC probes</th>
<th>大小 (bp)</th>
<th>Sure FISH probes</th>
</tr>
</thead>
<tbody>
<tr>
<td>8</td>
<td>122F23</td>
<td>7,984,903</td>
<td>8,129,058</td>
</tr>
<tr>
<td>13</td>
<td>328P06</td>
<td>8,630,922</td>
<td>8,818,498</td>
</tr>
<tr>
<td>19</td>
<td>122M01</td>
<td>25,119,452</td>
<td>25,264,995</td>
</tr>
<tr>
<td>36</td>
<td>122F03</td>
<td>23,199,386</td>
<td>23,269,766</td>
</tr>
</tbody>
</table>

Clones were selected from the CHORI-82 canine BAC library (www.bacpac.chori.org/library.php?id=253) to represent all three aberrant regions as well as a neutral region on CFA 8. The BAC addresses and clone detail are indicated. Custom SureFISH probes representing overlapping regions of the genome targeted by the BACs were generated in collaboration with Agilent Technologies (Santa Clara, CA) and the details indicated

Human oCGH data

oCGH-segmented data from 205 human urothelial tumors were accessed via the Cancer Genome Atlas (TCGA). Affymetrix Oncoscan FFPE Express Platform (Santa Clara, CA) data for an additional 80 cases were downloaded from Gene Expression Omnibus (GEO), GSE44323 (Karolchik et al. 2004; Chekaluk et al. 2013). Both data sets were imported and merged into a single project using Nexus copy number v7.5 (BioDiscovery, CA).

Isolation of regions of overlapping copy number aberration

For isolating regions of CNA shared between human and dog, two input files were created for each species: a reference file and a file containing aberration call data. The reference file contained syntenic regions of canine and human chromosomes, where each syntenic region was indexed using a “link ID” to identify the region throughout aberration analysis. The intersect option in Bedtools was used to first find intersecting regions between the aberration call data and the reference file for both the dog and human, using a threshold of 1 Mb (Quinlan and Hall 2010). The output was a list of overlapping aberrant regions between the canine and human data sets as mapped to the reference file. These aberrant regions were then matched to the respective link IDs between the two organisms, yielding a set of syntenic genomic regions sharing copy number loss or gain. Finally, overlapping link IDs were mapped to chromosomal regions with aberration calls, and the frequency of the specific aberration per species was evident in >5 % of the cohort. The TCGA data set was not associated with raw data values and so excluded from this component of the analysis. Regions with contiguous CNAs were selected, and the probe median values for each region calculated. Overlap analysis of the raw data sets was then performed to isolate regions of sequence similarity that have shared aberrations between canine and human data sets. The probe median values for each aggregate region for human and canine data were calculated and used to visualize the data as concentric Circos plots.

Humanization of canine oCGH data for comparative analysis

Canine oCGH data were recoded into “virtual” human genome format as described previously (Thomas et al. 2011; Poomman et al. 2014). Using these recoded coordinates, the tumor/reference signal intensity data for each canine data set were reprocessed to output the oCGH profile according to their virtual human chromosome locations. All human and humanized canine data were imported into a single experiment in Nexus copy number v7.5 to allow a direct comparative analysis.

Generation of call data and probe median values for comparative assessment

Raw data (log2 ratios) for the 80 human oCGH profiles deposited in GEO and the 31 canine profiles generated in the current study were analyzed separately to generate species-specific lists of aggregate genome regions where DNA copy number aberrations (CNAs) were
calculated. Output data from the final step were merged to create a single file referenced by the link ID. Following this unsupervised analysis, the process was repeated to target seven a priori human bladder cancer-associated genes: *PPARG*, *EGFR*, *CCND1*, *MDM2*, *E2F3*, *CDKN2A*, and *RBI* (The Cancer Genome Atlas Research Network 2014), to provide verification that these genes also play a role in canine UC and suggest a shared pathogenesis.

Statistical validation of the gene list

Regions of conserved copy number aberrations between dog and human were extracted, ranked (using R) independently for each organism (based on the frequency) giving priority to regions of higher frequency, and validated by calculating the joint probability. For regions that tied for the same rank, the ties.method argument of R’s rank function was used (R Development Team 2010). After calculating the individual ranks for human and dog aberrations, the maximum rank between dogs and humans was calculated, focusing on regions ranked highest in both species. The probability of the ranks for both gains and losses for each organism was calculated and the joint probability derived by multiplying the probabilities across both species. Regions with a nominally significant joint probability (<0.05) were further assessed.

Comparative pathologic staging analysis

Comparative analysis was performed using all human (n=285) and canine (n=31) samples included in the genome-wide copy number analysis. Canine staging, performed as described above, resulted in two tumor groups; group A—Tis and T1, representing non-muscle invasive tumors, and group B—T2 and T3, representing muscle invasive tumors. Human cases were similarly grouped to allow a comparative analysis of the frequencies of CNAs identified across the two groups.

Results

Canine UC biopsies show recurrent DNA copy number aberrations

oaCGH data of DNA isolated from canine UC biopsy specimens revealed that all cases presented with numerous recurrent CNAs. Representative genome-wide profiles copy number are provided in Fig. 1. In all cases, the CNAs detected were primarily either whole chromosome or large segmental aneuploidy. In addition, there were one or more chromothripic-like events detected in 74% (23/31) of patients involving 21 chromosomes. A chromothripic-like event was defined as a cluster of sub-chromosomal regions showing alternating copy number gains and losses (Cai et al. 2014). An example of chromothripic events in a single patient is shown in Fig. 2. While most occurred sporadically throughout the genome, chromothripic-like events of CFA 36 were evident in almost 30% of the cohort, with involvement of CFA 10 and 16 at 16% and 13%, respectively, and CFA 4 and 7 approaching 10% (Fig. 3). Assessment of the evolutionarily conserved chromosome segments (ECCS) of these five chromosome segments in the human data indicated that while chromothripic-like events in the ECCS shared with CFA 36 were evident in only 1% of cases, such events involving genome regions conserved with CFA 4, 7, 10, and 16 were detected in 5–7% of cases.

FASST2-processed oaCGH data were compiled into two penetrance plots representing the frequencies of called aberrations detected among the DNA samples derived from the fresh frozen and fixed tissue specimens (data not shown). Evaluation of the two data sets using the “compare” tool of Nexus indicated a high degree of concordance of the specimen type; only six aberrations >100 kb in size differed significantly in their frequencies between the two sample types. The size of these six regions ranged from 103–330 kb (mean=200 kb) and totaled just 1.2 Mb of the genome. With a high degree of concordance between the two specimen types, a penetrance plot representing aberration frequencies of all 31 cases of canine UC was generated (Fig. 4).

The highest frequency CNAs (>75% of cases) involved large segmented regions of CFA 13 (gain), 19 (loss), and 36 (gain) (Fig. 4). A second tier of recurrent copy number changes (frequency >33%) was evident as copy number loss of regions of CFA 2, 5, 6, 10, 12, 26, 27, 28, and X and copy number gains involving regions of CFA 2, 4, 5, 6, 7, 10, 14, 17, 20, 23, 24, 30, 31, 35, 38, and X. A 1-Mb region located in CFA 8q12 represented a segment of the canine genome where no detectable copy changes were evident across the cohort and may thus be regarded as an indicator of copy number neutrality for assessment of ploidy status. Several highly penetrant gains or losses also exhibited subtle copy number changes in the opposing direction. To develop the most sensitive FISH-based assay designed to detect
and quantify single loci, regions for probe selection were therefore based on those with unidirectional CNAs. The peaks of unidirectional aberration on CFA 13, 19, and 36 had frequencies of 96.77% gain, 77.42% loss, and 83.87% gain, respectively (Table 3). Evaluation of individual cases indicated that a region of CFA 36 was the most notably aberrant with Log2 ratios approaching 5.0 in several cases, suggesting genomic amplification.

FISH analysis of FFPE tumor biopsies and urine sediment allows direct visualization of urothelial cell copy number status.

FISH analysis was performed in 5 μm sections of FFPE non-neoplastic (control) canine bladder specimens (n = 5). Currently, SureFISH probes are only available in three colors, limiting our analysis to only the aberrant target regions on CFA 13, 19, and 36. There was high concordance between data obtained from BAC and SureFISH probes; though the SureFISH probes were cleaner (had no visible background signal), had reduced variation and a marginally higher mean copy number (Fig. 5a). It is apparent from these data that the mean copy number of all probes tested was close to n = 2 but slightly elevated (n = 2.15–2.3). In most tissue types, the use of 5 μm sections results in degree of nuclear truncation, leading to a mean copy number of n < 2. The elevated mean values in bladder epithelium are to be expected due to the presence of binucleate and/or tetraploid transitional epithelial cells, common even in healthy urothelium (Sahrief et al. 1980).
Both probe sets were used to assess copy number status of the target loci in cells obtained from FFPE UC tumor biopsies (n=5) previously interrogated by oaCGH (Fig. 5b). In each case, the data confirmed copy number gain of CFA 13 and 36 and loss of CFA 19 as recurrent features, with subtle differences in enumeration between
Figure 2. Penetrance data of primary canine UC tumors reveal highly recurrent genomic alterations. a Penetrance plot of recurrent CNAs identified within the cohort of 31 canine UC cases. Genomic locations are plotted along the x-axis. The y-axis indicates the percentage of the corresponding cohort that demonstrated either copy number gain (shown in blue above the midline) or loss (shown in red below the midline) of the corresponding chromosome region. b Canine ideogram indicating regions of CNA. Blue and red shading to the right and left of each chromosome indicates the corresponding regions of the chromosomes exhibiting gain and loss, respectively. The horizontal size of the shading corresponds with aberration frequency in the population. In (a) and (b), the highest recurrent unidirectional changes are shown with arrows (black) on CFA 13, 19, and 36. The green arrowed region of CFA 8 was observed to exhibit a neutral copy number in all 31 cases and so represents a control region.

BAC and SureFISH probes. In individual cells, the loci on CFA 36 was often detected as >8 copies/cell, and the mean of all individual patient means was ~4.7. CFA 13 displayed more conservative copy number gains (mean = -3.72), and CFA 19 was present in one to three copies (mean = -2.2), depending on genome ploidy (Fig. 5b). The data from BAC and SureFISH probe sets were highly effective as a means to acquire enumeration data for all target loci. When used to probe transitional cells recovered from the urine sediment of UC patients (n=24), each case revealed the presence of one or more of the three target aberrations. The distribution of the mean copy numbers derived for each FISH probe along with the combined mean of all cases is presented in Fig. 5c, demonstrating the recurrent gain of CFA 13 and 36 and loss of CFA 19 detected by both BAC and SureFISH probes.

Copy number changes in canine UC—an aid to diagnosis

Analysis of DNA samples from pathologically verified non-neoplastic DNA samples (n=100) indicated normal copy number status (n=2) for each of CFA 13, 19, and 36. As such, the sensitivity, specificity, percent correctly classified, and the AUC values (with 95% CI) for each of the three regions were calculated, based on their aberration frequency in confirmed canine UC and the lack of detectable aberration in 100 non-neoplastic tissues. The measures of association and potential predictive performance calculated for each of these aberrations are presented in Table 4. To evaluate the potential predictive power of a multivariate model (using up to all three regions together, with gain and loss
information for all three regions included), a decision tree model was constructed. With or without cross-validation, the best tree only had a single variable included, copy number gain of CFA 13. This was the best model and adding the additional variables neither improved nor weakened the model. In addition, if two or more of these three aberrations are detected in cells from a urinary tract specimen, the sensitivity and specificity to indicate the presence of neoplasm is extremely high (>99%), based on the data set evaluated.

Canine and human urothelial tumors share copy number aberrations

The segmented canine oCGH data were humanized to allow direct gross genome-wide comparison of CNA penetrance with accessible human segmented data (Fig. 6). While the general directional pattern of CNA penetrance across the genomes of both species was similar, segment penetrance exceeding 33% was rarely seen across the large (n=285) human cohort, in line with
what has been reported previously (Karolchik et al. 2004; Chekaluk et al. 2013). However, the high
penetrance of the gains of CFA 13 and 36 and loss of CFA 19 in canine UC resulted in correspondingly high-

![Figure 2.6](image-url)

**Table 4** Summary statistics of the power of each of the three selected regions of CFA 13, 19, and 36 as predictors of the presence of canine UC.

<table>
<thead>
<tr>
<th>Measure</th>
<th>CFA 13</th>
<th>CFA19</th>
<th>CFA36</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Value</td>
<td>95% CI</td>
<td>Value</td>
</tr>
<tr>
<td>Relative risk</td>
<td>101</td>
<td>23.044</td>
<td>101</td>
</tr>
<tr>
<td>Sensitivity</td>
<td>0.968</td>
<td>0.873</td>
<td>0.968</td>
</tr>
<tr>
<td>Specificity</td>
<td>1</td>
<td>0.97</td>
<td>1</td>
</tr>
<tr>
<td>Misclassification rate</td>
<td>0.008</td>
<td>0.088</td>
<td>0.053</td>
</tr>
</tbody>
</table>

![Figure 2](image-url)

**Fig. 6** Visual comparison of “humanized” canine and human UC onCGH data. **a** Top, penetrance plot of 285 cases of human UC derived from two large public datasets: onCGH segmented data representing 285 human urothelial tumors produced by the Cancer Genome Atlas (TCGA) and an additional 80 cases of Affymetrix Oncoscan FFPE Expression Platform data downloaded from Gene Expression Omnibus (GEO), GSE44323 (Karolchik et al. 2004; Chekaluk et al. 2013). **Bottom,** the penetrance plot of human onCGH data from the 31 canine UC cases generated as part of the present study (canine data shown in Fig. 4a). **b** The high level of penetrance of DNA copy number gain of CFA 13 is reflected along segments of HSA 4 and 8 (ii, iii). The highly penetrant loss of CFA 19 corresponds to loss of regions of HSA 2 and 4 (i, ii), and the highly penetrant gain of CFA 36 corresponds to a large region of HSA 2 (i), immediately adjacent the region shared with CFA 19.
frequency regions of penetrance on HSA 2, 4, and 8 when presented in their pseudo-human format (Fig. 6b).

Copy number aberrations along HSA 2p are evident in cases of human UC at about 20 % loss and 4 % gain (Fig. 6b (i)). These events are consistent with changes observed in the canine genome. However, in HSA 2q, the extent of the CNAs in regions shared with the dog differs. Along much of the length of HSA 2q, the frequencies of copy number gain and loss are comparable (~10 %) until 2q32.2, distal to which there is a gradual decline in the frequency of gain and a concomitant increase in frequency of loss, resulting in a gain/loss frequency of 5/20 % by the telomeric end of HSA 2q. In the humanized canine data, the proximal region of 2q resembles that observed in human UC, but a 40-Mb region within 2q12–2q32.2 (HSA 2;114–154 Mb) is lost in 77 % of canine patients. Adjoining and immediately distal to this region (HSA 2q23.3–32.2) is a 36-Mb (HSA 2;154–190 Mb) span of copy number gain present in ~83 % of cases. This alternating CNA event spans 76 Mb of HSA 2q and is the result of shared conserved chromosome segments with the two highly penetrant CNAs we identified in canine UC: loss of CFA 19 (distal, 33 Mb of the chromosome) and gain of CFA 36.

Low-level CNAs along the length of HSA 4 impact the full length of the chromosome in human UC (~8 % gain and ~14 % loss), as is largely the case with the humanized canine data (Fig. 6b (ii)). However, the canine data demonstrate that the highly penetrant gain of CFA 13 is represented by a 97 % gain of HSA 4p14–q13.3 (4;41–75 Mb), while the loss observed at 4q26–q31.21 (4;121–144 Mb) is due to the highly penetrant loss of CFA 19 (proximal, 22 Mb of the chromosome).

In human UC, HSA 8p shows a DNA copy number loss in ~27 % spanning 8p23.3–8p12, which reduced to ~13 % within 8p12–8p11.1 (Fig. 6b (iii)). A subtle gain (4 %) along 8p was noted, with the exception of 8p12–8p11.1, where the frequency of gain increased to 17 %. A similar pattern was evident in the canine data. In human UC, 8q is subject to a gain in ~25 % of cases at 8q11.1, but this frequency increases along the length of the q-arm so that by 8q22.1, the gain is seen in 34 % of cases. The canine data demonstrate CNA frequencies of 10 % gain and 4 % loss across 8q11–8q22.1, but 97 % gain and 0 % loss between 8q22.1 and 8q24.3 (the final 40 Mb of 8q). This highly penetrant gain of 8q in humanized data is due to the conserved segment shared with CFA 13.

To quantify the data and facilitate direct comparison of aligned shared genomic CNAs, a higher resolution comparative assessment was conducted using informatics analysis of genomic sequence based on syntenic regions of both genomes. Gains and losses were assessed separately. Shared region lengths were generated from a data-driven approach, ensuring that the novel observations made were compelled by the actual data and not influenced by prior knowledge. The first step of the informatics analysis was to find intersecting regions between the aberration call data and the reference file for both the dog and human, using a threshold of 1 Mb. (Quinan and Hall 2010). During the subsequent steps of the informatics approach, the shared regions are systematically reduced in size to define those where aberrations are shared between canine and human samples. With this process, a total of 2729 shared copy number gains and 2057 shared copy number losses were identified. For both shared gains and losses, 19 and 22 %, respectively, had a joint-rank probability of ≤0.05. In both cases, approximately 75 % of all shared regions were small at <100 kb in size. However, for joint gains, 19 % had a length of 100 kb and only 2 % were >100 kb. For the losses, ~1 and 25 % of the shared loss regions had a length of 100 and >100 kb, respectively.

Details of the top nine regions of shared rank across both human and canine UC are presented in Table 5 and visualized as concordant Circos plots in Fig. 7. The highest ranked regions of genomic overlap shared between canine and human UC cases included copy number gains of CFA 13/HSA 8 in 97/52 % of patients, respectively (Table 5). The specific region of overlap derived from our comparative approach resulted in a segment of HSA 8 that was just 3 kb in size and segment of CFA 13 that was 17.9 kb. This overlapping region had a joint-rank probability of 4.5×10⁻⁷ and indicates a highly significant role in UC for both dog and human. Within this shared region is the gene polyadenylate-binding protein 1 (PABPC1; HSA 8;101,698,044–101,735,037).

Additional significant joint-ranked regions (p<0.05) were further evaluated to determine if specific genes associated with CNAs in human UC were also evident in canine UC. Shared gains of S100A9, S100A8, and ID1 and shared loss of CDRN2A were identified by our pipeline, supporting this data-driven comparative analysis. In both the data-driven and a priori comparative analyses, the region surrounding HSA 8:101 Mb/CFA...
<table>
<thead>
<tr>
<th>Region number</th>
<th>Human region size (bp)</th>
<th>Human region aberration frequency (%)</th>
<th>Human rank probability</th>
<th>Dog region size (bp)</th>
<th>Dog region aberration frequency (%)</th>
<th>Dog rank probability</th>
<th>Joint-rank probability</th>
<th>Called aberration</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>3648</td>
<td>41</td>
<td>3.70E-04</td>
<td>cfa13:54000000-5417899</td>
<td>17,889</td>
<td>97</td>
<td>1.20E-03</td>
<td>4.50E-07</td>
</tr>
<tr>
<td>2</td>
<td>30,915</td>
<td>38</td>
<td>5.00E-04</td>
<td>cfa11:44260000-44276795</td>
<td>15,797</td>
<td>26</td>
<td>0.3</td>
<td>1.30E-04</td>
</tr>
<tr>
<td>3</td>
<td>185</td>
<td>39</td>
<td>4.10E-03</td>
<td>cfa22:42456000-42500000</td>
<td>5366</td>
<td>68</td>
<td>0.06</td>
<td>2.00E-04</td>
</tr>
<tr>
<td>4</td>
<td>4888</td>
<td>22</td>
<td>0.04</td>
<td>cfa24:53000000-34217250</td>
<td>17,250</td>
<td>52</td>
<td>0.05</td>
<td>2.20E-03</td>
</tr>
<tr>
<td>5</td>
<td>1790</td>
<td>32</td>
<td>0.05</td>
<td>cfa7:45309000-45330877</td>
<td>23,912</td>
<td>52</td>
<td>0.08</td>
<td>4.30E-03</td>
</tr>
<tr>
<td>6</td>
<td>2430</td>
<td>11</td>
<td>0.6</td>
<td>cfa9:43930000-44000000</td>
<td>60,999</td>
<td>61</td>
<td>0.02</td>
<td>0.61</td>
</tr>
<tr>
<td>7</td>
<td>1989</td>
<td>29</td>
<td>0.09</td>
<td>cfa9:43091000-4378901</td>
<td>68,811</td>
<td>35</td>
<td>0.1</td>
<td>0.014</td>
</tr>
<tr>
<td>8</td>
<td>48,124</td>
<td>20</td>
<td>0.43</td>
<td>cfa8:53000000-5312099</td>
<td>12,099</td>
<td>48</td>
<td>0.09</td>
<td>0.04</td>
</tr>
<tr>
<td>9</td>
<td>11,857</td>
<td>11</td>
<td>0.8</td>
<td>cfa2:66900000-6999021</td>
<td>99,021</td>
<td>84</td>
<td>0.04</td>
<td>0.04</td>
</tr>
</tbody>
</table>

Regions of shared copy number aberration were determined using human and dog-as-human (humanized) data. For each species, the shared aberrant region on both human and canine chromosomes is listed, as well as the frequency of that aberration in the sample cohort. Overlapping analysis resulted in nine regions with statistical significance (P<0.05). Statistical validation was performed by ranking and calculating rank probabilities.
Discussion

Canine UCs exhibit diagnostically relevant copy number aberrations

A major challenge in the management of canine UC is deferred diagnosis. Due to non-specific clinical symptoms mimicking cystitis and the invasive nature of
current diagnostic techniques, canine patients with progressive UC often remain undiagnosed for weeks to months (Mutsaers et al. 2003). The use of imaging techniques, such as radiography and ultrasonography, may identify the presence of abnormal growths in the urinary tract but cannot evaluate malignancy. Evaluation of urine sediment by routine cytology can lead to misdiagnosis; neutrophilic infiltration and degranulation due to bacterial cystitis can cause altered urothelial morphology, mimicking neoplastic cells. Similarly, neoplastic cells in patients with UC and concurrent bacterial cystitis may elude pathologic detection. As a result, biopsy and histopathologic evaluation is considered the “gold standard” of UC diagnosis (Mutsaers et al. 2003).

Biopsy may be performed by surgery, cystoscopy, or traumatic catheterization, all of which are considered invasive procedures. However, disturbance of the primary tumor may result in seeding of malignant epithelial cells throughout the urinary tract (Anderson et al. 1989). The complications associated with tumor seeding, combined with the invasiveness of biopsy techniques, support the need for safer and more efficient diagnostics.

The development of a non-invasive early screening technique to confirm the diagnosis of a UC would provide a means to mitigate the seemingly rapid progression of the disease by facilitating early therapeutic intervention. The propensity of neoplastic cells to shed into the bladder lumen is one worth exploiting, especially upon examination of the data presented in this study. Three particular CNAs, CFA 13 gain, CFA 36 gain, and CFA 19 loss, were highly recurrent in the canine UC cohort with at least one of these three aberrations detected in 100% of canine patients. Detection of transitional cells in the urine possessing one or more of these aberrations may therefore be considered highly suspicious for UC. Statistical evaluation of our data indicated that, with detection of two or more of these aberrations in cells recovered from a canine urinary tract, the sensitivity and specificity to indicate neoplasia is extremely high (≥99%). Furthermore, the detection of just the two most frequent aneuploidies (gains of CFA 13 and 36) provides an OR of 422.230, a RR of 33.817, and misclassification of 0. The use of FISH as the gold standard of copy number detection and enumeration was shown in this study to be an effective means to identify aberrant cells in urine sediment from dogs that presented with a UC. The use of custom SureFISH probes provided a more reliable, cleaner and effective alternative to conventional genomic (BAC) clone-based FISH probes. This study was based on an evaluation of FFPE and urine specimens from UC confirmed and also grossly healthy canine specimens. To establish true sensitivity and specificity values for a clinically valuable diagnostic assay, similar analyses should be performed using a
Canine UC is of comparative value to human medicine. Previous studies have documented HSA 8q as gained in copy number in 37% of human UC patients (Richter et al. 1998). In fact, gain of 8q22 has been identified as the most frequent copy number gain in urothelial carcinomas (Van Duin et al. 2005; Heldenblad et al. 2008) and is generally associated with poor clinical outcome (Chen et al. 2011). Results obtained by combining CNA data from 285 human UC in the present study further supported these reports, with 41% of tumors possessing a gain of 8q. The same aberration is also observed in our canine data set as gain of syntenic CFA 13, albeit with remarkably high frequency at 97% of canine UC cases. We propose that the high prevalence of the HSA 8q/CFA 13 gain in UC highlights its importance in both species and simultaneously suggests an evolutionarily conserved molecular pathogenesis in UC tumor development.

Despite the prevalence of the CFA 13 gain in UC, CFA 13 is gained in a number of other canine cancers, including sarcomas and carcinomas (Thomas et al. 2003c, 2014; Angstadt et al. 2011, 2012; Hedan et al. 2011; Poorman et al. 2014). The frequency of this aberration in general canine cancers makes it an unlikely candidate as a single UC-specific marker. Nevertheless, the high prevalence of CFA 13 gain in canine cancer suggests it is likely fundamental to oncogenesis. Comparative analysis of CFA 13 and HSA 8 indicates that if UC indeed shares an evolutionarily conserved mechanism of pathogenesis, genes associated with UC development in both species reside in the proximal half of HSA 8q, the region syntenic with CFA 13.

Using joint-rank probabilities to refine alignment of the canine and human genomes reduced the extent of the shared regions of HSA 8/CFA 13 to a region of the human genome that spans just 3 kb. Within this segment of 8q22.2 is the gene PABPC1, whose protein function is to bind the poly-A tail of messenger RNA (mRNA) in the nucleus and shuttle the transcript to the cytoplasm for translation (Eliseeva et al. 2013). PABPC1-associated poly-A tails stimulate translation and stabilize mRNA in the cytoplasm and increased levels of PABPC1 may lead to excessive and oncogenic increases in the mRNA half-lives and thus increased potency of even a normal number of transcripts. Previous studies have shown that PABPC1 is overexpressed in superficial bladder cancer samples (Chen et al. 2011). Gain of the PABPC1 locus in superficial and invasive UCs of both canine and human suggests an early and conserved role for the gene in UC progression. In contrast to human UC, the majority of canine tumors are invasive (T1–T4) at the time of diagnosis and thus at the time specimens are available for genomic analyses. This simple yet stark difference in the clinical behavior of canine and human UCs might explain the increased frequency of the aberration in dogs compared with humans, while underlining a potential role of PABPC1 in tumor progression.

Deletions of HSA 9 are known to be the first genomic alterations associated with human UC development (Simoneau et al. 2000). Specifically, loss of HSA 9q characterizes superficial bladder tumors and becomes less prevalent with increased tumor stage, while gains of HSA 8 become more prevalent (Castillo-Martin et al. 2010). The second most significant shared region according to our joint-rank probability analyses was a region of HSA 9q/CFA 11 that contains CDKN2A. This is a widely studied locus in human cancer research as it codes for the p16 tumor suppressor protein, deletion of which has been associated with muscle invasive UC in humans, presumably due to the rapid accumulation of mutations advantageous to tumor progression (Schulz 2006). In fact, the multicolor FISH-based Urovysion® (Abbott Technologies) human bladder cancer diagnostic assay includes CDKN2A as one of four regions detected and quantified. Along with focal deletion of CDKN2A, a lowered expression of the gene is also observed in human UC samples (Goe bell and Knowles 2010; The Cancer Genome Atlas Research Network 2014). The canine UC genome recapitulates the loss of the CDKN2A locus at a similar frequency (26% vs. human 38%), suggesting consequences to expression might also be disrupted in canine UC.

The third- and fourth-ranked regions harbor no apparent coding sequences for genes associated with cancer. The fifth-ranked region was located on HSA 1/CFA 7 (a shared gain) and is immediately flanked by S100A8 and S100A9. When the ranked region was expanded to include the two S100 genes, the aberration penetrance was unchanged. S100A8/9 are two damage-associated molecular pattern proteins (DAMPs) released by monocytes and neutrophils in response to hypoxia, as occurs in a proliferative tumor environment (Bansal et al.)
Both S100 proteins have been linked previously to UC development, with overexpression being diagnostic for the disease and higher levels suggestive of higher-grade tumors (Ebbing et al. 2013). Similarly, overexpression of S100A9 is associated with increased proliferation and migration of bladder cancer cell lines (Kim et al. 2011, 2014). The increased frequency of S100 loci gain in dogs (52 %) compared with humans (32 %) may be a reflection of the later time of diagnosis and also may contribute to the more aggressive tumor phenotype seen in canine patients.

Ranked regions with striking interspecies penetrance disparity include loss of HSA 2/CFA 19 and gain of HSA 2/CFA 36 (Table 5, regions 6 and 9). In these regions, copy number aberrations were observed at high frequency in canine UCs, while in humans, the corresponding genomic regions were aberrant only in a small percentage (11 %) of tumors. The discrepancy may be a feature of the different differential genome architecture of canine and human karyotypes, in so far as CFA 36 is a small chromosome but is evolutionary conserved as a single 36-Mb segment within the center of HSA 2q (2q23.3–32.2). CFA19 and 36 are juxtaposed on syntenic HSA 2 and are each interrupted by breakpoints which have been clearly documented in human UC at HSA 2q21 and q31, respectively (Fadl-Elmuhal 2005). Assuming breakpoints are observed in syntenic canine regions, these particular breakpoints would represent fragile sites in CFA 19 and 36 predisposed to duplication, deletion, and translocation. As canine cells evolve to malignancy, rapidly proliferative cells succumb to inherent genomic fragility, resulting in chromosome breaks and copy number aberrations (Richards 2001; Ma et al. 2012; Hosseini et al. 2013).

The prevalence of chromothripsis-like events on CFA 36 confirms suspected fragility of the chromosome. Chromothripsis, a chromosome-shattering phenomenon, leads to extreme genomic rearrangements, deletions, and amplifications within a concentrated chromosomal region (Stephens et al. 2011). In canine UC, 74 % of tumors showed distinct chromothripsis-like signatures, most commonly involving CFA 36 (30 %). The amplification of involved regions of CFA 36 interrupted by small regional losses, suggests that, at some point in tumor evolution, CFA 36 sister chromatids were subjected to numerous double-stranded breaks and rejoined imperfectly, leading to the gain of some shattered regions and loss of others. Our results of FISH on neoplastic urothelial cells support this notion, demonstrating massive amplification of CFA 36 signal within a small nuclear region. Such chromosomal damage may be induced by many means, including mutagenic carcinogens implicated in canine and human UC development. The relatively low frequency of chromothripsis-like events reported in human UC may be attributed to a more compact human genome, composed of 23 chromosome pairs, when compared with the 39 chromosome pairs of the canine karyotype, which leads to increased terminal chromosome surface area available for damage initiation and rejoicing.

The aberrant regions on CFA 19 and 36 contain genes of interest to canine UC. The gene LRPPC lies within the breakpoint on CFA 19 (Prazeres et al. 2011). LRPPC possesses tumor suppressor function, decreasing the numbers of PDGF and urokinase receptors on the cell membrane and halting external proliferation stimuli. Loss of LRPPC is associated with high-grade urothelial carcinoma in humans and may thus be of equal prognostic significance in dogs (Langbein et al. 2002). The fragile site on CFA36 contains the HOXD gene cluster and a relevant micro-RNA, miR-10b (Calin et al. 2004). Dysregulated expression of HOX genes leads to increased cell proliferation and neoplastic transformation, whereas upregulation of miR-10b has been associated with invasive and metastatic abilities in numerous carcinomas, including the bladder (Bhatlekar et al. 2014; Xiao et al. 2014).

Comparative staging analysis allowed comparison of the molecular landscape throughout tumor progression. Gains of HSA 1 and 8 and loss of X are the most frequent aberrations in non-muscle invasive human tumors, and their syntenic counterparts, CFA 7, 13, and X, are similarly aberrant in canine tumors with absence of muscle invasion. These results reaffirm the importance of HSA 8/CFA 13 gains, in particular, in UC development. Loss of the X chromosome, however, is replaced by a chromosome gain in muscle invasive tumors of both canine and human cases. Previous human UC studies have noted a decrease in the number of tumors demonstrating an X chromosome monosomy and an increase in polysomy with increasing tumor grade (Panani and Roussos 2006). Our results, combined with previous findings regarding tumor grade, suggest the involvement of the X chromosome in UC muscular invasion.

A major limitation to our staging analyses was the superficial nature of canine biopsies. Due to their acquisition via cystoscopy, rarely were all histological layers
of the bladder present in the biopsied tumor sample. In these cases, tumors were staged using available layers only. Therefore, it is likely that numerous canine tumors were at a more advanced stage in vivo than we were able to ascertain by biopsy, underrepresenting late-stage tumors and potentially obscuring aberration differences among stages. However, the association with X chromosome copy number status also adds confidence to the historical definitions provided. In future studies, more thorough staging analyses should be performed with more complete canine biopsies. Additionally, the same set of veterinary and human pathologists did not review the human cases, thereby precluding consistency among all human and canine cases.

Using PANTHER, the prevalence of genomic aberrations in regions containing critical groups of genes highlights the ways in which chromosomal aberrations contribute to neoplastic transformation. Our analysis adopted an integrative approach that identified genes and pathways that may play a role in UC progression in both canine and human cancers. Using the PANTHER classification system and inputting genome-wide CNAs across both species, we can identify ontological classes that are over- and under-represented in shared genomic regions of canine and human UC samples (SOM Fig. 1). The genes in our analysis are enriched across three major ontological components, including signaling pathways in metabolic processes, cellular processes, and biological regulation. The role of the pathways can now be investigated further by experimental manipulation on both primary UC samples and UC cell lines from both canines and humans. Advances of this nature will lead to new drug discoveries for UC and/or investigation of already available drugs not currently being considered for UC but targeting these same pathways. This type of pathway analyses will be strengthened in the future by combining other comparative genomic data from UC tumors such as sequence mutation, gene expression, and even epigenetic alterations.

Conclusion

The genomic landscape of canine UC is highly aberrant and of enormous diagnostic relevance. The copy number data generated in this study provided the foundations on which to develop a diagnostic assay for canine UC with close to 100% sensitivity and specificity. Furthermore, the non-invasive nature of a free-catch urine-based assay offers an attractive alternative to current options, which will promote earlier detection and thus maximize opportunity for prolonged life. The comparative value of a cross-species approach to cancer gene discovery has been demonstrated for UC with the identification of numerous aberrant genome intervals shared between human and dog. Through direct comparison of canine and human data as well as an informatics approach to highlight the highest ranked changes common to both species, we have demonstrated that dogs and humans share major cytogenomic aberrations that impact signaling pathways. Further evaluation of such genes and pathways as potential therapeutic targets may now be pursued.

Acknowledgments

The canine oatCGH and FISH data generated in this study were funded by the NCSU-UCVM Cancer Genomics Fund (MB). SS was supported in part by a Graduate Fellowship from the NCSU Comparative Biomedical Sciences Graduate Program, an NIH-T35 grant, a Triangle Community Foundation award, a George Hitchings New Investigator Award in Health Research, and the NCSU-UCVM Cancer Genomics Fund (MB). We gratefully acknowledge support of Skippy Frank Fund for Life Sciences and Translational Research/ Rockefeller Philanthropy Advisors (awarded to MB/JS), whose funding supported SR as a Skippy Frank Translational Postdoctoral Fellow. J.D.S. holds the Edward B. Clark, MD Chair in Pediatric Research, and is supported through the Primary Children’s Hospital (PC3) Pediatric Cancer Program funded by the Intermountain Healthcare Foundation and the Primary Children’s Hospital Foundation. We thank Rachael Thomas for assistance with humanization of canine CGH data and Clint Mason for valuable informatics advice.

References

crus transplation of a urinary bladder transitional cell carc
inoma of the urinary bladder in a dog. Cornell Vet 79:263–266
tive genomic hybridization and RT-qPCR: signatures of ge


Dobson JM (2013) Breed-predispositions to cancer in pedigree dogs. ISRN Veterinary Science 2013


Comparative cytogenomics of urothelial carcinoma


Figure 2.9. (SOM Figure 1). Gene ontology analysis highlighted gene function categories frequently affected by shared copy number aberration in human and canine UC.

Gene ontology analysis highlighted gene function categories frequently affected by shared copy number aberration in human and canine UC. A GO analysis was performed as a part of the pathway analysis done in PANTHER. Conserved copy number gains and losses are shown as the inner and outer donut plots, respectively, with number of genes affected shown in each category. These data indicated that genes associated with metabolic processes (GO:0008152), cell processes (GO:0009987), and biological regulation (GO:0065007) were the most prominent among human and canine UC. Each of these three processes is highlighted in the corresponding donut plot.
CHAPTER 3: A CULTURED APPROACH TO CANINE UROTHELIAL CARCINOMA RESEARCH: CHARACTERIZATION OF FIVE CELL LINES

Abstract

Urothelial carcinoma (UC), also known as transitional cell carcinoma (TCC), of the bladder is the most common neoplasm affecting the canine urogenital system. To facilitate study of the disease in vitro, UC cell line models have been established from primary tumors. Their resemblance to the primary disease, however, has not been well defined. In the present study, we evaluated five canine UC cell lines via oligonucleotide array comparative genomic hybridization (oaCGH), fluorescence in situ hybridization (FISH), and gene expression analysis. Comparison of genome-wide DNA copy number aberrations of the cell lines with primary biopsy specimens revealed redundancies in genomic aberrations, indicating that the cell lines retain the gross genomic architecture of primary tumors. As in the primary tumors, gain of canine chromosomes 13 and 36 and loss of chromosome 19 were among the most frequent aberrations evident in the cell lines. FISH analysis revealed chromosome structural aberrations, including tandem duplications, bi-armed chromosomes, and chromosome fusions, suggesting genome instability during neoplastic transformation. Gene expression profiling highlighted numerous differentially expressed genes, including many previously shown as dysregulated in primary canine UC and human bladder cancer. Pathway enrichment analysis emphasized pathways suspected to be at the crux of UC pathogenesis, including xenobiotic and lipid compound metabolism. These data support valid use of the canine UC cell lines evaluated by confirming they provide an accurate and practical means to interrogate the UC at a molecular level. Moreover, the cell lines may provide a valuable model for furthering our understanding of aberrant metabolic pathways in UC development.
Introduction

Urothelial carcinoma (UC), also referred to as transitional cell carcinoma, is the most common bladder neoplasm in the dog (Mutsaers, Widmer, & Knapp, 2003). While precise lifetime risk and incidence numbers in pet dogs are unknown, UC is estimated to affect more than 20,000 dogs each year in the United States (Knapp et al., 2014). Due to the uncomfortable and potentially fatal consequences associated with a rapidly growing bladder tumor, the diagnosis of UC conveys a guarded prognosis and is associated with considerable dog owner concern (Hamilton, Sarcormrattana, Illiopoulou, Xie, & Kitchell, 2012). Additionally, over 90% of canine bladder tumors are invasive with metastatic potential; approximately 20% of canine patients show overt metastases at the time of diagnosis and over 60% at death (Knapp et al., 2014). The devastating clinical course of disease, combined with a high rate of metastasis, emphasizes a need for early detection of the tumor. With improved detection and a better understanding of the disease, therapeutics may prove more efficacious, extend patient survival, and improve quality of life.

Previous studies have shown that the genomic landscape of canine UC is highly aberrant, with recurrent chromosome copy number aberrations affecting gene dosage on, most notably, canine chromosomes (CFA) 13, 19, and 36 (Shapiro et al., 2015). In addition, overwhelming breed predispositions suggest the risk for UC development is at least partially genetic. The inbred history of purebred dogs, resulting in reduced levels of genetic variation and enrichment in breed-associated loci, provides a biologic basis for breed susceptibility (Dobson, 2013). Scottish terriers, for example, are diagnosed with UC 20 times more frequently than the average dog, and beagles, Shetland sheepdogs, wire hair fox terriers, and West Highland white terriers are affected three to five times as frequently (Mutsaers et al., 2003). The uniquely homogenous genetic population of purebred dogs makes the dog
an excellent model for teasing out genetics that are potentially important in cancer of the highly heterogeneous human population. Furthermore, studies have found that canine UC closely resembles that of the human counterpart, in histopathological features, clinical behavior, and genomic aberrations, extending the value of the dog to human medicine (Knapp et al., 2000; Knapp et al., 2014; Shapiro et al., 2015).

In addition to genetic risk factors, UC is associated with numerous environmental factors, including flea and tick dips, obesity, pesticides, and cyclophosphamide treatment (Glickman, Raghavan, Knapp, Bonney, & Dawson, 2004; Glickman, Schofer, McKee, Reif, & Goldschmidt, 1989; Plotz et al., 1979). While correlations of UC incidence with each of these risk factors have been found, mechanisms of carcinogenesis have yet to be elucidated. Problems in cyclic compound metabolism have been proposed, but until the mechanisms of UC evolution are realized, provision of effective prevention and treatment strategies will remain challenging (J. Zhang et al., 2012). The study of potential molecular drivers and therapeutic targets would be greatly expedited by a biologically appropriate in vitro model.

Cell lines provide a useful in vitro model for the study of disease. By facilitating characterization and manipulation of all molecular facets without animal harm, cell lines enable researchers to evaluate highly experimental ideas and therapies. Although canine UC cell lines have previously been established for such use, their resemblance to the primary tumor in molecular behavior and genomic landscape has yet to be thoroughly evaluated (Dhawan, Ramos-Vara, Stewart, Zheng, & Knapp, 2009). Affirming the cell lines recapitulate molecular characteristics of the primary tumor would increase their clinical predictive value and enhance their significance in translational studies.

In the present study, we investigated the molecular profile of canine UC cell lines. Oligonucleotide array comparative genomic hybridization (oaCGH) and fluorescence in situ
hybridization (FISH) assessed cytogenetic changes in five canine UC cell lines. Clinical relevance of findings was validated by comparison of aCGH profiles of cell lines with those of primary tumor samples, which showed conservation of major genomic aberrations in both sample cohorts. Subsequent gene expression profiling quantified mRNA from copy number neutral and aberrant regions of the genome. KEGG pathway and gene ontology (GO) analysis of highlighted gene involved in lipid metabolism and cell cycle regulation to be enriched in differentially expressed genes (DEGs), highlighting a potential pathogenesis that is relevant to known risk factors. Our results affirm that the canine UC cell lines are genomically similar to the primary tumor, providing a relevant in vitro model for study of the molecular mechanisms of disease.

**Methods and Materials**

*Canine UC cell lines and urothelial controls*

Five tumor cell lines (Table 1) were established from pathologically-confirmed UC in dogs who were evaluated and treated in the Purdue University Veterinary Teaching Hospital. The biopsies used to establish the cell lines were collected via cystoscopy or at necropsy. All samples were obtained with informed pet owner consent and under an approved IACUC protocol. Once established, cells from each line were cryopreserved at various passages.

Control samples for expression analysis (healthy urothelium) were collected during necropsy of dogs with no clinical or histopathological signs of disease at North Carolina State University College of Veterinary Medicine (<1 hour post-euthanasia). After collection, bladder tissue was stored in transport media (RPMI supplemented with 10% FBS) until sample processing (<1 hour). Samples were split in half: one half was fixed in 10% neutral
buffered formalin (NBF) before paraffin-embedding (FFPE) to enable pathologic evaluation, and the second half was used for urothelial cell isolation. FFPE samples were sectioned and stained with hematoxylin and eosin (H&E) for review by a board certified veterinary pathologist. All control samples were found to be histopathologically normal prior to use in subsequent protocols. Urothelial cells were isolated by scraping the bladder mucosa three times with a glass slide and rinsing with 1x phosphate buffered saline (PBS), as described previously (Harris, Wade, & Handler, 1986). Urothelial scraping was performed in order to minimize contamination of urothelium by submucosal tissue layers. Cell scrapings were then analyzed under phase contrast to determine the proportion of epithelial cells to contaminating mesenchymal cells. Over 90% of isolated cells were confirmed as epithelial. RNA was immediately isolated from the healthy, fresh urothelial cells to minimize alterations in post-mortem and post-collection gene expression.

**Culture preparation**

Cells from each of the five lines were cultured in DMEM/F12 without glutamine (Mediatech, Manassas, VA) and supplemented with 10% fetal bovine serum (Mediatech), 1% GlutaMAX™ (Life Technologies, Carlsbad, CA), and 0.6% Primocin™ (InvivoGen, San Diego, CA) and grown to confluence, passaging when appropriate until a T75 flask of cells was obtained. For each line, cells from the same flask were split into three aliquots and used simultaneously for chromosome preparation, DNA isolation, and RNA isolation. Four of the cell lines (K9TCC-PU-An, K9TCC-PU-In, K9TCC-PU-Mx, K9TCC-PU-Sh) were available as low passage (<p7) and so were harvested at an earlier (<p10) and later (>p16) passage to evaluate genomic evolution during culture. One cell line (K9TCC) was available only from p14 and so was harvested at p16 only.
oaCGH

DNA from cell lines and frozen tumors was isolated using a DNeasy Kit according to manufacturer’s recommendations (Qiagen, Valencia, CA). Purified DNA was verified to be of high molecular weight and purity by agarose gel electrophoresis and spectrophotometry (Nanodrop™ 1000, Thermo Fisher Scientific, Wilmington, DE; 260:230>2.0 and 260:280>1.8). Sex-specific reference DNA samples were generated from mixed breed dogs, pooling equimolar quantities of DNA extracted from the whole blood of 10 healthy males or 10 healthy females using the QIAmp DNA Midi Kit (Qiagen). DNA was labeled using the Genomic DNA Enzymatic Labeling Kit (Agilent Technologies, Santa Clara, CA) as described previously (Poorman et al., 2014; Shapiro et al., 2015). Fluorescently labeled test and reference samples were hybridized to Canine G3 Sureprint 180,000 feature oaCGH arrays (Agilent, AMADID 025522) for 40 hours, as described previously (Bonberg N Fau - Pesch et al., 2014). Arrays were scanned at 3µm (Agilent, Model G2505C) and data extracted with Feature Extraction software (v10.9m Agilent) using the canFam2 genome build. CGH results were analyzed using the FASST2 algorithm in Nexus Copy Number (Biodiscovery, Hawthorne, CA). Significant copy number aberrations were defined by Log₂ ratios >0.201 (gain) or Log₂ ratios< -0.234 (loss). After aberration detection, cell lines and primary tumors were clustered based on aberrations using a complete linkage hierarchical clustering algorithm by Nexus Copy Number (Biodiscovery).

Genomic regions likely to be central to tumorigenesis were analyzed by the Genomic Identification of Significant Targets in Cancer (GISTIC) algorithm in Nexus Copy Number, which identifies regions unlikely to be aberrant by chance when taking aberration frequency and amplitude into account (Beroukhim et al., 2007). A G-score is computed to reflect the frequency and amplitude of the aberration, while a q-value suggests the likelihood
(probability) of that aberration occurring by chance when looking at the overall aberration pattern throughout the genome. Lastly, Aberrations in early and late passage cell lines were compared using the “Comparisons” tool in Nexus, using the early passage as baseline (p=0.05, differential threshold=1%).

oaCGH aberration profiles were evaluated for the presence of chromothriptic-like events, as described previously (Shapiro et al., 2015). Chromothriptic-like events were defined as discrete chromosomal regions displaying at least two juxtaposed and alternating significant gain/loss events.

**Primary tumor oaCGH data**

Previously published oaCGH data from 31 primary canine UCs was compared to cell line aberrations (Chapter 2, Shapiro et al., 2015). Using Nexus Copy Number, aberrations common to primary tumors and cell lines were compared using the “Comparisons” tool. Additionally, GISTIC analysis was repeated using primary tumor aberrations.

**Fluorescence in situ hybridization**

Cell from all five canine UC cell lines were harvested using conventional methods of colcemid-induced metaphase arrest (Life Technologies), hypotonic (KCl) treatment (Life Technologies), trypsinization (0.25% trypsin, Mediatech), and fixation, as described previously (Breen et al., 1999). Fixed cell suspensions were dropped onto clean glass slides and aged for three days at room temperature prior to ethanol dehydration (70%/90%/100%) and storage at -80°C.

Fluorescence *in situ* hybridization (FISH) was used to validate and visualize copy number aberrations indicated by oaCGH. Based on FASST2-called aberrations, 11 genomic regions were selected for FISH analysis: 10 aberrant regions of high amplitude and penetrance (>50% gain/loss) across the cohort and one balanced region on CFA 11,
selected as a copy number neutral control (Log$_2$=0) (Table 2). Probe DNA was extracted from clones of the CHORI-82 canine BAC library (http://bacpac.chori.org/library.php?id=253) containing the regions of interest and labeled with one of five spectrally-resolvable fluorochrome-conjugated dNTPs as described previously (Breen et al., 2004). Multicolor FISH reactions were performed first on DAPI-stained metaphase chromosome preparations of clinically healthy dogs to validate the unique and precise cytogenetic location of each probe, as described previously (Breen et al., 1999). Verified probes were hybridized to metaphase preparations of each cell line and visualized by using an Olympus BX61 fluorescent microscope (Olympus, Center Valley, PA) equipped with appropriate single pass filters. Cells (n≥30) exhibiting good chromosome separation were selected for assessment of numerical and structural organization and were counted to determine a) total chromosome number and b) number of signals for each fluorochrome (sequence of interest).

Using Log$_2$ ratios ascertained by oaCGH and also deduced from SLP probe enumeration, correlation coefficients were derived. Additionally, a Mann-Whitney U test (two-tailed, p<0.05) was performed using JMP Professional Statistical Software (v. 11, SAS, Cary, NC) to compare mean Log$_2$ ratios from both methods and among all cell lines.

**Gene expression analysis**

RNA was extracted from cultured cells or healthy (control) urothelial cells using the RNeasy Plus Mini Kit (Qiagen). RNA integrity was assessed using spectrophotometry (Nanodrop) and a 2100 BioAnalyzer (Agilent). Samples with 260:230 and 260:280 > 2.0 and RIN > 8.0 were used for microarray analysis and qRT-PCR validation of aberrant mRNA levels. RNA was labeled using the Quick Amp Labeling kit (Agilent). Purified RNA probes were hybridized to a one-color expression microarray (Agilent SurePrint G3 Canine 4x44k
Expression Array). Arrays were scanned at 3µm (Agilent, Model G2505C). Analysis was performed using the GeneSpring advanced platform (Agilent, v.11.5, 2011) and Nexus Expression (BioDiscovery, v.2.0, 2010). Samples were normalized between and within arrays. Expression levels were compared between RNA isolated from the cell lines and from urothelium of two histologically confirmed healthy dog bladders. Cell lines and reference gene expression profiles were clustered using an agglomerative hierarchical clustering algorithm in Nexus Expression (Biodiscovery).

In preparation for qRT-PCR validation of array data, primers were designed for a stably expressed control gene (RPL32) and for a gene within each of the loci assessed by FISH and dysregulated according to the expression array. Primer template specificity was confirmed by agarose gel electrophoresis of PCR products (single product) and primer melt curve analysis (single peak). An efficiency curve based on five ten-fold dilutions was constructed to evaluate the performance of each primer pair. Primers with efficiency between 90-110% and linear correlation coefficients over 0.95 were selected for qRT-PCR analysis (seven aberrant regions of interest plus a control gene, Table 3). cDNA was created using the QuantiTect Reverse Transcription Kit (Qiagen). The QuantiFast SYBR Green qPCR Kit (Promega, Madison, WI) was used for real time quantification of mRNA on an iCycler iQ™ Real Time Detection System (Bio-Rad, Hercules, CA). Fidelity was ensured by inclusion of no reverse transcriptase and no template negative controls. Gene fold changes were calculated by the Pfaffl method, accounting for variations in primer efficiency between reference and target genes (expression ratio=Efficiency_{TARGET}^{ΔCt(control-cell_lines)} / Efficiency_{REFERENCE}^{ΔCt(control-cell_lines)} ) (Pfaffl, 2001). Ratios less than 1 were converted into negative fold changes by -1/ratio. A correlation coefficient relating data from qRT-PCR and expression array was calculated using fold change values from genes evaluated using
both methods. Gene ontology (GO) and pathway analysis was conducted on DEGs using the online tool DAVID to extract biological relevance from the data (Huang et al., 2009a, b).

Results

*Canine UC cell lines display recurrent chromosome copy number and structural aberrations.*

Metaphase preparations from each of the five canine UC cell lines revealed a high degree of aneuploidy, with an excess of 100 chromosomes in four of five cell lines (Table 1). Additionally, oaCGH profiles revealed specific DNA copy number gains and losses across the genome of each cell line (Figure 1A). Highly recurrent aberrations (>80% frequency) involved loss of regions of CFA 1, 2, 5, 6, 9, 10, 12, 19, 20, 21, 22, 26, 28, 33, 34, and X, and gains of CFA 4, 5, 6, 8, 9, 10, 11, 13, 15, 16, 17, 27, 34, and 36. Copy number losses present on CFA 2, 6, 10, 21, 26, and 28 and gains of CFA 6, 8, 10, and 13 were observed in all five cell lines (100%). High amplitude changes (Log$_2$>4) were noted on CFA10, 36, and 38, suggesting DNA amplification in these regions. The “Comparisons” analysis in Nexus Copy Number of early versus late passage cell lines showed only three genomic regions with a greater than 1% difference in aberration frequency between oaCGH between early and later passage of the same cell lines (gain in late passage): CFA1q22, CFA2q24.1, and CFA9q22.3 (p<0.01).

Cell line oaCGH profiles were compared to those of 31 previously published primary tumors (Figure 1B). Hierarchical clustering of chromosome copy number aberrations among cell lines and primary tumors yielded a dendogram in which there was no segregation between the cell lines and primary tumors (Figure 1C). Comparison analysis of primary tumors and cell lines highlighted only 17 regions, constituting less than 0.2% of the genome (4.5Mb), aberrant in either the primary tumors or cell lines, while normal in the other (Table
GISTIC analysis of aberrations among primary tumors and cell lines showed that five of seven (71.4%) specific cancer-associated genomic regions altered in the cell lines (Table 5) were similarly altered in the primary tumors (Appendix A), including amplified regions on CFA10, 36, and 38 and gains of CFA13 (Q-bound<0.006, G-score>5).

Chromothriptic-like events were noted throughout the genome of all five of the cell lines, most notably on CFA 9 and 10 (>80% of cell lines) (Figure 2). CFA 5, 10, 19, 35, and 36 had an average of over five chromothriptic-events per cell line, with CFA10 chromothriptic-like events occurring an average of 10 times per cell line (range=1-20).

Targeted FISH analysis of genomic loci identified as aberrant by oaCGH confirmed the presence of abnormal DNA copy number in individual cells and enabled enumeration of exact copy number (Table 6). A Mann-Whitney U test (two-tailed, α<0.05) of average oaCGH and FISH Log$_2$ ratios for each cell line showed no significant differences between the two methods of copy number determination (Table 6, p>0.56 overall). Additionally, correlation analysis showed a strong correlation between FISH- and oaCGH-determined Log$_2$ ratios ($R^2=0.91$). FISH further enabled identification of several recurrent structural aberrations involving interrogated regions (Figure 3), including tandem duplications, translocations, chromosome fusions, and bi-armed chromosomes (Table 1). Tandem duplications were noted on CFA 10, 36, and/or 38 in all five cell lines (Figure 3A & C), coincident with high level amplification indicated by the corresponding oaCGH aberration profiles. An excess of 30 copies of the targeted locus of CFA 36 were noted in the K9TCC-PU-Mx line, confirming the expected Log$_2$>4. The region probed on CFA 38 demonstrated a translocation to a position adjacent to the CFA 36 locus in two cell lines (K9TCC-PU-An and K9TCC-PU-Sh). In K9TCC-PU-Sh, tandem duplications of CFA 36, juxtaposed with tandem duplications of CFA 38, were present twice on a single aberrant chromosome structure (Fig.
Additionally, centromeric fusion of CFA 13, resulting in a bi-armed aberrant chromosome, was noted in two lines (K9TCC-PU-An and K9TCC-PU-In; Fig. 3B). Other aberrantly metacentric chromosomes were noted in all five cell lines, affecting multiple chromosomes.

Gene expression profiles of cell lines differ from those of normal urothelium.

When profiled alongside healthy urothelium, the UC cell lines showed highly differential gene expression levels (examples shown in Table 7). Of 43,803 probes represented on the array, 8,688 displayed altered levels of expression with absolute fold change greater than 2.0 when normalized and compared to reference urothelium. Hierarchical expression clustering confirmed a vastly different expression landscape between tumors and control urothelium, each recognized as a separate outgroup based on DEGs (Figure 4).

qRT-PCR analysis of select genes verified expression array results. All seven genes analyzed for each of the five cell lines had a direction of fold change in agreement with the expression data, with log fold change ratios calculated from qPCR results showing a very strong correlation to expression array log fold change \( R^2=0.96 \), Figure 5). In addition, the magnitude of expression fold change calculated by qRT-PCR correlated strongly and positively with magnitude of copy number loss/gain, as calculated by oaCGH \( R^2=0.76 \), Figure 5).

Using the DAVID tool for gene ontology (GO) and pathway analysis, DEG enrichment in functional pathways was evaluated. Among underexpressed genes (relative fold change ≤ -2), the GO term “lipid metabolism” was the only GO term enriched \( p=0.03 \) in our dataset, with 189 genes underexpressed in the cell lines. Among overexpressed genes (relative fold change ≥ +2), “DNA replication, recombination, and repair” was the most highly
enriched term (p=0.01), with 13 overexpressed genes. When evaluating all DEGs, “DNA replication and repair” was the single most commonly dysregulated functional ontology, represented by 10% of all cell line DEGs.

KEGG pathway analysis in DAVID highlighted four pathways enriched for underexpressed genes: metabolism of xenobiotics by cytochrome p450, glutathione metabolism, drug metabolism, and fatty acid metabolism, resulting in a KEGG pathway clustering of these pathways with an enrichment score indicative of biological significance (Bonferroni=0.05, enrichment score=2.34). Additionally, 4% of genes with five-fold greater expression than normal urothelium were involved in cell cycle regulatory pathways (p=0.0004), as were 1.8% of all upregulated genes (p=0.00003). DAVID chromosome association analysis found 4.7% of overexpressed genes (68 genes) were found on CFA 13 and 4.2% on CFA 10 (61 genes). Similarly, 3.6% (66 genes) of underexpressed genes were located on CFA 12 and 1.2% on CFA 19 (23 genes).

Discussion

Canine UC represents the most common urogenital cancer in dogs and, due to the aggressive nature of the tumor, diagnosis conveys a guarded prognosis for the patient (Mutsaers et al., 2003). To date, curative therapies are lacking for UC, which is likely due in part to delayed tumor diagnosis and a lack of molecular understanding of tumor pathogenesis. It is known, however, that canine UC closely resembles prognostically unfavorable invasive human UC, making research in canine bladder cancer potentially valuable in improving the outlook for human UC patients (Dhawan et al., 2009; Knapp et al., 2000; Knapp et al., 2014; Mutsaers et al., 2003). As a result, a valid in vitro cell line model would be advantageous to both species and could potentiate investigation of more precise
molecular mechanisms involved in UC pathogenesis, including the development of therapies to target them. Previously, five canine UC cell lines were established for *in vitro* study of the disease, and four of five retain tumorigenicity in mice (Dhawan et al., 2009). With the potential to facilitate translational studies, care should be taken to determine that these cell lines provide a high clinical predictive value. Our study sought to evaluate: 1) the genomic similarity between primary UC and cell lines, 2) the genomic integrity of the cell lines over time, and 3) the gene expression landscape of canine UC cell lines.

Our results show that, although variation exists in the frequency of copy number aberrations between the cell lines and primary tumors, the most frequent copy number gains and losses are preserved in the cell lines, specifically loss of regions of CFA 19, and gain of regions of CFA 13 and 36 (Shapiro et al., 2015). Among primary tumors, 100% of the canine UC cases possess at least one of these aberrations, 94% possess two, and 68% possess all three aberrations, emphasizing the diagnostic potential of copy number aberrations and a potentially critical role of these chromosomes in tumorigenesis. Similarly, all five cell lines had at least one of these aberrations (100%), four had at least two (80%), and two had all three aberrations (40%). When oaCGH-called aberrations among the 31 primary tumors and five cell lines were clustered, no cell line outgroup was seen, suggesting the cell lines were more like primary tumors than one another other and demonstrating a lack of overwhelming culture artifact in their genomic profiles. Thus, the cell lines maintain the signature aberrations of the primary tumor and provide an *in vitro* tool with which to further investigate the significance of these aberrations in canine UC.

The relevance of the gains on CFA13 and 36 are further emphasized by GISTIC analysis. GISTIC analysis, which focuses on regions containing genes repeatedly implicated in neoplastic transformation and tumor growth, highlighted only seven regions aberrant in
the cell lines: amplified regions on CFA10, 36, and 38 and gains on CFA13. In primary
tumors, however, GISTIC analysis implicated numerous regions, including the majority
(~71%) of those seen in the cell lines. These results not only emphasize the importance of
genes highlighted by GISTIC analysis, but may also suggest that a number of the mutations
seen in the primary tumor are likely passenger mutations unessential for tumor maintenance
in the cell lines. Thus, we may be able to utilize the less genomically complex cell lines to
hone in on driver mutations crucial for UC pathogenesis, maintenance, and progression.

In addition to major chromosome aberrations, chromothriptic events represent
another manifestation of copy number change. First described in 2011, chromothriptic-like
events represent small genomic regions comprised of numerous adjacent and alternating
copy number changes (P. J. Stephens et al., 2011). Chromothriptic-like events were
previously found to be prevalent throughout the genome of primary canine UC, particularly
on CFA36, 10, 16, 4 and 7 (Shapiro et al., 2015). Similarly, the cell lines show a high
frequency and number of chromothriptic-like events throughout the genome, with 21 of 39
chromosomes demonstrating recurrent chromothriptic-like events in at least two cell lines.
These 21 chromosome include all of those with a high frequency of chromothripsis in
primary tumors, except CFA16. The prevalence of chromothriptic-like events in the cell line
genomes suggests inherent genome instability along with a massive chromosome shattering
event. For example, tandem duplications observed on CFA10, 36, and 38, as well as
translocations among these chromosomes (t(36;38)), may have arisen due to such
chromothriptic events early in or leading to neoplastic transformation. Although arising from
an unknown etiology, current hypotheses of chromothripsis in cancer implicate mutagenic
exogenous compounds, which induce double-stranded DNA breaks throughout the genome,
and chromosome missegregation (Holland & Cleveland, 2012; Philip J. Stephens et al.,
This type of chromosome shattering can lead to massive regional amplification, as well as losses and translocations, due to improper chromosome rejoining. Particularly when considering the potential etiology of risk-associated carcinogenic compounds, chromothripsis is a hugely interesting phenomenon in UC.

In addition to validating oaCGH-called aberrations, metaphase FISH of the cell lines allowed us to explore karyotypic reorganization occurring in canine UC, including recurrent tandem duplications responsible for CFA36 amplification and a CFA13 duplication and centromeric fusion often responsible for increases in CFA13. In our cell line analyses, copies of CFA10, 36, and 38 per cell were often too numerous to accurately count (>10-30 clearly distinguishable signals, particularly in K9TCC-Pu-Mx). Copy number aberrations of CFA13 and 19, however, are more conservatively aberrant in and cell lines, as is observed in primary tumors (Shapiro et al., 2015). Furthermore, in all cell lines, abnormally metacentric chromosome were seen, suggesting prior mitotic dysfunction and/or breakage-fusion-bridge cycles and leading to the formation of new hybrid chromosomes.

The karyotype of the cell lines, excluding K9TCC, further confirmed previous findings that canine UCs are often tetraploid (>50%) (Clemo, DeNicola, Carlton, Morrison, & Walker, 1994). Since oaCGH is blind to ploidy, the correlation between oaCGH-called aberrations and chromosome copy number requires knowledge of ploidy status, making a copy number neutral control critical to appropriate copy number interpretation. CFA11 was identified as copy number neutral using oaCGH values and FISH analysis; CGH values suggestive of neither a loss nor gain (Log2≈0 for all cell lines) and chromosome structure appeared normal. In polyploid cell lines, four copies of CFA11 were consistently observed, supporting a tetraploid genome. Similarly, when oaCGH implied a copy number loss, one to three copies were observed, showing a relative, but often not absolute, loss of genetic material. In
the case of CFA19, only in diploid K9TCC did the chromosome count consistently reflect an absolute copy number loss \((n<2)\). Relative to a duplicated genome, however, two copies of CFA 19 (K9TCC-PU-An) reflected a loss rather than a normal diploid number of \(\log_2=0\).

Cells were repeatedly passaged (>10 passages) in order to determine the stability of the UC genomic profile over time. No to little change was evident in oaCGH profiles or chromosome structure of cell lines after passaging cells from early to mid-late stage. Only three regions on CFA1, 2, and 9 were significantly different between early and late passages (gained), representing less than 0.006% of the entire genome in length. Although the region on CFA9 contains no known genes, the regions on CFA1 and 2 contain genes potentially relevant to the culture environment: insulin-like growth factor 2 receptor \((IGF2R, CFA1)\) and a protocadherin cluster \((PCDH, CFA2)\). Increased \(IGF2R\) and \(PCDH\) are both potential adaptations to the culture environment, allowing improved utilization of media sugars and culture flask adhesion, respectively. Nevertheless, such few aberrations acquired over 10+ passages represent a very minor effect of cell culture and repeated passage. This characteristic of the cell lines allows experimentation and analysis over time without concern of culture-induced changes, a valuable feature for translational studies.

We should note that several factors might influence our results. One is that cell lines are \textit{ex vivo} models. Thus, some aberrations detected in cell lines may be due to the altered culture environment. We were unable to analyze the primary tumor from which the cell lines were derived, precluding our ability to detect initial culture-related genomic changes. Secondly, since oaCGH is a cell population-based analysis, it is plausible that rare aberrations evaded detection by oaCGH, but would become more prominent with continued neoplastic cell proliferation. However, our cell lines retained the major copy number changes
seen in primary tumors, even after ten or more passages, leading us to conclude cell lines represent a good model for UC in vitro.

Factors incriminated in the development of UC include exposure to cyclic amines, including carcinogens in cigarette smoke, estrogen, and cyclophosphamide, as well as obesity (Brody, 2012; Glickman et al., 2004; Knapp et al., 2000; Knapp et al., 2014; Lawson, Vasilaras, De Vries, Mactaggart, & Nicol, 2008; Mutsaers et al., 2003; Talaska, 2003; Y. Zhang, 2013). Interestingly, DAVID analysis of underexpressed genes in our dataset revealed GO enrichment of genes essential to the metabolism of such lipophilic cyclic compounds. Underexpressed genes were also enriched in metabolic KEGG pathways, most notably pathways involved in xenobiotic metabolism. Decreased expression of metabolic genes suggests that toxic compounds are not being handled properly by the bladder epithelium, potentially leading to a buildup of toxic compounds in the urine and increased exposure of the urothelium to carcinogens. In particular, carboxylesterase-1 (CES-1; CFA2: 60.3 Mb, CanFam3.1; fold change = -633.5) is involved in metabolism of organophosphate and pyrethroid insecticides, both associated with UC development (Nishi et al., 2006; Wheelock et al., 2006; Yang et al., 2009). Similarly, decreased to undetectable levels of urothelial CES1 are associated with UC in humans (Pontén, Jirström, & Uhlen, 2008). In addition, CES enzymatic activity is highly variable among human individuals and may be related to canine breed-associated UC susceptibilities (Merali, Ross, & Pare, 2014; Sanghani, Sanghani, Schiel, & Bosron, 2009).

Of further interest, Cytochrome P450 2C19 (CYP2C19; CFA28:8.7 Mb; fold change= -8.1) is a potentially relevant metabolic gene central to the enriched cytochrome p450 KEGG pathway. CYP2C19 is one of the most important cytochrome p450 enzymes and is responsible for the metabolism of numerous cyclic amine xenobiotics, including the
pyrethroid-derivatives of hydantoin present in common insecticides (Suzuki et al., 2004). Similar to CES1, genetic polymorphisms of CYP2C19 are associated with reduced metabolic capacity in humans, suggesting similar phenomena may occur among genetically distinct dog breeds (Ruiter et al., 2010; Tamer et al., 2006). Our data corroborate previous speculations regarding the involvement of metabolic pathways in canine UC development and highlights a need for closer investigation.

Among overexpressed genes, GO terms were enriched for DNA replication, recombination, and repair. Similarly, KEGG analysis highlighted cell cycle regulator pathways, in which 4% of genes with expression five-fold above that of normal urothelium were involved (1.8% of all overexpressed genes). Of note is one of the most highly overexpressed genes in our dataset and a key gene in DNA replication and cell cycle regulation—PTTG1 (CFA4: 50.3 Mb; fold change= +57.5). As a result of its functions as a sister chromatid securin, microtubule nucleation regulator, and AKT activator, among others, PTTG1 overexpression potentiates tumor proliferation and invasion, as well as chromosome instability (Li et al., 2013; Moreno-Mateos et al., 2011; Yoon et al., 2012). Interestingly, activating mutations in FGFR3, an upstream regulator of AKT activity, are implicated in constitutive activation of the AKT pathway commonly seen in human UC (Juanpere et al., 2012). PTTG1 has the potential to be a major driver of AKT activation in canine UC, providing a valuable therapeutic target and highlighting pathway dysregulation similarities in human and dog UC.

Our expression data also emphasized the potential role of chromosome copy number on gene dosage and, therefore, gene expression. When analyzing genes located in regions of copy number aberration, it was shown that gene expression often varied directly with copy number change ($R^2=0.76$). DAVID chromosome analysis showed enrichment of
underexpressed genes on CFA19, which is frequently lost in UC cell lines. CFA 19 contains the gene for histamine N-methyltransferase (HNMT; CFA19: 40.7 Mb), a gene highly under expressed in our cell lines (fold change = -29.6). Although unreported in bladder cancer, reduced HNMT transcription has been reported in other human carcinomas and posited to be involved in tumorigenesis (Kierska, Szymanska, & Maslinski, 1992). Decreased levels of HNMT lead to local increases of angiogenesis-promoting PTGS2 (COX2). PTGS2 levels are increased in both canine and human UC, and this gene is similarly overexpressed in our cell line data (fold change= +76.9), providing a further mechanistic role for COX-2 inhibitors in UC treatment (Deborah W. Knapp et al., 1994; Mohammed et al., 1999). Additionally, COX2 is a downstream effector of an activated AKT pathway, again suggesting a conservation of AKT overactivation in both canine and human UC development.

Similarly, gain of CFA 13 led to chromosome enrichment of overexpressed genes (4.7% of overexpressed genes), including the oncogenic transcription factor MYC (CFA13: 25.2 Mb). Responsible for expediting the cell cycle and bypassing critical checkpoints, MYC has been implicated in numerous human cancers, including bladder cancer (Sauter et al., 1995) and is overexpressed in the canine UC cell lines (fold change=+8.5), along with downstream targets cyclin D1 and D2 (fold change=+2.2 and +14.2, respectively). The increase in downstream targets suggests amplified MYC is not only over transcribed, but also translated and functionally active. Furthermore, our expression data suggests a high level of conservation in DEGs between human and canine UC (Table 6), providing a potentially valuable model of the human disease and reinforcing previous findings that canine and human UC are molecularly, clinically, and histopathologically similar.

Despite strong correlations between copy number gain/loss and differential gene expression (Figure 6), it is not known if increased numbers of the genomic transcript lead
directly to increased mRNA levels. In fact, PABPC1, a gene located in the region of shared high frequency gain on HSA8/CFA13 in human and canine UC, was actually underexpressed (average fold change = -2.6) in the cell lines (Shapiro et al., 2015). However, PABPC1 is highly expressed, at both mRNA and protein levels, in normal urothelium, potentially obscuring subtle differences in expression while reducing their physiologic significance (Mutch, Berger, Mansourian, Rytz, & Roberts, 2002; Uhlén et al., 2015). Additionally, while human expression studies have shown relative overexpression of PABPC1 at the mRNA level, protein studies have shown a relative decrease in protein, espousing the need for protein evaluation alongside mRNA quantification (Chen, Feng, & Xu, 2011). We cannot discount the importance of epigenetic factors in determining expression. Moreover, particularly in the case of tandem duplications and structural aberrations, failure to duplicate or disruption of gene promoters and enhancers may alter predicted gene expression. Post-transcriptional gene regulation may also play a role due to possible alterations in silencing RNA. Regardless, it is reasonable to assume genomic gene dosage plays a role in determining corresponding mRNA levels.

**Conclusions**

The recurrence of specific genomic aberrations in canine UC emphasizes the importance of continued molecular research, particularly in predisposed dog breeds with reduced genetic variation and in relation to carcinogenic risk factors. A treatment found to be effective in canine cell lines, which then is effective in dogs, increases the clinical predictive value of the cell lines and, therefore, their value to biomedical research (Dhawan et al., 2009). The preservation of primary tumor aberrations in the cell line model provides an accessible and accurate means of performing genetic experiments *in vitro*, making the cell
lines a valuable resource for translational UC research. Future research should involve utilizing the UC cell lines in a functional manner to elucidate mechanisms of UC pathogenesis.

Acknowledgements

This study was supported by the NCSU Cancer Genomics Fund (MB). Additional support for SS came from an NIH-T35 grant, a Triangle Community Foundation Hitching's Young Investigator award, and the Comparative Biomedical Sciences DVM/PhD Program at NC State University College of Veterinary Medicine.

References


Table 3.1. Characteristics of five canine UC cell lines.

Five cell lines, derived from primary biopsy specimen, were evaluated in this study. Cell line identifier, initial passage, breed, and sex are shown. Chromosomes from metaphase spreads (n=30) of each cell line were counted to determine an average chromosome number. Recurrent structural changes realized upon FISH evaluation are indicated (TD=tandem duplication, t=translocation, f=fusion) along with the associated chromosome (CFA#).

<table>
<thead>
<tr>
<th>Cell Line ID</th>
<th>Initial Passage</th>
<th>Breed</th>
<th>Sex</th>
<th>Avg. Chromosome #</th>
<th>Specific Structural Changes</th>
</tr>
</thead>
<tbody>
<tr>
<td>K9TCC-PU-An</td>
<td>p3</td>
<td>Scottish Terrier</td>
<td>FS</td>
<td>131.6</td>
<td>TD(10,36), (36;38), f(13;13)</td>
</tr>
<tr>
<td>K9TCC-PU-In</td>
<td>p1</td>
<td>German Shepherd</td>
<td>FS</td>
<td>103.3</td>
<td>TD(10,36), f(13;13)</td>
</tr>
<tr>
<td>K9TCC-PU-Mx</td>
<td>p5</td>
<td>German Shepherd</td>
<td>FS</td>
<td>134.6</td>
<td>TD(10,36)</td>
</tr>
<tr>
<td>K9TCC-PU-Sh</td>
<td>p6</td>
<td>Collie</td>
<td>FS</td>
<td>105.5</td>
<td>TD(36,38), f(36;38),</td>
</tr>
<tr>
<td>K9TCC</td>
<td>p14</td>
<td>Mixed Breed</td>
<td>FS</td>
<td>76.2</td>
<td>TD(10)</td>
</tr>
</tbody>
</table>
Ten BAC clones were selected to probe regions shown to be highly aberrant in the cell lines (>50% penetrant). A copy number neutral region on CFA11 was chosen as an internal control to ensure correct interpretation of locus copy number. The BAC address, genomic location, and associated fluorochrome for each probe are shown.

<table>
<thead>
<tr>
<th>BAC Address</th>
<th>Region Probed</th>
<th>Fluorochrome</th>
<th>oaCGH-called Aberration</th>
</tr>
</thead>
<tbody>
<tr>
<td>326N03</td>
<td>chr1:35846825-36021610</td>
<td>Red</td>
<td>Loss</td>
</tr>
<tr>
<td>307I06</td>
<td>chr6:49193262-49385361</td>
<td>Green</td>
<td>Loss</td>
</tr>
<tr>
<td>326H08</td>
<td>chr10:13936488-14128322</td>
<td>Cy5</td>
<td>Amplification</td>
</tr>
<tr>
<td>126F01</td>
<td>chr12:52174704-52401789</td>
<td>Gold</td>
<td>Loss</td>
</tr>
<tr>
<td>186I06</td>
<td>chr13:38344516-38552798</td>
<td>Aqua</td>
<td>Gain</td>
</tr>
<tr>
<td>332N02</td>
<td>chr19:31345273-31534341</td>
<td>Red</td>
<td>Loss</td>
</tr>
<tr>
<td>313D22</td>
<td>chr21:5212350-5387435</td>
<td>Green</td>
<td>Loss</td>
</tr>
<tr>
<td>307D14</td>
<td>chr33:5409680-5589439</td>
<td>Red</td>
<td>Loss</td>
</tr>
<tr>
<td>199F16</td>
<td>chr36:7057670-7276062</td>
<td>Cy5</td>
<td>Amplification</td>
</tr>
<tr>
<td>328D10</td>
<td>chr38:25164963-25365710</td>
<td>Gold</td>
<td>Amplification</td>
</tr>
<tr>
<td>326L05</td>
<td>chr11:20496757-20706739</td>
<td>Aqua</td>
<td>Neutral</td>
</tr>
</tbody>
</table>
mRNA levels of genes located within seven regions of copy number aberration were evaluated by qRT-PCR to evaluate the relationship between genomic copy number and expression. The target gene, genomic location, and primer pair used are shown.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Location</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>RNPC3</td>
<td>chr6:50072550-50098045:-1</td>
<td>5’-GGGGCGACCGGCCCTTCTA-3’</td>
<td>5’-GACAGGACCGGACTG-3’</td>
</tr>
<tr>
<td>MDM2</td>
<td>chr10:13920606-13946580:1</td>
<td>5’-ACGGCAGAGAAAGGCCAAATT-3’</td>
<td>5’-GGCTCTCTGGTACTCAGT-3’</td>
</tr>
<tr>
<td>EIF2C2</td>
<td>chr13:38222456-38271623:-1</td>
<td>5’-CAAGGCAGTCCAGGTTC-3’</td>
<td>5’-GGCACTGTGCTGACTG-3’</td>
</tr>
<tr>
<td>RALB</td>
<td>chr19:32842484-32862087:-1</td>
<td>5’-GGGTCCCTGCTCTCTCAAC-3’</td>
<td>5’-CACAACCTCCACACAAG-3’</td>
</tr>
<tr>
<td>CLNS1A</td>
<td>chr21:24140052-24163815:1</td>
<td>5’-CCCTGGTCCTGCTGGCTCTG-3’</td>
<td>5’-GCTCGGTTCTCCAGGCTG-3’</td>
</tr>
<tr>
<td>STX19</td>
<td>chr33:4727847-4728760:-1</td>
<td>5’-CTGCTATTTTCGGCCAAATTT-3’</td>
<td>5’-GCACCTTTTTTTCCAGCAACC-3’</td>
</tr>
<tr>
<td>TAGLN2</td>
<td>chr38:25240217-25241796:1</td>
<td>5’-TGCGGAACCTGGACAGATCTTG-3’</td>
<td>5’-ACACAGAACGGGCCATCT-3’</td>
</tr>
<tr>
<td>RPL32</td>
<td>chr18:10198892-10204028:-1</td>
<td>5’-ATGCCCAACATTGGTATGG-3’</td>
<td>5’-ATGCCCAACATTGGTATGG-3’</td>
</tr>
</tbody>
</table>
Table 3.4. **Comparisons analysis of primary tumors and cell lines.**

oaCGH data from primary tumors and cell lines were simultaneously analyzed and compared using the Comparisons algorithm (Nexus Copy Number, Biodiscovery). Few aberrant regions were evident in only the primary tumors or the cell lines without being present in the other.

<table>
<thead>
<tr>
<th>Region</th>
<th>Cytoband Location</th>
<th>Event</th>
<th>Region Length</th>
<th>% of Cell Lines</th>
<th>% of Primary Tumors</th>
<th>Difference</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>chr1:78,045,153-78,183,158</td>
<td>q25.3</td>
<td>CN Gain</td>
<td>138005</td>
<td>40</td>
<td>0</td>
<td>40</td>
<td>0.015873016</td>
</tr>
<tr>
<td>chr2:30,342,739-30,496,780</td>
<td>q22</td>
<td>CN Gain</td>
<td>154041</td>
<td>40</td>
<td>0</td>
<td>40</td>
<td>0.015873016</td>
</tr>
<tr>
<td>chr5:15,451,858-16,425,474</td>
<td>q13 - q14.1</td>
<td>CN Gain</td>
<td>973616</td>
<td>40</td>
<td>0</td>
<td>40</td>
<td>0.015873016</td>
</tr>
<tr>
<td>chr5:37,555,012-38,093,648</td>
<td>q22 - q23</td>
<td>CN Gain</td>
<td>538636</td>
<td>40</td>
<td>0</td>
<td>40</td>
<td>0.015873016</td>
</tr>
<tr>
<td>chr5:38,455,746-38,838,561</td>
<td>q23</td>
<td>CN Gain</td>
<td>382815</td>
<td>40</td>
<td>0</td>
<td>40</td>
<td>0.015873016</td>
</tr>
<tr>
<td>chr5:54,138,826-54,328,601</td>
<td>q31</td>
<td>CN Gain</td>
<td>189775</td>
<td>40</td>
<td>0</td>
<td>40</td>
<td>0.015873016</td>
</tr>
<tr>
<td>chr5:71,439,567-71,702,697</td>
<td>q33</td>
<td>CN Gain</td>
<td>263130</td>
<td>60</td>
<td>0</td>
<td>60</td>
<td>0.00140056</td>
</tr>
<tr>
<td>chr18:43,829,143-43,918,733</td>
<td>q24</td>
<td>CN Loss</td>
<td>89590</td>
<td>0</td>
<td>58.06451613</td>
<td>-58.06451613</td>
<td>0.045454545</td>
</tr>
<tr>
<td>chr18:50,966,845-50,979,662</td>
<td>q25.1</td>
<td>CN Loss</td>
<td>12817</td>
<td>0</td>
<td>54.83870968</td>
<td>-54.83870968</td>
<td>0.047258297</td>
</tr>
<tr>
<td>chr18:58,785,242-58,847,127</td>
<td>q25.3</td>
<td>CN Loss</td>
<td>61885</td>
<td>0</td>
<td>61.29032258</td>
<td>-61.29032258</td>
<td>0.016414141</td>
</tr>
<tr>
<td>chr20:6,150,393-6,591,988</td>
<td>q11</td>
<td>CN Loss</td>
<td>441595</td>
<td>40</td>
<td>0</td>
<td>40</td>
<td>0.015873016</td>
</tr>
<tr>
<td>chr26:28,058,352-28,168,920</td>
<td>q23</td>
<td>CN Loss</td>
<td>110568</td>
<td>0</td>
<td>61.29032258</td>
<td>-61.29032258</td>
<td>0.016414141</td>
</tr>
<tr>
<td>chr26:30,039,800-30,049,050</td>
<td>q24</td>
<td>CN Loss</td>
<td>9250</td>
<td>0</td>
<td>54.83870968</td>
<td>-54.83870968</td>
<td>0.047258297</td>
</tr>
<tr>
<td>chr36:12,594,587-12,681,903</td>
<td>q13</td>
<td>CN Gain</td>
<td>87316</td>
<td>0</td>
<td>70.96774194</td>
<td>-70.96774194</td>
<td>0.005310458</td>
</tr>
<tr>
<td>chr36:18,375,715-18,902,004</td>
<td>q14</td>
<td>CN Loss</td>
<td>526289</td>
<td>40</td>
<td>0</td>
<td>40</td>
<td>0.015873016</td>
</tr>
<tr>
<td>chr36:21,250,955-21,625,961</td>
<td>q14</td>
<td>CN Loss</td>
<td>375006</td>
<td>40</td>
<td>0</td>
<td>40</td>
<td>0.015873016</td>
</tr>
<tr>
<td>chr38:5,907,007-6,054,242</td>
<td>q12</td>
<td>CN Gain</td>
<td>147235</td>
<td>0</td>
<td>54.83870968</td>
<td>-54.83870968</td>
<td>0.047258297</td>
</tr>
</tbody>
</table>
Table 3.5. *Regions implicated in cell line GISTIC analysis represent regions relevant to neoplasia.*

Regions are shown indicating the called aberration (copy number (CN) loss or gain), Q-bound, and G-score. The G-score reflects the frequency as well as magnitude of the aberration in the cohort, while the Q-bound indicates significance (α=0.05). Aberrant regions were located on CFA10, CFA13, CFA36, and CFA38. CFA13 and CFA36 are of diagnostic relevance in primary UC and were similarly aberrant in cell lines.

<table>
<thead>
<tr>
<th>Genomic Region</th>
<th>Aberration Type</th>
<th>Q-Bound</th>
<th>G-Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>chr10:13,614,516-14,400,849</td>
<td>CN Gain</td>
<td>8.22E-06</td>
<td>9.606238</td>
</tr>
<tr>
<td>chr10:17,743,915-20,676,296</td>
<td>CN Loss</td>
<td>0.02737</td>
<td>7.847499</td>
</tr>
<tr>
<td>chr10:44,389,882-71,751,790</td>
<td>CN Gain</td>
<td>0.005379</td>
<td>5.952698</td>
</tr>
<tr>
<td>chr10:5,318,483-7,102,150</td>
<td>CN Gain</td>
<td>0.005379</td>
<td>5.990278</td>
</tr>
<tr>
<td>chr13:3,033,844-66,047,830</td>
<td>CN Gain</td>
<td>0.005379</td>
<td>5.576475</td>
</tr>
<tr>
<td>chr36:22,492,857-25,421,734</td>
<td>CN Gain</td>
<td>0.005379</td>
<td>5.578811</td>
</tr>
<tr>
<td>chr38:3,874,100-4,368,891</td>
<td>CN Gain</td>
<td>0.005379</td>
<td>5.403421</td>
</tr>
</tbody>
</table>
Table 3.6. Compiled FISH and oaCGH Log2 data. Log2 ratio values obtained from oaCGH analysis of cell lines and calculated by raw FISH data are shown.

For each cell line, a Mann-Whitney U test was conducted to evaluate consistency of data between both analysis methods (p<0.05). In all cases, the mean Log2 ratio obtained from oaCGH and FISH was not significantly different (p≥0.32 in all cases), validating oaCGH results and demonstrating the utility of oaCGH in large-scale copy number analysis. Shaded cells contain Log2 ratios demonstrating significant gains or losses (Log2<-0.234 or Log2>0.201).

<table>
<thead>
<tr>
<th></th>
<th>CFA1</th>
<th>CFA6</th>
<th>CFA10</th>
<th>CFA12</th>
<th>CFA13</th>
<th>CFA19</th>
<th>CFA21</th>
<th>CFA33</th>
<th>CFA36</th>
<th>CFA38</th>
<th>CFA11</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>K9TCC-Pu-An</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.49</td>
</tr>
<tr>
<td>oaCGH Log2</td>
<td>-0.40</td>
<td>-0.50</td>
<td>2.94</td>
<td>-0.30</td>
<td>1.23</td>
<td>-0.96</td>
<td>-0.40</td>
<td>-0.60</td>
<td>0.60</td>
<td>1.20</td>
<td>0.00</td>
<td></td>
</tr>
<tr>
<td>FISH Log2</td>
<td>-0.37</td>
<td>0.05</td>
<td>1.52</td>
<td>-0.33</td>
<td>1.14</td>
<td>-0.93</td>
<td>-0.36</td>
<td>-0.19</td>
<td>1.23</td>
<td>0.90</td>
<td>0.00</td>
<td></td>
</tr>
<tr>
<td>K9TCC-Pu-In</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.32</td>
</tr>
<tr>
<td>oaCGH Log2</td>
<td>-0.40</td>
<td>-0.53</td>
<td>1.40</td>
<td>0.00</td>
<td>0.52</td>
<td>-1.00</td>
<td>-0.40</td>
<td>-0.60</td>
<td>0.40</td>
<td>0.43</td>
<td>0.00</td>
<td></td>
</tr>
<tr>
<td>FISH Log2</td>
<td>3.40</td>
<td>3.73</td>
<td>11.00</td>
<td>4.07</td>
<td>5.62</td>
<td>2.50</td>
<td>4.10</td>
<td>3.00</td>
<td>6.87</td>
<td>6.77</td>
<td>3.79</td>
<td></td>
</tr>
<tr>
<td>K9TCC</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.65</td>
</tr>
<tr>
<td>oaCGH Log2</td>
<td>0.13</td>
<td>-0.30</td>
<td>2.40</td>
<td>-1.00</td>
<td>0.96</td>
<td>-1.00</td>
<td>0.00</td>
<td>0.07</td>
<td>0.00</td>
<td>0.60</td>
<td>0.00</td>
<td></td>
</tr>
<tr>
<td>FISH Log2</td>
<td>2.23</td>
<td>2.10</td>
<td>6.96</td>
<td>1.13</td>
<td>3.96</td>
<td>1.06</td>
<td>2.00</td>
<td>2.60</td>
<td>1.97</td>
<td>2.03</td>
<td>1.97</td>
<td></td>
</tr>
<tr>
<td>K9TCC-Pu-Mx</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.76</td>
</tr>
<tr>
<td>oaCGH Log2</td>
<td>0.04</td>
<td>0.19</td>
<td>0.04</td>
<td>-0.20</td>
<td>0.90</td>
<td>0.00</td>
<td>-0.20</td>
<td>-0.22</td>
<td>1.90</td>
<td>1.10</td>
<td>0.00</td>
<td></td>
</tr>
<tr>
<td>FISH Log2</td>
<td>3.80</td>
<td>4.80</td>
<td>4.30</td>
<td>2.88</td>
<td>6.61</td>
<td>3.90</td>
<td>2.50</td>
<td>3.57</td>
<td>11.97</td>
<td>5.71</td>
<td>3.88</td>
<td></td>
</tr>
<tr>
<td>FISH Log2</td>
<td>-0.03</td>
<td>0.31</td>
<td>0.15</td>
<td>-0.43</td>
<td>0.77</td>
<td>0.01</td>
<td>-0.63</td>
<td>-0.12</td>
<td>1.63</td>
<td>0.56</td>
<td>0.00</td>
<td></td>
</tr>
<tr>
<td>K9TCC-Pu-Sh</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1.00</td>
</tr>
<tr>
<td>oaCGH Log2</td>
<td>-1.00</td>
<td>-1.30</td>
<td>1.30</td>
<td>-1.00</td>
<td>0.93</td>
<td>0.00</td>
<td>-1.80</td>
<td>-1.05</td>
<td>0.20</td>
<td>-1.30</td>
<td>0.00</td>
<td></td>
</tr>
<tr>
<td>FISH Log2</td>
<td>1.93</td>
<td>1.25</td>
<td>6.33</td>
<td>1.47</td>
<td>5.34</td>
<td>3.14</td>
<td>0.64</td>
<td>2.00</td>
<td>6.19</td>
<td>2.13</td>
<td>3.68</td>
<td></td>
</tr>
<tr>
<td>FISH Log2</td>
<td>-0.93</td>
<td>-1.56</td>
<td>0.78</td>
<td>-1.32</td>
<td>0.54</td>
<td>-0.23</td>
<td>-2.52</td>
<td>-0.88</td>
<td>0.75</td>
<td>-0.79</td>
<td>0.00</td>
<td></td>
</tr>
</tbody>
</table>
Table 3.7. *Gene expression microarray results.*

Numerous genes were dysregulated in the cell lines when compared to the expression profile of healthy canine urothelium. The genes listed were selected based on their magnitude of fold change, oncogenic potential, and relevance in human UC (see asterisks). The specific cell lines differentially expressing each gene are listed.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Location</th>
<th>Lines Differentially Expressing</th>
<th>Average Fold Change</th>
<th>Gene</th>
<th>Location</th>
<th>Lines Differentially Expressing</th>
<th>Average Fold Change</th>
</tr>
</thead>
<tbody>
<tr>
<td>CES1*</td>
<td>chr2</td>
<td>ALL</td>
<td>-633.5</td>
<td>BIRC5*</td>
<td>chr9</td>
<td>In, An, Mx, K9TCC</td>
<td>+3.6</td>
</tr>
<tr>
<td>HNMT</td>
<td>chr19</td>
<td>In, Sh, Mx, K9TCC</td>
<td>-29.6</td>
<td>RAF1*</td>
<td>chr20</td>
<td>ALL</td>
<td>+4.1</td>
</tr>
<tr>
<td>DBC1*</td>
<td>chr11</td>
<td>ALL</td>
<td>-13.6</td>
<td>CLU*</td>
<td>chr25</td>
<td>ALL</td>
<td>+5.5</td>
</tr>
<tr>
<td>CDKN2B*</td>
<td>chr11</td>
<td>ALL</td>
<td>-11.8</td>
<td>CCNG2*</td>
<td>chr32</td>
<td>ALL</td>
<td>+6.3</td>
</tr>
<tr>
<td>FHI1*</td>
<td>chr20</td>
<td>ALL</td>
<td>-11.5</td>
<td>MDM2*</td>
<td>chr10</td>
<td>In, Sh, An, K9TCC</td>
<td>+6.4</td>
</tr>
<tr>
<td>CYP2C19*</td>
<td>chr28</td>
<td>ALL</td>
<td>-8.1</td>
<td>MYC*</td>
<td>chr13</td>
<td>ALL</td>
<td>+8.5</td>
</tr>
<tr>
<td>BCL2*</td>
<td>chr1</td>
<td>ALL</td>
<td>-6.6</td>
<td>LAMB3*</td>
<td>chr7</td>
<td>ALL</td>
<td>+12.4</td>
</tr>
<tr>
<td>SRPX*</td>
<td>chrX</td>
<td>In, An, Mx, K9TCC</td>
<td>-6.1</td>
<td>TGFB2*</td>
<td>chr23</td>
<td>ALL</td>
<td>+12.6</td>
</tr>
<tr>
<td>FMO5*</td>
<td>chr17</td>
<td>ALL</td>
<td>-4.9</td>
<td>CCND2*</td>
<td>chr27</td>
<td>ALL</td>
<td>+14.2</td>
</tr>
<tr>
<td>SPARC*</td>
<td>chr4</td>
<td>ALL</td>
<td>-4.5</td>
<td>AURKA*</td>
<td>chr24</td>
<td>ALL</td>
<td>+22.2</td>
</tr>
<tr>
<td>PTEN*</td>
<td>chr26</td>
<td>ALL</td>
<td>-4</td>
<td>TAGLN2*</td>
<td>chr38</td>
<td>ALL</td>
<td>+25.3</td>
</tr>
<tr>
<td>BMP7*</td>
<td>chr24</td>
<td>An, Mx, K9TCC</td>
<td>-3.4</td>
<td>PTTG1*</td>
<td>chr4</td>
<td>ALL</td>
<td>+57.5</td>
</tr>
<tr>
<td>DAPK1*</td>
<td>chr1</td>
<td>An, Mx</td>
<td>-2.8</td>
<td>CDK1*</td>
<td>chr4</td>
<td>ALL</td>
<td>+72.5</td>
</tr>
<tr>
<td>VHL*</td>
<td>chr20</td>
<td>ALL</td>
<td>-2.1</td>
<td>PTGS2*</td>
<td>chr7</td>
<td>ALL</td>
<td>+78.9</td>
</tr>
</tbody>
</table>
Figure 3.1. *Genomic aberrations of canine UC cell lines recapitulate those seen in primary tumors.*

Cell lines (n=5) were analyzed via oaCGH at an early stage (<p16) and aberrations called based on Log2 ratios relative to normal canine DNA (A). When compared with 31 primary tumors previously arrayed (B), aberrations, including the diagnostically relevant gains of CFA13 and 36 and loss of CFA19, were shared between the two cohorts, validating the use of canine UC cell lines in genomic studies. When clustered by oaCGH aberration profiles (C), the cell lines (asterisked; TCC2-6) clustered among the primary tumors (no asterisk; TCC7-50) rather than appearing as an outgroup, further showing their greater similarity to the primary tumors than one another and showing minimal effect of the culture environment on genome evolution.
The cell lines demonstrate evidence of chromothriptic-like events in tumor evolution.

Chromothriptic-like events were seen throughout the genomes of all five cell lines, involving all but six chromosomes (A). At least three of the cell lines displayed chromothriptic-like events (≥2) on CFA2, 5, 7, 9, 10, 18, 20, 27 and X. The maximum average number of events on a single chromosome was observed on CFA10, with an average of ten chromothriptic-like events per cell line (B). CFA2, 5, 19, 20, 35, and 36 all showed an average of over four events. Thus, complex chromothriptic-like activity is a common event in the UC cell lines, just as in primary tumors.
Tandem duplications, translocations, and fusions were noted throughout cell line karyotypes. According to oaCGH, CFA 10 (Cy5) shows a sharp spike of gain in the probed region containing the *MDM2* gene. FISH suggests this spike might be attributed to tandem duplications of the locus along the chromosome (A). CFA 13 (aqua) appears as two fused copies (B) present multiple times within a karyotype, in addition to what appear to be structurally normal copies and leading to an overall gain. Tandem duplication and locus translocation are both observed on CFA 36 (Cy5) and 38 (gold) (C). The pattern shows an overlap of duplication, in which a copy of the CFA 36 locus is flanked by CFA 38.
Figure 3.4. *Clustered heat map of cell line differential expression.*

Numerous genes were recognized as over or under expressed when comparing cell lines to normal urothelium. Green represents down-regulated genes, while red are up regulated. Clustering along the Y-axis is by probe set, and clustering along the X-axis is according to expression profile similarity. The control (normal) urothelium (columns 1 and 2) clearly segregates from the cell lines, suggesting a vastly different expression profile between normal urothelium and UC cell lines.
Figure 3.5. Gene expression arrays, qRT-PCR, and oaCGH results support one another and emphasize the role of genomic copy number on mRNA levels.

Seven differentially expressed genes located in copy number-aberrant regions (see Table 3) were selected for qRT-PCR analysis to validate microarray results. Correlation was very strong ($R^2=0.96$) between the two methods and direction of fold change was in agreement for all genes, confirming the gene’s differential expression is true. In addition, comparison of qRT-PCR and oaCGH Log2 ratios revealed a strong correlation ($R^2=0.82$), demonstrating the importance of genomic gene dosage on mRNA levels.
CHAPTER 4: INVASION OF THE CELL LINES: THE ROLE OF CANINE PTTG1 IN UC INVASION

Abstract

Urinary bladder cancer, or urothelial carcinoma (UC), represents the most common urinary cancer in both canine and human populations. The highly invasive behavior these tumors, particularly in canine patients, renders them aggressive and associated with a high propensity for metastasis. To date, no efficacious treatments exist for dogs or humans with metastatic disease, leading to a poor disease prognosis and low survival rates. PTTG1 is a carcinoma-associated oncogene whose overexpression initiates tumor cell invasion and metastasis. In UC, previous studies have elucidated the prevalence of PTTG1 overexpression in human tumors and canine cell lines. Here, evaluation of PTTG1 mRNA and protein levels in primary canine tumors, as well as cell lines, was performed. All canine tumors and cell lines overexpressed PTTG1, with protein expression enriched in phenotypically mesenchymal tumor cells. We hypothesized that PTTG1 is essential for initiation of the epithelial-mesenchymal transition (EMT) and the progression to invasion. To determine if PTTG1 plays a role in canine UC invasion, cellular levels of PTTG1 were reduced by siRNA targeting, in conjunction with Matrigel Invasion Chambers. Attenuation of PTTG1 reduced the number of invaded tumor cells, neoplastic cell invasiveness, and cell proliferation and curtailed the epithelial-mesenchymal transition, as assessed by levels of vimentin and E-cadherin. Treatment with vorinostat reduced PTTG1 levels in UC cell lines, as well as their proliferative ability. Our results suggest PTTG1 is involved in the highly invasive nature of canine UC and may, therefore, be a lucrative and readily translatable therapeutic target.
Introduction

Urinary bladder cancer is the fifth most common cancer in men and second most common genitourinary cancer in the United States (Siegel, Miller, & Jemal, 2015). Affecting almost 75,000 Americans each year, 90% of patients are afflicted by neoplasia of the bladder mucosa, or urothelium (Wu, 2005). Urothelial carcinoma (UC) manifests itself as one of two main tumor types: superficial, low-grade or invasive, high-grade. Although ~80% are superficial, the remaining 20% of tumors are invasive at initial diagnosis and carry an extremely poor prognosis; invasive tumors are associated with a high rate of fatal metastasis (>50%) and a 5-year survival rate of only 6% (Siegel et al., 2015). Thus, curtailing the invasive ability and preventing metastasis of neoplastic urothelial cells is at the crux of efficacious UC treatment.

Currently, invasive UC is treated by radical cystectomy, involving removal of the entire bladder and urethra, prostate in men, and uterus and vagina in women. To address metastases, standardized chemotherapeutic protocols exist, but despite high toxicity, 95% of patients fail treatment, leading to rapid clinical deterioration (Calderaro et al., 2014). Although acknowledged as a major research necessity, research in recent decades has failed to elucidate more efficacious therapies for invasive UC. In fact, UC is one of three cancers in the United States to have shown no improvement in the 5-year-survival rate since 1987 (Siegel et al., 2015).

Aside from humans, dogs are the only domestic mammals with a significant incidence of UC, representing ~2% of all canine cancer diagnoses. Over 90% of canine patients have invasive tumors, with nearly 60% demonstrating evidence of distant metastases by the time of death (Knapp et al., 2014). Similar to human invasive tumors, canine patients have a generally guarded prognosis, with an expected one-year survival.
Canine bladder tumors biologically duplicate those of humans, presenting with comparable histopathology, clinical behavior, response to treatment, and genomic signatures (Shapiro et al., 2015). Furthermore, risk factors, including carcinogen exposures, are shared. Consequently, the dog is an optimal model for the study of invasive UC with the potential of advancing the treatment and prognosis for both species.

Previous studies have elucidated a number of molecular targets with potential roles in UC invasion. Many candidate genes are involved in DNA integrity, microtubule stability, cell cycle control, or PI3K/AKT pathway regulation. One oncogene with a well-established role in each of these cellular processes is \textit{PTTG1}, known as securin (Liao et al., 2011; Yoon et al., 2012; Zhou, 2013). When localized to the nucleus, securin acts as a broad-targeting transcription factor increasing secretion of growth factors, including bFGF, VEGF, and IL-8, and the proto-oncogene \textit{MYC} (Yoon et al., 2012). In the cytoplasm, the main function of securin is to facilitate microtubule nucleation and cellular motility (Huang et al., 2012). Increased expression of \textit{PTTG1} is sufficient to induce cellular transformation and the epithelial-mesenchymal transition (EMT) \textit{in vitro and in vivo} and is associated with poor clinical outcomes, including rapid tumor growth and metastasis, in numerous human carcinomas (Huang et al., 2014; Lai, Fang, Chiu, & Huang, 2010; Mora-Santos et al., 2013; Solomon & Burton, 2008; Vlotides, Eigler, & Melmed, 2007; Xia et al., 2013; Yoon et al., 2012; E. Zhang et al., 2014). Thus, \textit{PTTG1} may play a critical role in the pathogenesis of invasive UC.

Although undetectable in healthy urothelium, \textit{PTTG1} is overexpressed in neoplastic urothelium of human patients (Lai et al., 2010). Similarly, we have previously shown that \textit{PTTG1} is highly overexpressed in canine UC cell lines and may, therefore, be overexpressed in the primary tumor (Chapter 3). \textit{In vitro} studies of \textit{PTTG1} in human
prostatic carcinoma, among others, have shown that gene knockdown reduces the proliferative and invasive ability of neoplastic cells and represents a viable therapeutic approach (Huang et al., 2012). Vorinostat, an FDA-approved histone deacetylase inhibitor (HDACi), has been shown to reduce levels of PTTG1 in carcinomas and may represent a rapidly translatable means of therapeutic PTTG1 targeting (Demeure et al., 2013; Hernández, López-Lluch, Navas, & Pintor-Toro, 2009).

Despite the potential relevance of PTTG1 in tumorigenesis, PTTG1 has not been investigated in veterinary medicine in any capacity. In addition, functional roles of PTTG1 in human UC have yet to be assessed. In the current study, we sought to characterize PTTG1 in canine UC and evaluate its viability as a therapeutic target. Levels of cellular PTTG1 were quantified by qRT-PCR, Western blot of cell line protein isolates, and immunohistochemistry (IHC) of formalin-fixed paraffin-embedded (FFPE) tumor specimens. Overexpression of PTTG1 mRNA and protein was observed in canine UC tumors and cell lines. siRNA-mediated knockdown of PTTG1 in canine cell lines resulted in decreased levels of mRNA, allowing analysis of the effect of PTTG1 on invasive ability in an in vitro model. PTTG1 knockdown significantly decreased neoplastic cell invasion, as assessed by Matrigel invasion chambers, and displayed a prominent inhibitory effect on proliferation. Our results not only espouse a role for PTTG1 in the invasion of canine bladder tumors, but elucidate a new therapeutic target in both canine and human UC.

Methods and Materials

Tissue specimens

Formalin-fixed paraffin-embedded (FFPE) biopsy specimens were acquired from previously diagnosed UC patients in the NCSU CVM Pathology archives (n=18, Table 1).
Dogs were of various breeds, including but not limited to those predisposed to UC development. For controls, bladder biopsies from recently euthanized (<1 hour) healthy dogs were fixed in 10% neutral buffered formalin (NBF), followed by paraffin embedding (n=3). In addition, healthy canine testicular tissue, which normally expresses high levels of PTTG1/securin, was acquired from recently euthanized dogs (n=2) and similarly fixed and processed to serve as a positive control throughout experiments. Hematoxylin and eosin stained (H&E) sections from each specimen (patient or control) were reviewed by a board-certified veterinary pathologist and confirmed to represent UC or healthy tissues, respectively. For UC specimens, FFPE tissues were macrodissected to enrich for neoplastic cells and minimize dilution by non-neoplastic cells.

For mRNA and Western blot analyses, bladders were collected from dogs (n=3) less than one hour post-euthanasia. Bladders were stored in transport media (RPMI media supplemented with 10% FBS) until processing (less than one hour post-collection). Bladders were then bisected; one half was formalin-fixed, paraffin embedded, and evaluated via histopathology to ensure the specimen was healthy (non-neoplastic, non-inflammatory) and retained the urothelial lining. Urothelial cells were isolated from the other half by scraping the mucosa three times with a glass slide and rinsing with 1x phosphate buffered saline (PBS), as described previously (Harris, Wade, & Handler, 1986). Urothelial scraping was performed in order to minimize contamination of urothelium by submucosal tissue layers. Cell scrapings were then analyzed with a hemocytometer under phase contrast to determine the proportion of epithelial cells to contaminating mesenchymal cells. Over 90% of isolated cells were confirmed to be epithelial.

Staging and metastasis information of the UC patients was obtained from either previous studies (Chapter 2, Shapiro et al., 2015) or clinical records and reported according
to the American Joint Committee on Cancer staging guidelines (American Joint Committee on Cancer, 2002).

**UC cell lines**

Five cell lines were available for *in vitro* experimentation. Cell lines were established from primary bladder tumors at diagnosis or necropsy at the Purdue University College of Veterinary Medicine. After storage in liquid nitrogen, cell lines were thawed and initiated in T25 culture flasks. Cells were cultured in DMEM/F12 without glutamine (Mediatech, Manassas, VA) supplemented with 1% GlutaMAX™ (Life Technologies, Carlsbad, CA), 0.1% Primocin (Invivogen, San Diego, CA) and 12% FBS (Mediatech). When ~80% confluent, cells were removed from the flask using 0.05% trypsin with EDTA (Mediatech) and passaged accordingly in a 1:3 ratio.

**qRT-PCR of PTTG1**

RNA was isolated from specimens utilizing the RNeasy Plus Kit (Qiagen, Valencia, CA; fresh tissues/cell lines) or the E.Z.N.A. Total RNA Kit (Omega Bio-Tek, Norcross, GA; FFPE tissues) with xylene deparaffinization. Fresh tissues and cell lines were homogenized by using the QiaShredder (Qiagen). Healthy/non-neoplastic tissues were isolated via both methods in order to evaluate potential differences in fold change attributed to tissue preparation or RNA isolation method. After RNA isolation, samples were evaluated by spectrophotometry to ensure adequate integrity for further analysis (260:280>2, 260:230>2). Purified RNA (50-100 ng) was converted to cDNA by using the QuantiTect Reverse Transcription Kit (Qiagen, fresh tissues/cell lines) or the SuperScript Vilo cDNA Synthesis Kit (Life Technologies, Carlsbad, CA, FFPE tissues), according to manufacturer’s recommendations.
Primers for \textit{PTTG1} quantitative reverse transcription PCR (qRT-PCR) were designed using the NCBI PrimerBLAST Program (Ye et al., 2012). The control gene \textit{RPL32} was selected for consistent levels of expression among all samples (within one critical threshold (Ct), standard deviation (S.D.) <1). \textit{PTTG1} (F: 5'-CTCAGATGACACCTATCCAGAA-3'; R: 5'-GAGAGGCACCTCCATTCAAGG-3') and \textit{RPL32} (F: 5'-ATGCCCAACATTGTTATGG-3'; R: 5'-ATGCCCAACATTGTTATGG-3') primer pairs were designed to amplify a 100-150 base pair region of the target gene and flanked an exon-exon boundary to minimize amplification of contaminant genomic DNA. Traditional RT-PCR followed by agarose gel (2%) electrophoresis was performed using a temperature gradient (55-65°C) to optimize the annealing temperature for each primer pair and ensure single product specificity. The optimal temperature was ~60°C, which was used for all further analyses. Following temperature determination, qRT-PCR primer efficiency curves were generated using a standard 10-fold dilution of cDNA to determine primer efficiency. \textit{PTTG1} and \textit{RPL32} primer pairs were calculated to be 95-105% efficient.

Using validated primers, qRT-PCR was performed on mRNA samples using 0.5 \( \mu \text{M} \) of each forward and reverse primer (IDT, San Jose, CA) along with the Roche LightCycler 480 SYBR Green Master Mix I (Roche, Nutley, NJ). Samples were amplified and analyzed using the LightCycler 480 (Roche) with a 10 minute preincubation at 95°C and 45 cycles consisting of the following: denaturation 95°C for 10s, annealing 60°C for 10s, and extension 72°C for 10s. No template controls were included to monitor contamination. Amplification plots and melt curves were evaluated to ensure product specificity and lack of amplification in the no template control wells. Ct values were determined utilizing the “Second Derivative Maximum” algorithm in the LightCycler 480 software (Roche). Fold changes were calculated using the 2\(^{\Delta\Delta \text{Ct}}\) method, where \(\Delta \text{Ct} = \text{Ct}_{\text{PTTG1}} - \text{Ct}_{\text{RPL32}}\) and \(\Delta\Delta \text{Ct} = \Delta \text{Ct}_{\text{TUMOR}} - \Delta \text{Ct}_{\text{CONTROL}}\).
Significant differences in *PTTG1* expression were detected by a Mann-Whitney U-Test (two-tailed, α=0.05) of ΔCt values (tumors, cell lines, and controls) and fold-changes (cell lines and tumors) using the JMP Professional Statistical Software (v. 11, SAS, Cary, NC).

**Immunohistochemistry**

Securin (*PTTG1* protein) immunohistochemistry (IHC) was performed on 5 µm sections of FFPE UC biopsies (n=15), healthy/non-neoplastic FFPE bladder specimens (n=3), and healthy FFPE testes (n=2), each mounted onto charged glass slides at the NCSU CVM Histology Laboratory. Heat-induced epitope retrieval was performed in Tris-EDTA (pH=9.0) using the Pascal Pressurized Heating Chamber (Dako, Carpinteria, CA) at 120°C for 30 seconds and cooled to 90°C. Sections were then blocked with 1% bovine serum albumin (BSA) in Tris-buffered saline (TBS) for 20 minutes at room temperature. Securin protein was detected by incubation with a mouse monoclonal anti-securin primary antibody (ab3305, Abcam, Cambridge, MA) diluted in 1% BSA (1/1000) for 30 minutes at room temperature. Primary antibody detection utilized the EnVisio™ Anti-mouse Polymer kit (Dako) for 30 minutes at room temperature, followed by 3,3’-Diaminobenzidine (DAB) staining for five minutes and hematoxylin counterstain.

The securin antibody chosen was human reactive but predicted to work with canine based on sequence homology (>85% amino acid homology). Thus, prior to bladder staining, the antibody was confirmed to be canine reactive by using a known securin-positive tissue (canine testes). After appropriate reactivity was confirmed, a canine testes slide was included in each run as a positive control. In addition, a negative control (stained with secondary antibody only) was included to ensure staining resulted from primary antibody interaction only.
Stained slides were evaluated by light microscopy with evaluation of 30 high power fields (HPF) per case. Note was taken of the number of positively stained cells per HPF, the cellular localization of securin (cytoplasmic, nuclear, membranous), and tissue localization. Using staging information (Table 1), tumors were evaluated for differences in number of positively staining cells or staining localization among tumor stages.

*Protein isolation and western blotting*

Whole cell lysates of cell lines were isolated using RIPA buffer (Boston Bioproducts, Ashland, MA) supplemented with HALT protease inhibitor (100x, Thermo Scientific, Rockford, IL), phosphatase inhibitor cocktail (#2, Boston Bioproducts), and phenylmethylsulfonyl fluoride (PMSF, Thermo Scientific). Cells were rinsed with ice cold 1X PBS thoroughly prior to lysis on ice. For T25 flasks of cell lines, 500 µL of RIPA was added. Following RIPA treatment, cells were incubated with shaking (200 RPM) on ice for 30 minutes. Cells were then scraped using a chilled cell scraper. Lysates were spun at 10,000 RPM for 10 minutes at 4°C, and then the supernatant removed to a clean, protease-free tube. The Pierce BCA Assay Kit (Thermo Scientific) was used to quantify protein, using dilutions of BSA to construct a standard curve. A spectrophotometer (Nanodrop 1000, BCA Module) was used to measure absorbance values of BSA standards and determine relative protein concentrations of each cell lysate.

Protein was prepared for Western blotting by diluting samples (20ug) 3:1 v:v in 4x Laemmli buffer (Bio-Rad, Hercules, CA) with β-mercaptoethanol. Prepared samples were heated at 95°C for 5 minutes, placed on ice 1 minute, and centrifuged 30 seconds (14000 RPM). Protein was then run on a 12% Mini-Protean TGX pre-cast gel (BioRad) under reducing and denaturing conditions at 40V for 10 minutes and 110V for one hour. Protein was transferred onto a PVDF membrane (0.2µm, Biorad) using the Genie Blotting System at
12V for one hour (Idea Scientific, Minneapolis, MN). Membranes were then blocked using 5% non-fat milk (NFM) in TBS supplemented with 0.05% Tween-20 (TBST) for one hour at room temperature with shaking. Securin primary antibody was the same as previously used for IHC (Abcam, 1/1000 in 5% NFM), and a mouse monoclonal beta-actin antibody (ab8226, Abcam) was included for loading control and band normalization (1/1000 in 5% NFM). Primary antibody interactions were detected by a horseradish peroxidase-conjugated rat anti-mouse secondary antibody (1/3000 in 5% NFM; ab131368, Abcam). Chemiluminescent substrate (Pierce ECL, Thermo Scientific) was applied to detect bound antibody prior to imaging on the ChemiDoc MP (Biorad). The bands for β-actin and securin were found at the appropriate molecular weights of 42 kDa and 27 kDa, respectively.

siRNA-mediated knockdown of PTTG1

After initial thawing, cells were cultured for at least two weeks prior to transfection. When cells were ~80% confluent, cells were trypsinized as above and prepared for knockdown experimentation. A transfection mix was created for each reaction consisting of 500µL of serum and antibiotic free DMEM/F12, 5µL of RNAiMAX Transfection Reagent (Life Technologies), and 10nM targeting or control siRNA (TriFECTa DsiRNA Kit for Canine PTTG1, IDT). The siRNA used in this experiment is a pre-annealed dicer-substrate interfering RNA duplex (DsiRNA) which is designed to initiate Dicer activation and ensure complete degradation of target mRNA. A 1:1 mixture of two DsiRNA complexes resulting in maximal knockdown of PTTG1 (Duplex 1: 5’-AAAUUCAAAACAUUUGCUAACAAUAG-3’, 3’-TTUAAGUUGUAACCAUGCAGAUCGU-5’; Duplex 2: 5’-AGGUGGCAAUUCACUAUCAGAGUCGA-3’, 3’-TCCACGUGUUAAGUGGUACGUUCAG-5’).
Prior to initial duplex transfection, transfection efficacy and optimization utilized a non-targeting validated TexasRed-conjugated (TEX-615™) oligonucleotide (10 nM, IDT) with charge equal to that of the targeting duplex. Upon transfection, oligonucleotides were able to be visualized within successfully transfected cells. Living transfected cells were then visualized with a fluorescent microscope (Nikon TE200, Melville, NY). Over 80% of cells were ensured to contain ample amounts of the fluorescent prior to proceeding with DsiRNA knockdown.

For PTTG1 knockdown optimization, cells were reverse transfected with one of three transfection mixes in duplicate wells of a 6-well culture plate: 1) transfection reagent only (transfection control), 2) non-targeting DsiRNA + transfection reagent (negative control), or 3) PTTG1-targeting DsiRNA + transfection reagent. Each transfection mix (500µL, described above) was first added to plate wells. The non-targeting negative control DsiRNA was not complementary to any known transcriptome sequence, enabling assessment of the effect of DsiRNA treatment alone on mRNA levels and cell proliferation. Then, 1 x 10^5 cells were added to each well in 2 mL complete media without antibiotics. At 42 hours post transfection (PTF), each treatment was analyzed for mRNA levels and proliferation. For qRT-PCR analysis, mRNA was isolated using the RNeasy Plus Kit (Qiagen), as described above. To determine proliferation, cells were removed from the culture dish by trypsin/EDTA treatment and counted, including viability analysis, using the Cellometer Auto T4 (Nexcelom, Lawrence, MA).

In order to determine the effect of DsiRNA treatment on knockdown, mRNA expression levels were compared among all treatment groups by using the 2^(-ΔΔCt) method, as above. Transfection control cells (tcontrol) were compared with negative control (neg) to ensure minimal effect of the general transfection process on gene specific knockdown.
(ΔΔCt<sub>NEG-CONTROL</sub>). Negative control cells were compared with knockdown cells to determine fold change evoked by gene-specific DsiRNA transfection (ΔΔCt<sub>KNOCKDOWN-NEG</sub>). Knockdown was only considered successful when exceeding 90%, as calculated by 100-(100% x 2<sup>-ΔΔCt</sup>). 

PTTG1 expression remaining was, therefore, <10% in all cases and similar to that of non-neoplastic cells.

Invasion analysis

Prior to use, Matrigel® Invasion Chamber (Corning, Corning, NY) membrane inserts were equilibrated to room temperature for one hour. Membranes were then rehydrated by adding 2mL of serum free media to both the top and bottom chamber compartments. The plate containing rehydrated membranes was placed in a 37°C humidified incubator for 2 hours. During this time, cells were trypsinized, as described above, centrifuged (1000 RPM x 5 minutes) to remove traces of FBS, and resuspended in 2mL of antibiotic and serum free media. Three transfection mixes were created as above: transfection control, negative control, and knockdown. After membrane hydration, chambers were transferred to a new six-well plate containing 2 mL of DMEM/F12 media with 12% FBS as the chemoattractant (antibiotic-free). When ready to plate, serum-free media from rehydration was removed carefully from the top well of the Matrigel Chamber and replaced by the appropriate transfection mix. Then, 1 x 10<sup>5</sup> cells in 2mL serum free media were reverse transfected and added to the top chamber to allow invasion, as described previously (Tamashiro, Furuya, Shimizu, & Kawamori, 2014). Plates were incubated at 37°C for 42 hours before invasion analysis. Simultaneously, a six-well plate was seeded as in previous transfection experiments, allowing analysis of mRNA among treatments to ensure successful knockdown.
After 42 hours, cells were removed from the top chamber by using a cotton swab in a scrubbing fashion. Membranes were initially scrubbed by moistening the swab with media from the chamber. After removal of the media, the scrubbing procedure was repeated twice with 1X PBS to ensure complete removal of non-invaded cells. To ensure potential alterations in invasion were not due to cell death, the viability of cells remaining within the upper chamber was assessed and confirmed to be in line with previous experiments (>85%). The transwell insert possessing invaded cells was then moved to a 6-well plate containing 1X PBS to remove traces of media for two minutes, followed by fixation in fresh 100% methanol for 10 minutes. Cells were then transferred to a 6-well plate containing crystal violet stain (0.1% crystal violet in 0.01% ethanol) for 10-20 minutes. Membranes were rinsed in a series deionized water series then placed at room temperature to dry for at least 24 hours. Dry membranes were removed from the inserts using a #11 scalpel from the bottom of the membrane and mounted bottom-down on a glass slide using VectaShield Mounting Media (Vector Laboratories, Burlingame, CA).

Mounted membranes were analyzed for invasion using a light microscope at 40X objective. The membrane was demarcated into quarters. Two opposing quarters were then counted, averaged, and multiplied by 4 to obtain the total number of cells on the membrane. Replicate treatment counts were averaged to obtain an average invasion number for each cell line. The number of cells invaded per treatment was then normalized to the total cell number, as determined by proliferation in the duplicate reaction of the accompanying six-well plate, to account for differences in proliferation associated with gene knockdown. An invasion index was then calculated for negative control (%invading\textsubscript{NEG\_CONTROL}/%invading\textsubscript{T\_CONTROL}) and knockdown (%invading\textsubscript{KNOCKDOWN}/%invading\textsubscript{NEG\_CONTROL}) cells, where %invading=\#invading/\# total cells.
Both number of cells invaded and invasion indices were compared between treatments by using a Mann-Whitney Exact Test (two-tailed, $\alpha = 0.05$) in JMP Professional (v. 11, SAS).

**qRT-PCR analysis of epithelial and mesenchymal cell markers**

To evaluate the effects of reduced levels of *PTTG1* on the epithelial-mesenchymal transition, qRT-PCR of epithelial marker E-cadherin (*CDH1*) and mesenchymal marker vimentin (*VIM*) was performed. Primers were designed to yield an E-cadherin amplicon of 112 base pairs (F: 5'-TGGAGAGGAGGATCAGGACTT-3'; R: 5'-ATACTGGGCACACTCAGGA-3') and a vimentin amplicon of 113 base pairs (F: 5'-GTCACCTTCGGGAGTACCAG-3'; R: 5'-AAGAGGCAGAGCAATCCTGC-3'). *RPL32* was used as the reference gene, as described above. Prior to qRT-PCR analysis, an annealing temperature gradient was performed and efficiency curves constructed utilizing a series of five ten-fold dilutions of neoplastic cDNA. The ideal annealing temperature was found to be 62°C for both primer pairs and efficiencies 95-105%. cDNA from negative control cells, *PTTG1* knockdown cells, and normal urothelium was analyzed and compared to determine the effects of knockdown on epithelial/mesenchymal markers. $\Delta Ct$ values were compared using a Mann-Whitney Exact Test ($\alpha = 0.05$) using JMP. Since primer efficiencies were found to be equivalent between test and reference genes, fold changes were calculated by the $2^{-\Delta\Delta Ct}$ method.

**Vorinostat treatment of *PTTG1*-overexpressing UC cell lines**

Established cell lines were seeded into 6-well plates ($1 \times 10^5$ cells/well) containing 3mL of DMEM/F12 media supplemented with 12% FBS. After 24 hours incubation at 37°C, media was replaced and vorinostat (2mg/mL in 100% ethanol vehicle, Biotang, Waltham, MA) added to treatment wells (2.5 µM). The dose of 2.5 µM has been shown to elicit maximal response *in vitro* and is twice the maximum plasma concentration ($C_{max}$) achieved
in human patients (Munchi et al., 2006; O’Connor, 2006). Simultaneously, a second well was treated with equivolume 100% ethanol (vehicle control) and a third well remained untreated. After a 48 hour incubation at 37°C, RNA was isolated from the monolayer and supernatant of wells, followed by cDNA conversion and PTTG1 qRT-PCR as described above. Significant differences in PTTG1 expression were identified using a Mann-Whitney Exact test of ΔCt_{PTTG1-RPL32} values among vehicle- and vorinostat-treated cells (α=0.05). Additionally, cell lines (K9TCC-Pu-An, K9TCC-Pu-In, K9TCC-Pu-Mx, and K9TCC-Pu-Sh) were similarly treated with 2.5 µM vorinostat or equivolume 100% ethanol (vehicle-only) and evaluated for differences in proliferation (total cell number). Viability was noted among all treatments.

A dose-response curve for proliferation was simulated using the K9TCC-Pu-An cell line. Cells were plated in each well of a 6-well plate with the addition of vehicle control (100% ethanol), 2.5 µM vorinostat, or 5 µM vorinostat in duplicate wells. Cells from each treatment were counted at 24 hours post-treatment (HPT) and 48 HPT.

**Results**

*PTTG1/Securin is highly overexpressed in primary canine tumors and cell lines and suggestive of the epithelial-mesenchymal transition*

Differences in PTTG1 mRNA levels among tumor and normal groups, as determined by qPCR, were analyzed for significance using nonparametric comparisons via a pairwise Mann-Whitney U test of ΔCt_{PTTG1-RPL32} values of tumor cell lines, FFPE biopsies, FFPE control bladders, and fresh control urothelium. ΔCt values of FFPE UC biopsies were significantly lower than those of either fresh or FFPE control urothelium (p=0.01, Figure 1A). Similarly, cell line ΔCt values were significantly lower than both control types (p=0.04) but
not statistically different from FFPE UC biopsies (p=0.63), indicating relative overexpression of PTTG1 in tumors and cell lines. Fresh and FFPE controls did not differ significantly from one another (p=0.66), validating their concurrent use as control for fold change calculation and confirming that the fixation process does not significantly alter detectable levels of PTTG1. Thus, ΔCt values of all six FFPE and fresh samples were used to calculate an average ΔCt\text{CONTROL} for tumor fold change analysis. After validating the use of both FFPE and fresh urothelium as controls, a ΔΔCt\text{TUMOR-AVG\_CONTROL} and relative fold change (2^{ΔΔCt}) was calculated for all neoplastic samples to determine the extent of gene dysregulation.

PTTG1 levels were increased by approximately 30-fold in neoplastic urothelium compared to normal urothelium (µ_{cell\_lines} = +31.1, µ_{primary} = +33.4), indicating a massive increase in PTTG1 expression in canine UC (Fig. 1B).

Immunohistochemistry enabled validation of PTTG1/securin overexpression at the protein level in primary tumor biopsies (FFPE). Prior to analysis of securin in urothelial tissues, we analyzed the ability of the antibody to detect securin protein in FFPE healthy canine testes, which naturally express securin at high levels. The vast majority of testicular securin was localized to the cytoplasm of spermatocytes, as is seen in human testes, demonstrating similarities in securin expression in normal tissues between humans and dogs and validating antibody reactivity in canine tissues (Fig. 2) (Uhlén et al., 2015).

In normal urothelium, no evidence of securin staining was observed when analyzing 30 HPF/FFPE section (Fig. 3A). In UC biopsies, however, significant securin expression was evident in 100% of analyzed cases (n=15). An average of 79.7 strongly stained cells/HPF were evident among biopsies when averaging counts from 30 HPF/section. In addition, securin expression was enriched in basal neoplastic cells and epithelial cells with a mesenchymal phenotype, including invaded neoplastic cells evident throughout the
submucosa and muscularis (Fig. 3B, C, D). Within each case, adjacent normal regions of the urothelium were completely devoid of securin immunostaining (Fig. 3B), serving as an internal control for non-specific staining and attaching biological relevance to regions of securin overexpression. Both nuclear and cytoplasmic partitions of Securin staining were evident among UC patients: 4 of 15 (26.7%) showed staining of both the cytoplasm and nucleus in the majority of positively-stained cells (>50%) while the remaining 11 showed mainly cytoplasmic staining only (73.3%). In addition, 8 of 15 (53.3%) showed strong staining of the nuclear membrane in >50% of positive cells. No significant differences in number of positively-staining cells (p=0.25, Mann Whitney U test) or cellular localization (p=0.14, Fisher’s exact test) among various tumor stages was observed (Table 1).

Similarly, Western blot analysis of cell line protein isolates demonstrated elevated levels of Securin in three of five cell lines compared to normal urothelium (Fig. 3E). In fact, cell lines displayed extremely high levels of securin, while normal urothelium had undetectable protein levels. Two cell lines (K9TCC and K9TCC-Pu-In) did not demonstrate significant levels of securin protein despite elevated levels of mRNA. Moreover, these two cell lines showed the lowest fold changes in mRNA expression (14.9 and 17.9, respectively) among cell lines. Similarities in levels of the loading control β-actin ensured valid interpretation of the data.

Knockdown of PTTG1 in canine UC cell lines results in decreased levels of PTTG1 mRNA

In order to assess knockdown efficacy, traditional RT-PCR and qRT-PCR were performed on transfection control cells, negative control cells, and knockdown cells. Using qPCR as the gold standard, knockdown efficiency was expected to be >90% based on \( \Delta\Delta C_{t}\) values, leaving <10% PTTG1 expression (Fig. 4A). In cases in which knockdown levels failed to meet these criteria, the experiment was optimized and repeated.
until such levels were achieved. Two cell lines (K9TCC-PU-In and K9TCC) failed to knockdown with >50% efficiency and were excluded from further analysis. Alongside knockdown experiments, PTTG1 levels in normal canine urothelium were assessed to evaluate the ability of gene knockdown to return cells to physiologically normal levels of PTTG1 (Fig. 4B). Although mRNA levels decreased greatly with DsiRNA treatment, knockdown cells retained approximately two-fold (1 Ct) elevated levels of PTTG1.

In all knockdown experiments, a non-targeting negative control duplex was included to ensure decreased levels of mRNA were gene specific and not a result of the transfection procedure. In all cases, it was shown that transfection with a non-targeting duplex resulted in very minimal to no reduction of cellular levels of the target gene (ΔΔCt\textsubscript{neg_control-trans_control}, as well as minimal change in proliferation (Fig. 6A). In addition, assessment of viability indicated minimal cell death (>85% viability) as a result of the transfection procedure or gene knockdown.

PTTG1 knockdown results in decreased cell proliferation and invasion in canine UC cell lines

The use of Matrigel Invasion chambers in conjunction with PTTG1 knockdown allowed investigation of the role of PTTG1 on tumor cell invasion in vitro. At 42 hours PTF, cells were fixed and subsequently analyzed for cell invasion through the Matrigel pseudo basement membrane compared to negative control. In the three cell lines analyzed (K9TCC-Pu-An, K9TCC-Pu-Mx, and K9TCC-Pu-Sh), knockdown of PTTG1 resulted in significantly decreased number of cells invaded through the basement membrane (p<0.001, Mann Whitney Exact test) (Fig. 5). In control cells, the average number of cells invaded through the basement membrane ranged from 1699.5 cells (K9TCC-Pu-Sh) to 3878.5 cells (K9TCC-
Pu-Mx) (Fig. 6B). With PTTG1 knockdown, the number of invaded cells was decreased, on average, by 81.0% compared to negative control (range 80.3-81.6%).

In addition to decreased invasion, PTTG1 knockdown resulted in significantly decreased total cell numbers (Fig. 6A). At 42 hours PTF, knockdown inhibited proliferation by 57.9% relative to negative control cells. Despite decreased cell numbers, cell viability was relatively unchanged (>85% among all controls and knockdowns), suggesting that rather than having a toxic effect on the cells, PTTG1 knockdown inhibits proliferation. Due to the inhibitory effect of PTTG1 knockdown on proliferation, it was necessary to correct the invasive ability of the cell lines for decreased proliferation in order to truly assess the effect of PTTG1 on the propensity of neoplastic cells to invade. Invasiveness was corrected for proliferation by determining the invasive index (% invaded<sub>Knockdown</sub>/% invaded<sub>NEG_CONTROL</sub>).

PTTG1 knockdown significantly decreased the invasive ability of cells relative to those in control experiments (average=45.4% of control, Mann-Whitney Exact Test, p=0.03, Fig. 6C, Table 2). These data demonstrate that PTTG1 influenced not only the total number of invading cells, but the invasive tendency of neoplastic cells.

**PTTG1 knockdown leads to reduced expression of mesenchymal markers**

To assess the effects of PTTG1 knockdown on the epithelial-mesenchymal transition, qRT-PCR quantification of epithelial marker E-cadherin and mesenchymal marker vimentin was performed. All transfection control cell lines had significantly increased levels of vimentin (average fold change= +17.6) and decreased levels of E-cadherin (average fold change= -2.6) compared to normal urothelium (Fig. 7A). PTTG1 knockdown resulted in significantly increased levels of E-cadherin relative to control cell lines (average fold change=1.3x control, p=0.04, Mann-Whitney Exact Test, Fig. 7B), as well as decreased levels of vimentin (average fold change= -1.9x control, p=0.03, Fig. 7C). K9TCC-Pu-Sh
showed the most dramatic changes in vimentin, with knockdown decreasing cellular levels by 2.9-fold, and K9TCC-Pu-Mx showed the greatest increase in E-cadherin (1.5-fold increase relative to control). Overall, knockdown resulted in slight, yet significant, corrections of aberrant gene expression (Fig. 7A).

**Vorinostat treatment reduced levels of PTTG1 and limited cell proliferation**

Due to the clinical availability of vorinostat and its ability to depress cellular PTTG1 expression in human carcinomas, each of five cell lines, including transfection-resistant K9TCC and K9TCC-Pu-In, were dosed with vehicle-only (100% ethanol) or vorinostat (2.5µM) to evaluate its effects on PTTG1. Levels of PTTG1 among vehicle- and vorinostat-treated cells were evaluated via qRT-PCR and compared for significance via a Mann-Whitney Exact test of ΔCt_{PTTG1-RPL32} values. Vorinostat treatment reduced levels of PTTG1 at 48 HPT relative to untreated cell lines (average fold change= -2.1, 95% C.I.= 1.3-3.03, p=0.05), suggesting that vorinostat has a similar PTTG1-inhibitory effects in dogs. K9TCC-Pu-An demonstrated the largest PTTG1 reduction with vorinostat treatment (fold change= -3.4 relative to vehicle-treated), with K9TCC-Pu-Mx also showing a significant reduction (fold change= -2.1). The other three cell lines (K9TCC-Pu-In, K9TCC, and K9TCC-Pu-Sh) demonstrated modest decreases in PTTG1 (fold changes= -1.7, -1.7, and -1.4, respectively).

Additionally, the effects of vorinostat treatment on cell proliferation were evaluated. A dose-response curve was generated using K9TCC-Pu-An, seemingly the most sensitive to vorinostat treatment based on PTTG1 expression response (Fig. 8A). Results showed a dose-dependent decrease in cell number at 24 (vehicle-treated=1.6 x 10^5 cells, 2.5µM vorinostat=1.5 x 10^5 cells, 5 µM=1.2 x 10^5 cells) and 48 (vehicle-treated=2.0 x 10^5 cells, 2.5µM=1.4 x 10^5 cells, 5 µM=1.0 x 10^5 cells) HPT. In addition, cell lines K9TCC-Pu-In,
K9TCC-Pu-Mx, and K9TCC-Pu-Sh were treated with vorinostat (2.5 µM) for 48 hours, followed by assessment of cell proliferation and viability. Vorinostat treatment resulted in significantly decreased numbers of cells at 48 HPT (p=0.04, Mann-Whitney Exact test, one-tailed, Fig. 8B). On average, vorinostat treatment reduced total cell number at 48 HPT by 48.2% and reduced cell viability by 12.2% (Fig. 8C). K9TCC-Pu-Mx showed the greatest reduction in cell number (76.5% decrease) and viability (23%), while K9TCC-Pu-An showed the least reduction in both cell number (30%) and viability (16%). K9TCC-Pu-In and K9TCC-Pu-Sh showed higher efficacies with a 42.3% and 43.8% decrease in cell number, respectively (Fig. 8B).

Discussion

Pituitary tumor-transforming gene 1 (PTTG1), also known as securin, is a recently characterized oncogene posited to play a major role in numerous carcinomas (Demeure et al., 2013; Huang et al., 2012; Yoon et al., 2012). Many roles have been proposed for PTTG1 in tumorigenesis, most notoriously in neoplastic cell chromosomal instability, transcriptional control of proto-oncogenes, proliferation, and invasion of neoplastic cells. In humans, levels of PTTG1 mRNA and protein are elevated in neoplastic compared to normal urothelium, which shows no protein and minimal mRNA expression (Lai et al., 2010; Uhlén et al., 2015). Similarly, gene expression studies have shown that PTTG1 is one of the most highly overexpressed genes in canine UC cell lines, with an average fold increase in mRNA levels of +57.5 (Chapter 3). Although known to be aberrantly expressed, functional characterization of PTTG1 in UC of either species is lacking. In addition, PTTG1 has not yet been studied in dogs in any capacity. We therefore sought to determine levels of PTTG1 in UC tumor biopsies and cell lines and to evaluate the potential consequences of PTTG1.
overexpression on neoplastic cell proliferation and invasion. If aberrant and functionally relevant, *PTTG1* might represent a new target for precision medicine in canine UC, as well as its human counterpart.

qPCR analysis of RNA isolated from five cell lines and FFPE tumor biopsies (n=18) confirmed overexpression of *PTTG1* in 100% of cases. On average, cell line levels of *PTTG1* were 31.1-fold greater than those of normal urothelium while FFPE biopsy specimens showed an average fold change of 33.4 relative to normal urothelium. Thus, cell line and primary tumor biopsies were found to express similar levels of *PTTG1*, reinforcing the utility of the cell line model through their preservation of molecular alterations *in vitro* and suggesting a requirement for *PTTG1* overexpression throughout tumor growth and maintenance. Furthermore, the cell lines provide a similar and valuable model for the *in vitro* study of *PTTG1* in canine UC.

Prior to analysis of securin in UC biopsies, we evaluated the expression and localization of securin in canine testes, one of few healthy tissues that constitutively expresses *PTTG1*/securin in humans and laboratory rodents (Talvinen et al., 2013). Our results agreed with previous studies, with canine testes showing strong cytoplasmic staining of securin in spermatocytes, demonstrating similarities in the protein localization and, therefore, potentially function in humans and dogs. Spermatocytes possess high levels of securin presumably due to high meiotic activity and cell turnover, emphasizing the potentially deleterious effects of ectopic overexpression of the gene in bladder urothelium.

When evaluating securin protein levels in canine UC, staining was evident in 100% of analyzed UC FFPE biopsies by IHC, providing ample evidence that *PTTG1* is overexpressed at both mRNA and protein levels in canine UC. IHC further revealed the intracellular localization of securin to be cytoplasmic in 100% of cases, as in healthy testes,
but also nuclear (>50% of cells) among 26.7% of UC patients. Increased nuclear localization is associated with aggressive tumor phenotypes and poor survival outcomes in other human carcinomas, likely due to the effects of securin on transcriptional control and p53 inhibition (Avoranta et al., 2011; Kim et al., 2006). Our data, however, did not suggest an association between tumor stage and the number of positively stained tumor cells or cellular localization, in agreement with what has been shown in human UC (Lai et al., 2010) (Table 1). We do acknowledge the limitations associated with canine UC staging, in which incomplete biopsies are often obtained. Thus with more complete biopsies and more accurate staging, an association or lack thereof may be more reliably assessed.

Previous studies have shown that increased levels of cytoplasmic securin lead to the acquisition of a mesenchymal cell phenotype along with decreased contact inhibition and increased intercellular spacing, indicative of the EMT (Yoon et al., 2012). We also observed this phenomenon; cells with cytoplasmic securin staining appeared mesenchymal when compared to their negatively staining neighbors, suggesting an association between securin and the EMT, a fundamental step in tumor cell invasion (Micalizzi et al., 2010). Securin is crucial to the EMT: it promotes the formation of invasion lamellopodia by controlling microtubule nucleation and, through activation of AKT, sustains polarized invasion (Shah et al., 2011). Thus, phenotypically mesenchymal neoplastic cells overexpressing securin and undergoing an EMT may promote tumor invasion. Furthermore, positively stained cells were more prevalent among basal urothelial cells, suggesting these undifferentiated urothelial stem cells may have a propensity to overexpress securin and undergo invasive EMT (Hatina & Schulz, 2012). Although not all tumors were staged as invasive yet all tumors demonstrated cytoplasmic securin localization, this may be indicative of tumors evolving to a
more invasive phenotype and demonstrative of the aggressive nature of canine UC or, again, reflective of incomplete biopsy.

IHC results, combined with high levels of PTTG1 in neoplastic cells and tumor localization, support a potential role of PTTG1 in canine UC invasion, warranting further investigation. Specifically due to its involvement in microtubule nucleation and dynamics, PTTG1 promotes tumor invasion and metastasis both in vitro and in vivo (Moreno-Mateos et al., 2011). PTTG1-depleted cells are unable to accurately polarize, severely impairing cell migration and suggesting that PTTG1 is necessary for tumor invasion (Yoon et al., 2012). We thus hypothesized that PTTG1 promotes the invasive behavior of canine UC tumors and evaluated the functional role of PTTG1 in canine UC by performing siRNA-mediated knockdown of PTTG1 in UC cell lines.

Dicer-substrate interfering RNA (DsiRNA) duplexes enabled efficient mRNA knockdown. DsiRNA duplexes are comprised of 27-mer RNA nucleotides, allowing natural dicer enzyme uptake and preferential processing of the siRNA duplex, yielding increased potency compared to traditional 21-mer siRNA. PTTG1 knockdown was >90% complete in all three cell lines analyzed, as assessed by qPCR. Simultaneously, transfection control samples (transfection reagent only) and negative control samples (transfection reagent and non-targeting DsiRNA) were analyzed to ensure target-specific effects of PTTG1 knockdown.

Following PTTG1 knockdown, proliferation and invasion were assessed in knockdown and control cell lines. Our results showed PTTG1 had massive effects on the proliferative ability of neoplastic urothelial cells. Cell counts at 42 hours PTF were significantly lower in PTTG1 knockdown cells than negative control cells (<50% of control). In fact, knockdown cells showed little proliferation 42 hours after plating, suggesting PTTG1
knockdown inhibited their ability to complete mitosis and proliferate. Cell viability, however, remained relatively constant (>85%) among control and knockdown cells, indicating the effects of PTTG1 on neoplastic cell number were due to inhibition of proliferation rather than toxicity and emphasizing tumor cell dependence on PTTG1 for cell proliferation and division. In addition, the minimal cellular toxicity associated with PTTG1 knockdown suggests in vivo targeted applications may produce less deleterious effects than current chemotherapeutic regimens, especially in light of the few tissues expressing high levels of PTTG1.

To assess potential effects of PTTG1 on tumor invasion, we combined PTTG1 knockdown with Matrigel invasion chambers. Matrigel invasion chambers are a widely used in vitro model for epithelial cell invasion. The experimental system, which resembles a culture transwell, has an upper chamber coated with extract from a murine sarcoma, simulating an in vitro basement membrane complete with collagens, laminins, and proteoglycans. Prior to gene knockdown, all UC cell lines demonstrated invasion through the Matrigel pseudo basement membrane. These results agree with the in vivo characteristics of the cell lines, in which all tumors were invasive and/or metastatic at diagnosis and cell line establishment (≥T1 with evident metastases at diagnosis or upon necropsy, Table 1). Following PTTG1 knockdown, the number of invaded cells was significantly decreased (~80%). In light of the inhibitory effects of PTTG1 knockdown on cell proliferation (42.1% of control), however, we further created an invasion index for each knockdown experiment, in which the number of cells invaded were corrected for the decrease in proliferation (total cell number). In all cases, the invasive ability was significantly decreased among PTTG1 knockdown cell lines (45.4% of control, on average). Our results demonstrate not only that PTTG1 knockdown is effective for reducing tumor growth, but also for reducing the propensity for neoplastic cell invasion. Since these cell lines have previously been found to
mimic the disease *in vivo*, reduction of *PTTG1* levels in combination therapy with tumor volume reducing agents, such as radiation, provides a potentially effective therapeutic approach.

After acknowledging the effects of *PTTG1* knockdown on tumor cell proliferation and invasion, we sought to determine if accompanying alterations in the transcriptome supportive of the EMT existed. Vimentin, a mesenchymal marker of the EMT, is an intermediate filament protein associated with the acquisition of a mesenchymal phenotype and behavior, including increased motility (Yun & Kim, 2013). We found that, compared to normal urothelium, levels of vimentin were significantly increased in the cell lines (average fold change= +17.6), in agreement with what has been noted in human UC (average fold change= ~6.0 in muscle invasive tumors) (Paliwal, Arora, & Mishra, 2012; Wan et al., 2015). Such increased levels of vimentin support an increased mesenchymal and, therefore, motile behavior of neoplastic urothelium. *PTTG1* knockdown resulted in significantly decreased levels of vimentin in the cell lines compared to negative controls (average fold change= -1.9 of control cell lines), suggesting *PTTG1* reduction has the ability to at least partially reverse the EMT. In contrast, levels of the epithelial cell adhesion marker E-cadherin were significantly reduced in cell lines (average fold change= -2.7), with *PTTG1* knockdown cells increasing levels relative to controls (average fold change= +1.3 of control cell lines). Thus, *PTTG1* knockdown helps restore cellular adhesions characteristic of epithelial tissue. Although *PTTG1* has been shown to inhibit the EMT in some human carcinomas, no such functional role of *PTTG1* in human UC has been shown. Our results in the canine model, combined with the similarities in clinical behavior and therapeutic response between invasive human and canine tumors, support a similar role of *PTTG1* in human UC.
Our results validate a role of *PTTG1* in tumor proliferation, the EMT, the propensity for tumor invasion, and the potential for metastasis. Thus, reduction of *PTTG1* by knockdown or targeting therapeutics presents a viable option for curtailing the invasive potential of canine, and potentially human, UC. Human *PTTG1* is a known target of the FDA-approved HDACi vorinostat. Most commonly used for the treatment of T-cell lymphoma, vorinostat significantly decreases *PTTG1* levels *in vitro* in carcinomas (Demeure et al., 2013). Vorinostat studies in dogs have shown it is extremely well tolerated, promoting the palliative goals of veterinary oncology (Kerr et al., 2010). Moreover, Vorinostat is synergistic with alternative chemotherapeutics, including mitoxantrone, the current standard treatment for canine UC, as well as radiosensitizing (Basseville et al., 2012; Baschnagel et al., 2009; Chen et al., 2010; Cohen, Powers, Amin, & Desai, 2004; Diss et al., 2014). Thus, we hypothesized that vorinostat would similarly reduce *PTTG1* levels in canine urothelial tumors and represent a viable therapeutic.

We treated five canine UC cell lines (K9TCC-Pu-An, K9TCC-Pu-In, K9TCC, K9TCC-Pu-Mx, and K9TCC-Pu-Sh) with vorinostat (2.5 µM). After 24 and 48 hours, qRT-PCR of isolated RNA was performed to compare *PTTG1* levels between cells with and without (vehicle-control only) vorinostat treatment. A significant decrease in *PTTG1* resulted from treatment (average fold change= -2.1 compared to control cell lines), implying that, as in humans, vorinostat treatment is able to target reduce transcript levels of the oncogene. Furthermore, a dose- and time-dependent reduction in cell proliferation was observed with vorinostat treatment (0-5 µM). These results suggest that vorinostat may be an effective means of reducing tumor *PTTG1* levels, thereby inhibiting tumor proliferation, invasion, and metastatic potential similar to *PTTG1* knockdown. Moreover, the efficacy of vorinostat, an
HDACi, suggests upstream inhibitors of PTTG1 may be deacetylated and, therefore, silenced in canine UC, warranting further investigation.

Although the role of the AKT pathway in human UC is well recognized, little research has supported similar activation of the AKT pathway in canine UC (Yoon et al., 2012). The PI3K/AKT pathway is known to play a central role in human UC; activation of the pathway is evident in at least 88% of UC patients (Calderaro et al., 2014). Hence, finding effective ways to target the PI3K/AKT pathway may prove an advantageous approach to the treatment of UC. PTTG1-mediated malignancy, as well as migratory and invasive behavior, is believed to at least partially occur due to AKT activation (Yoon et al., 2012). If PTTG1 similarly activates AKT in dogs, this provides a link not only between the gene expression changes of humans and dogs, but also between major cell signaling pathways at the crux of UC development. The dog may, therefore, be an even better therapeutic model than previously thought.

Our findings validate the prominence of PTTG1 expression alterations in invasive canine UC. Furthermore, our results further emphasize the value of canine UC cell lines in molecular and therapeutic advancement. Reduced levels of cellular PTTG1 resulted in a reduction in neoplastic cell proliferation and invasiveness. Additionally, treatment of PTTG1-overexpressing cells with the FDA-approved chemotherapeutic vorinostat successfully reduced levels of PTTG1, albeit less drastically. Vorinostat also reduced neoplastic cell proliferation in a dose-dependent manner. Thus, targeted PTTG1 therapy may represent an efficacious therapeutic target with a rapidly translatable chemotherapeutic. Future studies should test the value of PTTG1 targeted therapy in vivo, with the hope that such findings will prove applicable and valuable to impede canine UC invasion, with comparative applications to human medicine.
Acknowledgements

A huge thank you to the NCSU CVM histopathology department, especially Sandra Horton, for helping with antibody selection, protocol development, and IHC performance. Thank you to Dr. Vahbiz Shroff and Dr. Steve Suter for loaning their Western blotting equipment for my analyses. Thank you to Dr. Mac Law for use of his microscope with attached camera which enabled me to capture images of IHC slides and Matrigel membranes. Thanks to Jill Harned for taking the time to teach me about siRNA technology and helping me organize the optimization of my siRNA experiments. Lastly, thank you to Dr. Debbie Knapp at Purdue University for providing the cell lines used in this experiment.

References


Table 4.1. Signalment, tumor stage, and the presence of overt metastases of patients and cell lines included in PTTG1 analyses along with PTTG1/securin expression data.

<table>
<thead>
<tr>
<th>Patient ID</th>
<th>Breed</th>
<th>Sex</th>
<th>Stage</th>
<th>Metastases?</th>
<th>PTTG1 Fold Change</th>
<th># Securin +</th>
<th>Localization</th>
</tr>
</thead>
<tbody>
<tr>
<td>TCC20</td>
<td>Scottish Terrier</td>
<td>FS</td>
<td>T2</td>
<td>N</td>
<td>17.99261948</td>
<td>95</td>
<td>C</td>
</tr>
<tr>
<td>TCC38</td>
<td>Dachshund</td>
<td>MC</td>
<td>T3</td>
<td>Y</td>
<td>21.74582694</td>
<td>212</td>
<td>CM</td>
</tr>
<tr>
<td>TCC40</td>
<td>Doberman Pinscher</td>
<td>FS</td>
<td>T2</td>
<td>Y</td>
<td>25.38668298</td>
<td>122</td>
<td>CNM</td>
</tr>
<tr>
<td>TCC49</td>
<td>Shetland Sheepdog</td>
<td>FS</td>
<td>T1</td>
<td>N</td>
<td>35.34252802</td>
<td>15</td>
<td>C</td>
</tr>
<tr>
<td>TCC42</td>
<td>Dachshund</td>
<td>FS</td>
<td>T2</td>
<td>Y</td>
<td>52.70982511</td>
<td>104</td>
<td>CM</td>
</tr>
<tr>
<td>TCC33</td>
<td>Scottish Terrier</td>
<td>FS</td>
<td>T2</td>
<td>N</td>
<td>121.9376637</td>
<td>3</td>
<td>C</td>
</tr>
<tr>
<td>TCC41</td>
<td>Pembroke Welsh Corgi</td>
<td>FS</td>
<td>Tis</td>
<td>N</td>
<td>10.80279957</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>TCC34</td>
<td>West Highland White Terrier</td>
<td>FS</td>
<td>Tis</td>
<td>Y</td>
<td>38.14265419</td>
<td>21</td>
<td>CNM</td>
</tr>
<tr>
<td>TCC43</td>
<td>Pembroke Welsh Corgi</td>
<td>FS</td>
<td>T1</td>
<td>N</td>
<td>44.69324097</td>
<td>147</td>
<td>CNM</td>
</tr>
<tr>
<td>TCC50</td>
<td>Basenji</td>
<td>FS</td>
<td>Tis</td>
<td>N</td>
<td>16.07410679</td>
<td>95</td>
<td>CM</td>
</tr>
<tr>
<td>TCC45</td>
<td>Labrador Retriever</td>
<td>FS</td>
<td>T1</td>
<td>N</td>
<td>17.50869922</td>
<td>51</td>
<td>C</td>
</tr>
<tr>
<td>TCC35</td>
<td>Dalmation</td>
<td>FS</td>
<td>T1</td>
<td>N</td>
<td>26.47697993</td>
<td>43</td>
<td>C</td>
</tr>
<tr>
<td>TCC46</td>
<td>Miniature Schnauzer</td>
<td>FS</td>
<td>T3</td>
<td>Y</td>
<td>14.48679618</td>
<td>57</td>
<td>C</td>
</tr>
<tr>
<td>UC1</td>
<td>German Shepherd Dog</td>
<td>FS</td>
<td>T3</td>
<td>Y</td>
<td>31.34144952</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>TCC39</td>
<td>Keeshond</td>
<td>MC</td>
<td>T2</td>
<td>Y</td>
<td>34.05984584</td>
<td>82</td>
<td>CM</td>
</tr>
<tr>
<td>UC2</td>
<td>American Eskimo Dog</td>
<td>FS</td>
<td>T2</td>
<td>Y</td>
<td>22.83750807</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>TCC47</td>
<td>Unknown</td>
<td>FS</td>
<td>Tis</td>
<td>Y</td>
<td>22.83750807</td>
<td>73</td>
<td>CNM</td>
</tr>
<tr>
<td>TCC48</td>
<td>Unknown</td>
<td>FS</td>
<td>T1</td>
<td>N</td>
<td>47.2857421</td>
<td>75</td>
<td>CN</td>
</tr>
<tr>
<td>K9TCC</td>
<td>Mixed</td>
<td>FS</td>
<td>T1</td>
<td>Y</td>
<td>17.87659421</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>K9TCC-Pu-An</td>
<td>Scottish Terrier</td>
<td>FS</td>
<td>T1</td>
<td>Y</td>
<td>21.45635836</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>K9TCC-Pu-In</td>
<td>German Shorthaired Pointer</td>
<td>FS</td>
<td>T1</td>
<td>Y</td>
<td>14.87687908</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>K9TCC-Pu-Mx</td>
<td>German Shepherd Dog</td>
<td>FS</td>
<td>T1</td>
<td>Y</td>
<td>71.25898357</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>K9TCC-Pu-Sh</td>
<td>Collie</td>
<td>FS</td>
<td>T1</td>
<td>Y</td>
<td>29.92611993</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

- **Sex:** FS=female spayed, MC=males castrated
- **Stage:** Tis=No invasion, T1=invasion through basement membrane, T2=invasion into muscularis, T3=invasion through muscularis (American Joint Committee on Cancer, 2012)
- **Metastases:** Y=yes, N=no
- **PTTG1 mRNA fold change values calculated relative to healthy (control) urothelium by qRT-PCR**
- **# Securin +:** average number of cells per high power field, as assessed by IHC, positive for securin staining
- **Localization:** site of the majority (>50%) of securin IHC staining C=cytoplasmic, N=nuclear, M=nuclear membrane
Table 4.2. Data acquired from Matrigel Invasion analyses, including total cell number, average number of invaded cells, average invasive indices, and associated p-values.

Average invasive indices were calculated by $\%_{\text{invaded}}^\text{negative}/\%_{\text{invaded}}^\text{control}$ for negative control cell lines and $\%_{\text{invaded}}^\text{knockdown}/\%_{\text{invaded}}^\text{negative}$ for knockdown cell lines. p-values were calculated based on total cells invaded per experiment (n=4) using a Mann-Whitney Exact test ($\alpha=0.05$).

<table>
<thead>
<tr>
<th></th>
<th>Total # Cells</th>
<th>% Proliferation</th>
<th>Avg. # Invaded (±S.D.)</th>
<th>Avg. Invasion Index</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>K9TCC-Pu-An</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Negative Control</td>
<td>350000</td>
<td>0.93</td>
<td>3154.5 (±256.4)</td>
<td>0.98</td>
<td>0.03</td>
</tr>
<tr>
<td>Knockdown</td>
<td>170000</td>
<td>0.49</td>
<td>585.75 (±87.7)</td>
<td>0.38</td>
<td></td>
</tr>
<tr>
<td><strong>K9TCC-Pu-Mx</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Negative Control</td>
<td>285000</td>
<td>1.08</td>
<td>3878.75 (±251.9)</td>
<td>0.97</td>
<td>0.03</td>
</tr>
<tr>
<td>Knockdown</td>
<td>112000</td>
<td>0.39</td>
<td>713 (±367.0)</td>
<td>0.47</td>
<td></td>
</tr>
<tr>
<td><strong>K9TCC-Pu-Sh</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Negative Control</td>
<td>352000</td>
<td>1.10</td>
<td>1699.5 (±83.0)</td>
<td>0.96</td>
<td>0.03</td>
</tr>
<tr>
<td>Knockdown</td>
<td>135000</td>
<td>0.38</td>
<td>333.8 (±64.8)</td>
<td>0.51</td>
<td></td>
</tr>
</tbody>
</table>
Figure 4.1. *PTTG1* is highly overexpressed in canine UC cell lines and tumor biopsies.

Canine UC cell lines (n=5) and FFPE tumor biopsies (n=18) were analyzed via qRT-PCR. Health fresh (n=3) and FFPE bladder (n=3) was included as control to allow ΔCt comparison. Results showed that ΔCt values between FFPE and fresh urothelium were not significantly different, validating their use as control for neoplastic samples (A). Moreover, ΔCt values between FFPE biopsies and cell lines were not significantly different, yet significantly (**p<0.01) different from both controls. Fold-change calculations revealed massive overexpression of *PTTG1* in cell lines (fold change=+31.1) and primary tumors (fold change=+33.4) (B). Error bars represent one standard error about the mean.
Figure 4.2. *Securin expression in canine testicular tissue recapitulates that seen in humans.*

IHC of canine testes with anti-securin antibody revealed the cytoplasmic localization of securin in a canine tissue that constitutively expresses the gene. Primary spermatocytes contained the majority of securin staining, likely due to the high meiotic activity of the cells.
Figure 4.3. IHC and Western blot validate protein expression of securin in canine UC tumors and highlight its potential role in the epithelial-mesenchymal transition.

Healthy control canine urothelium and submucosal layers are devoid of securin staining (10X magnification, A). In UC, however, securin immunostaining is evident in neoplastic regions (red arrow), while absent from normal urothelium of the same patient (black arrow) (B). Additionally, securin staining is evident and strong throughout regions of the tumor which have invaded into the submucosa (red arrow). In discrete regions of invasion, securin-positive cells are seen lining the invasive tumor mass (blue arrow) (C). In regions that have yet to invade through the basement membrane, the majority of staining is observed in basal-most urothelial cells (green arrow). Upon higher magnification (60X), securin-positive urothelial cells appear phenotypically mesenchymal, possessing elongated lamellopodia with intense cytoplasmic staining (D, magenta arrow). Similarly, Western blot analysis of protein isolates from UC cell lines (1-5; K9TCC-Pu-An, K9TCC-Pu-Mx, K9TCC, K9TCC-Pu-Sh, and K9TCC-Pu-In, respectively) demonstrated relatively elevated levels of securin protein in three of five UC cell lines, as assessed by Western blot, compared to normal urothelium (C1, C2). Ruler bars represent 200 µM (10x magnification) or 50 µm (60x).
Figure 4.4. *DsIRNA knockdown of PTTG1 in canine UC cell lines successfully reduced cellular levels of PTTG1 to more physiologically-normal levels.*

After knockdown, qRT-PCR of PTTG1 was performed to determine the efficiency of gene knockdown. In all cell lines, >93% knockdown was achieved, leaving <10% of PTTG1 in knockdown neoplastic cells (+) remaining when compared to negative control cells (-) (A). When compared with normal urothelium, *PTTG1* levels remained elevated in knockdown cells, but were much diminished (B). The reference gene *RPL32* was included in analyses to correct for difference in the amount of total RNA from the cells, as well as potential off-target effects.
Figure 4.5. *PTTG1*-knockdown cells demonstrated decreased invasion relative to *PTTG1*-overexpressing neoplastic cells.

Non-targeting or *PTTG1*-targeting DsiRNA treatment was performed in conjunction with Matrigel Invasion Chambers to assess the role of *PTTG1* in the invasiveness of K9TCC-Pu-An (A, B), K9TCC-Pu-Mx (C, D), and K9TCC-Pu-Sh (E, F) cells. After knockdown, cell lines demonstrated significantly reduced numbers of invaded cells (red arrows) on the bottom of the Matrigel membrane (B, D, F) compared to negative control cells (A, C, E), validating that *PTTG1* overexpression contributes to the propensity for invasion of canine UC cells. (40X magnification, ruler bar=100µM)
Figure 4.6. *PTTG1* knockdown results in reduced proliferation and invasion of canine UC cell lines.

Canine UC cell lines were treated with transfection reagent only ("X", transfection control), non-targeting DsiRNA with transfection reagent ("X-", negative control), or *PTTG1*-targeting DsiRNA with transfection reagent ("X+", knockdown). Cells treated with *PTTG1* DsiRNA showed a significantly reduced cell number at 42 hours post-transfection (PTF) compared to transfection and negative controls (A). Additionally, knockdown reduced the number of cells invaded through a Matrigel membrane (B). Due to the reduction in total cell number, we calculated an invasion index to determine the relative invasiveness of negative control and knockdown cells (C). Still, invasion indices of knockdown cells were significantly lower than control cells, showing *PTTG1* had a true effect on both proliferation and the invasiveness of UC cells. (*p<0.05) Error bars represent one standard error about the mean.
Figure 4.7. Expression markers of EMT are altered in UC cell lines and partially recovered by PTTG1 knockdown.

mRNA levels of an epithelial (E-cadherin) and mesenchymal (vimentin) marker were evaluated to determine the effect of PTTG1 knockdown on the EMT. Prior to knockdown (-), levels of E-cadherin (CDH1) and vimentin (VIM) were significantly decreased and increased, respectively, relative to normal urothelium, indicative of tumor cell acquisition of mesenchymal cell properties (A, points represent individual cell line treatments). Reduction of PTTG1 levels by DsiRNA knockdown (+) curtailed aberrant expression of both markers, reducing the relative fold change when compared to normal urothelium. Knockdown of PTTG1 resulted in significantly increased levels (decreased ΔCt_{CDH1-RPL32}) of E-cadherin mRNA relative to negative control cell line levels in all three cell lines (p=0.04, Mann-Whitney Exact Test) (B). Conversely, levels of vimentin mRNA were significantly decreased as a result of PTTG1 knockdown (increased ΔCt_{VIM-RPL32}, p=0.04) suggesting reversal of the EMT (C). Thus, we conclude PTTG1 contributes to the mesenchymal and invasive character of canine UC cell lines. (*p<0.05). Error bars represent one standard error about the mean.
Figure 4.8. Vorinostat treatment reduces cell proliferation in canine UC cell lines.

Since vorinostat treatment significantly reduced levels of PTTG1 in canine UC, we hypothesized vorinostat treatment to have similar effects on cell proliferation. K9TCC-Pu-An cells were treated with vehicle-only (ethanol), 2.5 µM vorinostat, or 5 µM and counted at 24 and 48 hours. A time- and dose-dependent reduction in cell proliferation was noted (A). At 48 hours post treatment (HPT), cells treated with 5 µM vorinostat showed no increase in cell number from time 0, whereas vehicle-treated cells doubled. Cells of three additional cell lines—K9TCC-Pu-In, K9TCC-Pu-Mx, and K9TCC-Pu-Sh were then dosed with 2.5 µM vorinostat (+) or equivolume ethanol (-) and counted at 48 HPT (B). Among cell lines, significant decrease in the number of neoplastic cells was evident after vorinostat treatment (C). Error bars represent one standard error about the mean.
CHAPTER 5: THERAPEUTIC TARGET PRACTICE: INVESTIGATION OF MIR-10B AND BRAF^{V600E} IN CANINE UC

Abstract

Canine urothelial carcinoma (UC) represents the most common cancer of the canine urogenital tract and one of the most invasive tumors in veterinary medicine; approximately 90% of canine UCs are invasive. Invasive canine UC closely resembles the highly fatal invasive human disease, providing a model for therapeutic study and advancement. The identification of druggable targets, including differentially expressed molecules and actionable genomic mutations, is paramount, particularly for inhibiting tumor invasion and subsequent metastasis. Dysregulated miRNA and constitutively activated kinases represent two types of druggable targets. In the present study, we evaluated levels of the oncogenic and metastasis-related miRNA 10b (miR-10b) in canine UC. Although located in a region of chromosome 36 (CFA) recurrently amplified in UC, we found levels of miR-10b are significantly decreased, suggesting increased genetic dosage does not lead to increased expression, invasion, and metastasis. An alternative target commonly implicated in invasive human tumors is \textit{BRAF}, a MAPK/ERK pathway kinase constitutively activated by a single valine to glutamic acid amino acid switch at codon 600 (V600E). To evaluate the mutation status of \textit{BRAF} in canine urogenital tumors, we sequenced prostatic (n=17) and urothelial carcinomas (n=45), as well as five UC cell lines. Approximately 70% of all tumors and cell lines were positive for the mutation, a T→A transversion at codon 450 equivalent to the prevalent human V600E mutation. Since therapeutics have been developed to inhibit V600E activity in human patients, we investigated the potential efficacy of BRAF^{V450E} reduction on canine UC cell proliferation and invasion by siRNA knockdown of \textit{BRAF}. Although a slight reduction in proliferative ability was seen, a minimal and unpredictable change in tumor cell
invasiveness resulted, suggesting potential inhibitor resistance in vivo, as is seen in humans. Thus, although likely uninvolved in the invasive ability of canine UC, miR-10b and BRAF<sup>V450E</sup> represent differential characteristics between healthy and neoplastic tissues, suggesting they may be diagnostically and pathologically relevant in UC. Additionally, dogs may represent a model of BRAF inhibitor resistance, aiding development of inhibitors with improved efficacy for both humans and dogs.

**Introduction**

Cancer of the transitional epithelium of the urinary bladder, or urothelial carcinoma (UC), is the second most common cancer of the urogenital tract in humans (Siegel et al., 2015). Although the majority of tumors (~80%) are superficial in nature and prognostically favorable, invasive tumors (20%) show a high rate of metastasis (~50%) and are largely unresponsive to current chemotherapeutics, with a 5-year survival rate of only 6% (Lerner, Schoenberg, & Sternberg, 2006; Siegel et al., 2015). Thus, development of more efficacious therapeutics for invasive UC is critical.

One of the most efficient and reliable means for drug development includes use of an animal model. Fortunately, a natural model of invasive human UC occurs in the pet population; UC is the most common urogenital cancer of dogs. The vast majority (>90%) of canine tumors are invasive at diagnosis, providing a valuable model of the less common yet fatal variant of human UC (Knapp et al., 2014). Unlike laboratory rodents in which induced tumors fail to metastasize, canine tumors demonstrate rates of metastasis in excess of 20% at diagnosis and 60% by death, mirroring the metastatic rate of invasive human UC (Knapp et al., 2014). Furthermore, the response of human and canine UC to chemotherapy is similar. Thus, dogs not only provide a spontaneous model of the disease, but also one that
behaves clinically much like human UC and may facilitate a more comprehensive understanding of UC. With a greater understanding of the disease comes a greater opportunity to identify pathogenesis-related molecular targets, an increasingly desirable option in clinical management of cancer. The oncogenic microRNA (miRNA) miR-10b and the MEK kinase *BRAF* represent two druggable targets of interest in canine UC and, due to similarities with human UC, may be similarly relevant in treatment of the human disease (Decker et al., 2015; Shapiro et al., 2015).

miRNAs represent a class of endogenous small, non-coding RNAs which function to target and degrade or inhibit the translation of complementary mRNA. When the targeted mRNA is that of a tumor suppressor, miRNA complementarity results in reduced levels of tumor suppressor genes, leading to tumorigenesis. miR-10b is overexpressed in numerous tumor types and is believed to be at the crux of tumor invasion and metastasis via targeted degradation of *HOXD10*, a suppressor of cell invasion (Liu et al., 2012; Nakayama et al., 2013; Parrella et al., 2014; Xiao et al., 2014). In addition to *HOXD10* effects, studies of miR-10b in bladder cancer have shown that increased miR-10b leads to decreased levels of *KLF4* and E-cadherin, reducing epithelial adherence and initiating the epithelial-mesenchymal transition (Y. Lu & Cheng, 2014). The potential role of miR-10b in UC makes it an attractive therapeutic target of antisense (ASO) therapeutics to curtail deleterious effects of miRNA overexpression (Broderick & Zamore, 2011; van Rooij, Purcell, & Levin, 2012; Yu & Cho, 2015). Furthermore, miRNA is incredibly stable in blood, urine, and formalin-fixed paraffin-embedded (FFPE) tissues, providing a stable and readily assessed therapeutic marker (J. S. Hall et al., 2012).

Our previous work has highlighted the prevalence of genomic miR-10b amplifications in canine UC. Located on canine chromosome 36 (CFA36:22985925-22986034, canfam2),
the miR-10b locus is gained in 84% of all canine UCs (Chapter 2, Shapiro et al., 2015). Levels of mature miRNA transcripts have been found to correlate strongly to genomic copy number, suggesting miR-10b genomic amplification may also increase effective targeting miR-10b levels (L. Zhang et al., 2006). If miR-10b is increased in UC and plays a role similar to that seen in other carcinomas, miR-10b targeting may be a valuable therapeutic approach.

In addition, dysregulation of the MAPK pathway is often implicated in tumor growth, invasion, and metastasis, particularly in carcinomas (Horbinski, 2013; Warrick & Tomlins, 2015). BRAF is a serine/threonine kinase integral to initiation of the MAPK signaling cascade. With only two activation domains, mutations readily convert BRAF into a constitutively active form. Consequently, BRAF mutations can potentiate the entire MAPK signaling cascade and result in neoplastic transformation, cell proliferation, and invasion. The vast majority of BRAF mutations (>92%) occur in the activation domain at exon 15, codon 600, nucleotide 1799, and induce a valine to glutamic acid amino acid switch (BRAF<sup>V600E</sup>) (Spittle et al., 2007).

Although prevalent in epithelial tumors, including melanoma, previous studies have shown that BRAF mutations in human bladder UC patients are infrequent (2 in 30, or 6.7%) with rates in prostatic UC of ~10% (Boulalas, Zaravinos, Delakas, & Spandidos, 2009; Wen et al., 2011). Furthermore, mutation rates are increased in tumors of higher stage and grade. Canine studies, however, indicate that up to 90% of bladder UC patients exhibit the V600E mutation (Decker et al., 2015). To date, the prevalence of BRAF mutations in canine prostatic carcinomas has yet to be investigated. Pharmacologic BRAF<sup>V600E</sup> inhibitors are highly efficacious in an array of human tumors harboring the mutation. Thus, the detection of
BRAF$^{V600E}$ among canine patients may link canine UC to precision medicine protocols, in addition to highlighting MAPK pathway dysfunction in human and canine UC.

In the current study, we aimed to evaluate the viability of two potential and druggable molecular targets—miR-10b and BRAF$^{V600E}$—in canine UC. Using qRT-PCR, levels of mature miR-10b in canine UCs ($n=20$) were determined. Despite high level amplifications of CFA36, a significant reduction in miR-10b was observed among cases and without correlation to CFA36 Log2 ratios, suggesting miR-10b overexpression does not play a role in UC invasion. In addition, canine UCs ($n=45$), prostatic carcinomas ($n=17$), and UC cell lines ($n=5$), were evaluated for the BRAF$^{V600E}$ (canine V450E) mutation by Sanger sequencing. The mutation was highly prevalent ($\sim70\%$) tumors and cell lines and thus potentially relevant to disease pathogenesis. The effect of BRAF$^{V450E}$ on canine UC proliferation, invasion, and metastasis, as evaluated by siRNA knockdown in UC cell lines, however, suggested that BRAF$^{V450E}$ does not promote invasion in canine UC. Our results show that, although miR-10b and BRAF$^{V450E}$ may be pathologically relevant in canine UC, the discovery of potential physiologic implications requires further investigation.

**Methods for miRNA analysis**

*Case collection*

Formalin-fixed paraffin-embedded (FFPE) UC biopsies ($n=20$), in addition to healthy bladders as control ($n=10$), were included in miRNA analysis. UC biopsies were obtained via cystoscopy or cystotomy with owner consent prior to fixation in formalin and paraffin embedding. Healthy bladders were collected from freshly euthanized dogs at necropsy and immediately placed in transport media (RPMI media). Healthy bladders were then sectioned, fixed in 10% neutral-buffered formalin (NBF) for 24 hours, and paraffin embedded. Prior to
inclusion in this study, all bladder samples were confirmed to be UC (patients) or healthy (controls) by evaluation of hemotoxylin and eosin stained (H&E) sections by a board-certified veterinary pathologist. Prior to miRNA analysis, UC tissue sections were macrodissected to enrich for neoplastic cells and minimize dilution by normal cells.

miRNA isolation

Total RNA was extracted from all FFPE sections using the miRNeasy FFPE Kit (Qiagen, Valencia, CA). Eight to ten 5-µm sections were deparaffinized using xylene and then the protocol followed according to manufacturer’s recommendations. Following isolation, RNA samples were evaluated via spectrophotometry for isolate purity (260:230 and 260:280≥2) and concentration. Total RNA samples were analyzed with the Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA) using an RNA 6000 Nano total RNA Kit to ensure efficient miRNA extraction (peak evident at ~20-25 nucleotides) and evaluate RNA integrity (RIN) for further analysis. RIN value, however, has been shown to have no effect on detectable miRNA concentrations in miRNA analyses (Jung et al., 2010).

miRNA TaqMan® Assays

Analysis of four different miRNA was performed during this study: three possible control miRNA (miR-181b, miR-874, and miR-148b) and one test miRNA (miR-10b). For each target, a miRNA-specific TaqMan® MicroRNA Assay (Life Technologies, Carlsbad, CA) was acquired, including cfa-miR-181b, hsa-miR-874, hsa-miR-148b, and mmu-miR-10b. All miRNA assays include miRNA-specific stem-loop primers for cDNA synthesis with optimal sensitivity and specificity, cDNA-specific qRT-PCR primers, and TaqMan miRNA probes (5’ FAM reporter, 3’ NFQ-MGB quencher) for qRT-PCR detection and quantification.
**miRNA cDNA Conversion**

For each miRNA analyzed, miRNA-specific cDNA was created with the miRNA-specific TaqMan® Assay using the TaqMan® MicroRNA Reverse Transcription Kit (Life Technologies) miRNA-specific reverse transcriptase primers included. Total RNA (10 ng, as calculated from spectrophotometry concentration) was added to a 15 µL reverse transcription reaction containing 100mM dNTPs, MultiScribe™ Reverse Transcriptase 50U, 10x RT buffer, and 3.8 U RNase Inhibitor. Reverse transcription cycling conditions were as follows: 30 minutes at 16°C, 30 minutes at 42°C, and 5 minutes at 85°C.

**TaqMan qRT-PCR**

miRNA-specific TaqMan Small RNA Assays (20X, Life Technologies) were used for miRNA qRT-PCR analysis of the four different canine miRNAs. cDNA product (1 uL) was added to the miRNA Small RNA Assay mix, along with miRNA cDNA-specific primers, a miRNA-specific TaqMan probe tagged with a 5'-6-FAM reporter and 3'-MGB-NFQ quencher, and TaqMan Universal Master Mix II (no UNG, Life Technologies). All reactions were performed in triplicate and repeated twice on two different qRT-PCR machines to ensure result consistency: the ABI StepOne™ Plus (Life Technologies) and the LightCycler® 480 (LC480, Roche, Indianapolis, IN). Cycling conditions were as follows: 95°C enzyme activation for 10 minutes, followed by 40 cycles of 95°C for 15 seconds and 60°C for 60 seconds. Critical threshold (Ct) values were determined used in the Second Derivative Maximum algorithm in the LC480 software (Roche). Significant differences in miR-10b expression ($\Delta C_{t_{miR-10b-reference}}$) among samples were determined by a Mann-Whitney U Test (two-tailed, $\alpha=0.05$) in JMP Professional Statistical Software (v.11, SAS, Cary, NC). The 2$^{-\triangle\Delta C_{t}}$ method was used to determine fold change of miR-10b in tumor samples relative to
control samples, where \( \Delta \Delta Ct = \Delta Ct_{\text{TUMOR}} (Ct_{\text{miR-10b}} - Ct_{\text{REFERENCE}}) - \Delta Ct_{\text{CONTROL}} (Ct_{\text{miR-10b}} - Ct_{\text{REFERENCE}}) \).

Reference miRNA analysis

Ct values were determined for all 30 samples (10 normal, 20 tumor). A mean Ct value, along with standard deviation (SD) was calculated for each potential reference gene. Genes with a SD <1 among all samples were considered potential reference genes. Ct values were then converted to a linear scale using \( 2^{-\Delta Ct} \) and analyzed using the NormFinder (Aarhus University Hospital, Denmark) add-on for Excel (Microsoft, Redmond, WA). NormFinder determines a relative stability value (SV) for each reference gene/combination of reference genes from 0-1, with values close to zero defining the most stably expressed reference gene (Claus Lindbjerg Andersen, Jensen, & Ørntoft, 2004).

mRNA isolation and qRT-PCR of miR-10b target HOXD10

mRNA levels of the miR-10b target HOXD10 (cfa36: 22951600-22954004, CanFam2) were evaluated to evaluate the targeting effects of miR-10b. mRNA was isolated from FFPE tumor biopsies using the E.Z.N.A FFPE RNA Kit (Omega Biotek, Norcross, GA) using xylene deparaffinization and following manufacturer’s recommendations for isolation. Following RNA isolation, all samples were analyzed via spectrophotometry (Nanodrop, 260:230 and 260:280>2). mRNA (1 ug) was then converted to cDNA using the SuperScript Vilo cDNA Synthesis Kit according to manufacturer’s recommendations (Life Technologies, Carlsbad, CA).

Primers were designed to target canine HOXD10 mRNA (F: 5’-AGCAAAGAGGAAATCAAGTCTGA-3’, R: 5’-TGGTGCTTAGTGTAAGGGCA-3’) and the validated reference gene RPL32 (F: 5’-ATGCCCAACATTGGTTATGG-3’, R: 5’-CTCTTTCCACGATGGCTTTG-3’) (Chapter 3, 4). Prior to qPCR analysis, HOXD10 primer
annealing temperature optimization was carried out using a temperature gradient RT-PCR (55-65°C), with the optimal temperature found to be 62.5°C. Optimal conditions for RPL32 were used as previously described (Chapter 3, 4). After primer optimization, an efficiency curve was constructed utilizing a set of five ten-fold dilutions of neoplastic RNA. An efficiency of 95% was observed, ensuring reliable qPCR interpretation. For qPCR, KAPA SYBR® Fast Universal Master Mix with ROX (KAPA Biosystems, Wilmington, MA) was used, along with 0.5 µM of each forward and reverse primer. Reactions were run on the ABI StepOne Plus™ Real Time PCR System (Applied Biosystems) with the following conditions: 95°C 3m, (95°C 20s, 62.5°C 20s, 72°C 20s) x 40 cycles. A relative fold change was calculated by comparing HOXD10 expression between normal and neoplastic specimens using the $2^{-\Delta\Delta C_t(neoplastic\text{-}control)}$ method, where $\Delta C_t = C_{t_{HOXD10}} - C_{t_{RPL32}}$.

oaCGH/fold change correlation analysis

To determine if genomic levels of CFA36 lead to increased levels of the contained miRNA, Log2 ratios, as previously determined by oligonucleotide array comparative genomic hybridization (oaCGH, Chapter 2) were compared to miR-10b expression (Shapiro et al., 2015). oaCGH values for the miR-10b locus were available for 19 of 20 patients (Table 1). Using a simple linear regression analysis for two continuous variable in JMP Pro 11, the Log2 ratios and Log2 fold change values of miR-10b for these 19 patients were compared and fitted with a linear regression line, including the $R^2$ value for goodness of fit.

Methods for BRAF analysis

Case collection

Biopsies were collected from histopathologically-confirmed cases of bladder urothelial or prostatic carcinoma obtained via cystoscopy or cystotomy with approved owner
consent. Samples were then either flash-frozen in liquid nitrogen (n=24 bladder) and stored at -80°C or fixed in 10% NBF for 24 hours, followed by paraffin embedding (FFPE, n=20 bladder, 17 prostate). Prior to inclusion in analysis, tumor FFPE sections were macrodissected to enrich for neoplastic regions. In addition, one free-catch urine sample (500 µL) was collected from a dog diagnosed with UC via ultrasound (urethral invasion and metastasis noted), urine cytology, and FISH (+13/-19/+36) (Shapiro et al., 2015). Five previously established cell lines (K9TCC-Pu-An, K9TCC-Pu-In, K9TCC, K9TCC-Pu-In, and K9TCC-Pu-Sh) were included. FFPE samples of healthy/non-neoplastic (control) bladder (n=4) and prostatic (n=5) urothelium obtained from nine recently (<2 hours) euthanized dogs was included following histopathologic confirmation of healthy (non-neoplastic, non-inflammatory) tissue.

**DNA isolation**

For sequencing, DNA was isolated from tissue specimens and cell lines using either the DNeasy Blood and Tissue Kit (frozen tissues, cell lines; Qiagen) or the QIAmp DNA FFPE Tissue Kit with xylene deparaffinization (FFPE, Qiagen). For isolation of urine DNA, the QIAmp Viral RNA Mini Kit (Qiagen) was used following urine concentration using an Amicon Ultra-15 mL 30K concentrating column (4000 RPM x 20-40 minutes, volume-dependent; Milipore, Billerica, MA). Following isolation, all samples were analyzed via gel electrophoresis and spectrophotometry (260:230 and 260:280≥1.8).

**Primer design, PCR, and targeted Sanger Sequencing**

Canine BRAFV600E-specific primers were designed by interrogating the genomic region syntenic to the location of the human BRAFV600E mutation. The canine BRAF gene is located at CFA16:8,222,913-8,318,179 (CanFam3.1). The V600E mutation is in exon 15 of the gene at codon 450 of the isoform 1 transcript, at nucleotide position 1349, leading to
recognition of the V600E mutation as V450E in the dog. Flanking forward (5’- GTCACATATCGGAAATAGAACC-3’) and reverse (5’-ATTGATTTTTGTGAATACTGGG-3’)
primers were designed at CFA16:8206013-8296034 and 8296470-8296449, respectively, yielding a BRAF exon 15 amplicon of 458 base pairs.

PCR conditions were optimized using an annealing temperature gradient. The optimal annealing temperature was found to be 58°C, at which temperature all further PCR reactions were performed. Apex™ REDTaq DNA Polymerase Master Mix (Genesee Scientific, San Diego, CA), along with 0.5 uM of each forward and reverse primer, was used in each PCR reaction. Reaction cycling conditions were as follows: 95°C 5 min, (95°C 20s, 58°C 20s, 72°C 20s) x 30 cycles, and a final elongation step of 72°C for 5 minutes. PCR products from all reactions were analyzed by agarose gel electrophoresis for appropriate BRAF product amplification and product specificity (single band ~458 bp). The remainder of the PCR reaction was purified using the GeneJET PCR Purification Kit (Thermo-Fischer Scientific, Waltham, MA) with elution in ultrapure water. Purified PCR product (10 ng) was combined with 6.4pmol of forward primer and subjected to Sanger sequencing at the North Carolina State University Genomic Sciences Laboratory using the ABI 3730xl DNA Analyzer (Life Technologies).

**Sequencing analysis**

Sequence data were analyzed using the CLC Sequence Viewer (CLC Bio, Boston, MA) and 4Peaks (NucleobYtes, Amsterdam, Netherlands) software. Electropherograms were closely interrogated at the V450E locus to enable detection of mutated alleles less prevalent than wild type.
Cell culture

Three previously transfected cell lines (K9TCC-Pu-An, K9TCC-Pu-Mx, K9TCC-Pu-Sh; Chapter 4) were thawed from storage in liquid nitrogen using a rapid thaw technique and then grown in DMEM/F12 media (Mediatech, Manassas, VA) supplemented with 12% FBS, 1.0% GlutaMAX (Life Technologies), and 0.2% Primocin (Invivogen, San Diego, CA). Prior to transfection and knockdown analysis, cell lines were grown for at least two weeks to ensure consistent growth and vitality.

DsRNA-mediated BRAF knockdown

siRNA-mediated knockdown of BRAF was accomplished by using a single dicer-substrate interfering RNA (DsRNA) complementary to BRAF mRNA at exon 5 with the following sequences: 5’-rGrGrArArCrArUrArUrArGrArArGrCrCrCrUr-3’ and 5’-rGrUrCrCrArArGrGrCrUrUrCrUrArU-3’ (CFC.RNAI.X532749.12.2, Integrated DNA Technologies, Coralville, IA). A transfection mix was first created, consisting of 500µL serum-free DMEM/F12 media, Lipofectamine® RNAiMAX (Life Technologies) (1% (K9TCC-Pu-An, K9TCC-Pu-Mx) or 1.5% (K9TCC-Pu-Sh)), and one of three DsRNA treatments 1) no duplex (transfection control), 2) non-targeting negative control duplex (negative control), or 3) BRAF-targeting duplex (knockdown). The transfection mix (500 µL) was then added to the wells of a six-well plate in duplicate. Cells (1x10^5) in 2 mL of antibiotic free media were then seeded in each well of a six-well plate and incubated at 37°C for 42 hours. Duplex concentrations of 10nM (K9TCC-Pu-An, K9TCC-Pu-Mx) or 20nM (K9TCC-Pu-Sh) were found to elicit maximal BRAF knockdown, as assessed by qRT-PCR (see below).

RNA isolation, cDNA conversion, and qRT-PCR analysis

Prior to qRT-PCR quantification of knockdown experiments, mRNA-specific primers were designed to target BRAF at a region outside of the V450E mutation to eliminate
differences in qPCR efficiency among genotypes (F: 5’-
AATGATTAAGTGACACAGGAACA-3’; R: 5’-GTGTATTCTTCATAGGCTCCAGA-3’). The
optimal annealing temperature and product specificity was assessed by gradient RT-PCR,
with an annealing temperature of 62°C chosen for further qRT-PCR analysis. BRAF primer
efficiency curves were constructed using a series of five, 10-fold dilutions of cDNA, which
showed BRAF primers to be 101% efficient. RPL32 primers and reaction conditions were as
described above in HOXD10 analyses. cDNA was combined with 0.5 µM of forward and
reverse primer and with LightCycler 480 SYBR Green I Master Mix (Roche) with the
following cycling conditions: 10m 95°C and (95°C 10s, 60°C 10s, and 72°C 10s) x 45 cycles.
Simultaneous melt curve analysis allowed confirmation of single product amplification and
specificity. Ct values were determined by the second derivative maximum algorithm on the
LC480 Software (v. 1.5, Roche).

At ~42 hours post transfection (PTF), RNA was isolated from each well of the six-
well plate using the RNeasy Plus Mini Kit (Qiagen) in-well lysis protocol according to
manufacturer’s recommendations, including use of the QiaShredder (Qiagen) for cell
homogenization. cDNA was then synthesized using from purified RNA (100 ng) and the
QuantiTect Reverse Transcription Kit (Qiagen). Synthesized cDNA was diluted 1:1 with
ultrapure water and then combined with 0.5 µM of forward and reverse primer, along with
LightCycler 480 SYBR Green I Master Mix (Roche). Cycling conditions and data analysis
were as in efficiency curve calculation. All reactions were performed in triplicate with no
template negative controls included to monitor reagent contamination. The percent gene
expression remaining after knockdown was calculated according to % remaining expression
=100 - (100% x 2^ΔΔCt). Maximal knockdown achieved was ~75-80% in all instances, leaving
<25% expression remaining at 42 hours PTF. In addition, alterations in cell proliferation
(total cell number) due to \textit{BRAF} knockdown were determined by counting total cells at 42 hours PTF (transfection control, negative control, knockdown) using the Cellometer Auto T4 and noting cell viability (Nexcelom, Lawrence, MA).

\textit{Invasion analysis}

To access the effect of \textit{BRAF} knockdown on cell invasion, six-well plate Matrigel Invasion Chambers (Corning, Corning, NY) were used. In the upper chamber of the transwell plate, $1 \times 10^5$ cells were reverse transfected and seeded in serum and antibiotic-free media, along with non-targeting or \textit{BRAF} targeting DsiRNA and transfection reagent plated in triplicate, as described previously (Tamashiro et al., 2014). To the bottom well, 2 mL of media containing 12\% FBS was added, serving as chemoattractant for cell invasion. After 42 hours at 37$^\circ$C, unininvaded cells were removed from the upper surface of the Matrigel membrane by scrubbing with a cotton swab soaked in 1X phosphate-buffered saline (PBS) then removing the media, adding 1X PBS, and repeating the scrub/wash twice. After the third scrub and PBS removal, the upper transwell was transferred to a six-well plate of 1X PBS and rinsed for 3 minutes to remove residual FBS. Then, transwells were moved to 100\% methanol for fixation for 10 minutes, followed by 10 minutes in a crystal violet stain (0.1\% crystal violet, 0.01\% ethanol). Membranes were rinsed in a series of three beakers of ultrapure H$_2$O and then left to dry for at least 24 hours. After 24 hours, membranes were removed from the transwell inserts and mounted onto a clean class slide (bottom side-down) using VectaShield (Vector Laboratories, Burlingame, CA) topped with a coverslip. For invasion analysis, the membrane was divided into quarters. Two opposing quarters were counted, averaged, and multiplied by four to determine the total number of invaded cells per membrane.
BRAF knockdown experiments were compared to negative control experiments to account for differences in invasion due to the transfection procedure per se. To account for differences in total cell number, an invasion index was further calculated for each cell line (invasion index= \( \frac{\text{% invaded}_{\text{knockdown}}}{\text{% invaded}_{\text{negative control}}} \), where \( \text{% invaded}=\frac{\# \text{ invaded}}{\text{total \# cells}} \)) Significant differences in invasion were determined by a Mann-Whitney Exact Test (two-tailed, \( \alpha=0.05 \)) of the number of cells invaded and invasion indices (JMP Pro 11).

Results

A combination of reference miRNA provides the most stable reference for quantitative miRNA analysis.

Ct values for each reference gene (miR-874, miR-181b, miR-148b) were compared among all samples (control bladders and tumors). The standard deviation (SD) of Ct values for each gene was calculated, with genes of SD<1 being deemed adequate reference genes. Only one single gene—miR-148b—showed stable expression (SD<1). Three gene combinations showed stable expression: 1) miR-871/-181b, 2) miR-148b/-181b, and 3) miR-148b/-871/-181b. Of these, Ct values of miR-874/-181b showed the least deviation from the mean (SD=0.73).

Ct values were then linearized (\( 2^{-\text{Ct}} \)) to allow comparison by the reference gene selection software NormFinder. NormFinder assigns a stability value based on the standard deviation and accumulated standard deviations from a set of potential reference genes (C. L. Andersen, Jensen, & Orntoft, 2004). The combination of all three miRNA references—miR-148b/-871/-181b—gave the most stable expression according to NormFinder, with a stability value=0.083 (Figure 1). The stability value of no other gene or combination of genes
was <0.1. Therefore, the average Ct values of miR-148b/-871/-181b were most appropriately used for relative fold change determination of miR-10b (reference average).

*miR-10b is significantly downregulated in canine UC.*

To evaluate significant differences in miR-10b levels, $\Delta Ct_{miR-10b-reference\_avg.}$ were compared among all healthy and neoplastic bladders. $\Delta Ct$ values among control urothelium was significantly lower than those of neoplastic, implying significantly decreased levels of miR-10b in canine UC ($p<0.0001$, Mann Whitney U test, Figure 2A). The fold change of miR-10b in bladder tumor samples was then compared to normal bladder samples using the 2$^{-\Delta\Delta Ct}$ method. To obtain a value for $\Delta Ct_{\text{CONTROL}}$, the average Ct values for miR-10b among all healthy controls were averaged (avg.Ct$_{\text{miR-10b}}$), as well as the average Ct values for the average of all three miRNA reference genes for each control sample (miR-148b/-874/-181b) for normalization (avg.Ct$_{\text{reference\_avg.}}$), to give $\Delta Ct_{\text{control}}$=avg.Ct$_{\text{miR-10b}}$-avg.Ct$_{\text{reference\_avg.}}$. In addition a fold change was calculated for individual control samples to ensure a fold change ~+/-1 (no change) when compared to the average of all controls, demonstrating control homogeneity and validating UC patient differential expression ($p<0.0001$, Fig. 2B). miR-10b fold change values for UC biopsies were significantly lower (mean fold change= -3.9, SD=2.5, $p<0.0001$, Table 1) than control urothelium (mean fold change= -0.6, S.D.=1.2). Furthermore, 16 of 20 tumors (80%) showed a significant ($\geq$ 2-fold decrease) in miR-10b levels.

To determine potential miR-10b functionality, mRNA levels of the miR-10b downstream target *HOXD10* were determined by qPCR. Among tumors, 18 of 20 (90%) showed a significant ($\geq$2-fold) increase in *HOXD10*. Thus, *HOXD10* levels were significantly elevated in neoplastic urothelium relative to healthy (average fold change= +9.7, S.D.= 8.3, Table 1), supporting that miR-10b did not have increased targeting and degradation of
HOXD10. These levels, however, did not correlate to levels of miR-10b ($R^2=0.038$) or levels of CFA36 ($R^2=0.04$).

Tumor miR-10b Log2 fold changes were further compared to their respective CFA36 oaCGH-derived Log2 ratios, when available (19 of 20 cases). Using a simple linear regression analysis, the dependence of miR-10b levels on CFA36 copy number was determined. The linear fit displayed an $R^2=0.013$, indicating that only 1.3% of fold change variation may be explained by miR-10b Log2 values. Therefore, relative levels of CFA36 are not predictive of mature miR-10b levels (Fig. 3).

**BRAF V600E (V450E) mutations are prevalent in canine bladder and prostate carcinomas and UC cell lines.**

Sanger sequencing of BRAF exon 15 elucidated a high prevalence of the equivalent V600E (V450E) mutation in 30 of 45 canine bladder urothelial carcinomas (67%) and 13 or 17 prostatic carcinomas (76%), yielding an overall prevalence of 71.5% in carcinomas of the lower urogenital tract. In both bladder and prostate carcinomas, all recognized mutations were characterized by a T$\rightarrow$A (GTG$\rightarrow$GAG) nonsynonymous substitution in codon 450 of the **BRAF** gene, resulting in an amino acid switch from valine to glutamic acid (Fig. 4A). All **BRAF**$^{V450E}$ patients were, however, heterozygous for the mutation (Fig. 4B, C), with the wild type allele often predominant (Fig. 4B). No other mutations in **BRAF** exon 15 were observed. In addition, 4 of 5 previously characterized (Chapter 3) canine UC cell lines (80%) demonstrated the V450E mutation (K9TCC-Pu-An, K9TCC-Pu-In, K9TCC, and K9TCC-Pu-Mx). No control bladder or prostate tissues indicated the presence of a **BRAF** exon 15 mutation. Thus, the overall prevalence of **BRAF**$^{V450E}$ among urogenital tumors and cell lines was 70.1%.
Knockdown of BRAF minimally reduces cell proliferation and unpredictably and minimally affects invasion.

DsiRNA technology was utilized to reduce cellular levels of BRAF, including BRAF\textsuperscript{V450E}. To ensure both wild type and mutant BRAF genotypes would be targeted, the knockdown duplex targeted non-mutated regions of the BRAF gene, specifically exon 5. To analyze knockdown efficiency, qRT-PCR quantified levels of BRAF mRNA at 42 hours PTF. Knockdown efficiency was calculated using the Ct values for knockdown experiments and control (non-targeting DsiRNA) cells by Efficiency = % gene expression knockdown = 100 - (100% x 2^{\Delta\Delta C_t(knockdown-control)}). Knockdown efficiency was found to be >75% in all cell lines included in analysis (Figure 5).

Efficient transfection allowed further analysis of the role of BRAF by comparing the effects of BRAF knockdown on BRAF\textsuperscript{V450E} (K9TCC-Pu-An, K9TCC-Pu-Mx) and wild type (K9TCC-Pu-Sh) cell lines. Among the cell lines, BRAF knockdown resulted in insignificantly reduced cell numbers at 42 hours PTF compared to non-targeting oligonucleotide control (negative control) (p=0.40, Mann-Whitney Exact Test): 18% decrease in K9TCC-Pu-An and 17% in K9TCC-Pu-Mx and K9TCC-Pu-Sh (Table 2, Figure 6A). Then, BRAF knockdown was combined with Matrigel invasion chambers, an in vitro means of assessing cell invasion and metastatic potential. Upon comparing the numbers of cells invaded through the Matrigel membrane, no significant difference between negative control (non-targeting duplex) and the respective knockdown cells was observed (K9TCC-Pu-An p=0.66, K9TCC-Pu-M p=0.08, K9TCC-Pu-Sh p=0.08; Mann Whitney Exact Test). Similarly, when comparing invasion indices (\%invaded\textsubscript{knockdown}/\%invaded\textsubscript{neg_control}), BRAF knockdown showed a minimal effect on the invasiveness of individual neoplastic cells (Table 2, Figure 6B): K9TCC-Pu-Mx showed the greatest mean decrease in invasion with DsiRNA BRAF knockdown (mean=18.1%
decrease, 81.9% of control), while K9TCC-Pu-An showed an increase in invasion potential (106% of control). K9TCC-Pu-Sh (BRAF wild type) showed a minimal decrease in invasiveness (13.4% decrease, 86.7% of control). No significant difference in invasive ability was observed between cell lines with and without the BRAFV604E (p=0.24, comparing invasion indices or percent of invaded cells in mutant and wild-type cell lines).

Discussion

miR-10b in canine UC

In both human and veterinary medicine, invasive urothelial carcinoma (UC) poses a therapeutic challenge. In addition to demonstrating a high rate of metastasis (~50% in both species), efficacious therapeutics to curtail invasion and metastasis and extend survival are severely lacking, leading to the association of invasive UC with a poor prognosis. Although less common than prognostically favorable superficial tumors in humans (approximately 20% and 80%, respectively), dogs rarely develop superficial UC, with over 90% invading the basement membrane by the time of diagnosis (Knapp et al., 2014). Thus, the canine represents a spontaneous and valuable model in the search for improved treatments for invasive human UC. The detection of actionable biomarkers, including dysregulated and mutated genes, would augment the number of viable therapeutics and expand precision medicine in UC.

miRNA constitute a class of biomarkers ideal for both cancer detection and targeted treatments. Mature miRNA are small RNA molecules (~22 nucleotides) which serve as single-stranded RNA “guides” for the type III RNase Dicer. When a miRNA complementary to a messenger RNA is present, the association of Dicer with miRNA leads to targeted degradation of the complementary transcript, resulting in reduced transcript levels or
translation inhibition (Carthew & Sontheimer, 2009). If the targeted gene is a tumor suppressor, overexpression of a complementary miRNA can result in reduced levels of the tumor suppressor and tumorigenesis. In addition to its functional relevance, miRNA is extremely stable in fixed tissues and bodily fluids, including urine, making differential expression readily detectable. If elevated levels of a particular miRNA exist in neoplastic cells, patient-tailored ASO therapy can effectively reduce miRNA levels in vivo (Sicard, Gayral, Lulka, Buscail, & Cordelier, 2013; Ohno et al., 2013). Thus, miRNA has the potential to impact several aspects of cancer management.

One particular miRNA, miR-10b, has been implicated both human and canine UC. miR-10b is overexpressed in numerous human cancers, including bladder, and promotes aggressive, invasive phenotypes via targeted degradation of the anti-invasion factor HOXD10 (Z. Liu et al., 2012; Nakayama et al., 2013; Parrella et al., 2014; Xiao et al., 2014; A. Zaravinos et al., 2012). Consequent loss of inhibitory HOXD10 leads to increased expression of pro-invasion RhoC, facilitating tumor invasion and metastasis (Ueno et al., 2012; Xiao et al., 2014). Hence, miR-10b overexpression is associated with the development of metastases in bladder cancer, among others (Xiao et al., 2014). Other studies, however, have shown reduced expression of miR-10b in primary tumors compared to surrounding normal urothelium (Zaravinos et al., 2012).

In dogs, miR-10b is located in a peak region of CFA36 amplified in >83% of UC patients (Fig. 7) (Shapiro et al., 2015). Furthermore, miR-10b is located within the HOXD cluster of CFA36 expressed during embryogenesis and necessary for adult urogenital function (Redline, Williams, Patterson, & Collins, 1992). Increased levels of the transcriptionally active genomic region during adulthood might result in increased expression of miR-10b. We hence hypothesized that increased genomic levels of the miR-
10b locus lead to overexpression of the pro-invasion oncoMIR miR-10b. To test this hypothesis, we compared levels of the mature miR-10b transcript among neoplastic and healthy urothelium.

Prior to miR-10b analysis, it was necessary to select a suitable reference gene for intersample normalization. In previous canine miRNA studies, the small RNA RNU6B has been used for miRNA normalization; however, cellular RNU6B levels are unpredictable and result in deceptive miRNA expression interpretations (Ratert et al., 2012; Wotschofsky et al., 2011). RNU6B is 106 base pairs in length, making it approximately five times longer than mature miR-10b and consequently less stable, more impacted by formalin fixation, and more efficiently extracted than miRNA (Ratert et al., 2012). Furthermore, use of RNU6B requires cDNA conversion and real-time detection by methods other than TaqMan miRNA assays, adding an additional variable to expression analysis. To improve the reliability miRNA analysis in canine UC, we sought a suitable miRNA or combination of reference miRNAs for valid qRT-PCR analysis. We analyzed three miRNA stably expressed in human UC—miR-181b, miR-874, and miR-148b—for similar stability in canine UC (Ratert et al., 2012). Using TaqMan miRNA cDNA conversion and qRT-PCR assays, the expression of each miRNA in normal and neoplastic urothelium was determined. By comparing miRNA Ct standard deviations and stability values, as calculated by NormFinder, the combination of all three miRNAs provided the most stable reference values and was reliably used for future analyses. We further advocate this reference miRNA combination as preferable to the current standard RNU6B.

We then determined levels of miR-10b in healthy and neoplastic canine bladder specimens using a TaqMan Canine miR-10b qRT-PCR assay normalized to the average of all three reference miRNA. When comparing expression between normal and neoplastic
bladders, 80% of tumors showed significantly reduced levels (>2-fold decreased) of miR-10b (mean fold change= -3.9), contrary to our hypothesis. In agreement, we found HOXD10, a major miR-10b mRNA target, to be significantly overexpressed (≥2-fold) in 90% of tumors compared to normal bladder (average fold change= +9.7), supporting reduced degradation due to reduced levels of the miRNA. We cannot ignore, however, that HOXD10 is located only ~34 kb upstream of miR-10b and included within the genomic region of high copy number gain, suggesting genomic copy number may augment levels of HOXD10. If indeed gain of the HOXD10 locus is involved in overexpression, a genomic or epigenetic alteration between the HOXD10 and miR-10b loci, potentially due to genomic anomalies such as chromothripsis, may be responsible for the discordance in expression levels between the two genes. Additionally, not all tumors with decreased miR-10b showed increased levels of HOX10, suggesting that downregulation of miR-10b alone is not responsible for HOXD10 overexpression. Nevertheless, our results support that miR-10b is decreased in primary canine UC, supporting previous human studies showing reduced levels of miR-10b in the primary tumor. Furthermore, anti-invasion factor HOXD10 is increased in canine UC, suggesting UC invasion occurs via a pathway unaffected by inhibitory HOXD10. miR-10b levels in canine UC metastases should, however, be investigated to further draw parallels between human and dog and augment our understanding of miR-10b in UC.

Despite the roles of miR-10b in invasion via downregulation of HOXD10, recent studies have elucidated an alternative role of miR-10b in invasion inhibition (Guo et al., 2015). Upregulation of miR-10b is associated with decreased cell invasion, while miR-10b downregulation correlated with increased cell invasion in endometrial adenomyosis. Via interactions not yet well understood, decreased miR-10b leads to increased activation of PI3KCA and, subsequently, AKT. Thus, decreased levels of miR-10b may amplify the
already heightened level of PI3K/AKT activity in UC, leading to cell invasion and tumor dissemination, and should be further investigated.

Regression analysis of miR-10b levels and respective CFA36 Log₂ ratios showed no correlation ($R^2<<1$) existed between genomic levels of miR-10b and transcript levels. Thus, amplification of CFA36 in canine UC does not lead to tumor invasion via increased miR-10b. Numerous explanations may be made for this discrepancy in genomic and transcript levels, beginning with the genomic makeup of CFA36. As we have previously elucidated, CFA36 is subject to recurrent and widespread chromothriptic-like events—chromosome shattering and inaccurate rejoining that results in localized regions of chromosome gain and loss (Chapter 2, Shapiro et al., 2015). Chromothripsis rearranges affected areas, as well as removes promoter regions essential for expression. The disruption of genomic composition in chromothriptic regions may alter transcriptional dynamics, leading to reduced or increased gene expression dependent on juxtaposed regions. Furthermore, miR-10b in contained within a region of CFA36 syntenic to the cancer associated fragile site at human chromosome (HSA) 2q31, predisposing the region to generation of double-stranded DNA breaks and subsequent genomic amplification, deletions, and chromosome rearrangements (Calin et al., 2004; Ma et al., 2012). Thus, disruption of the miR-10b genomic locus may contribute to unexpected expression. Additionally, epigenetic modifications, such as methylation, may attempt to compensate for genomic amplification, leading to silencing of target genes (Kurscheid et al., 2015).

In the current study, only levels of mature miRNA transcripts were quantified. These mature miRNA, however, reflect an edited version of the transcribed genomic locus. miRNA transcripts go through two distinct phases of processing, from a transcribed primary miRNA (pri-miRNA), through a precursor miRNA (pre-miRNA), at which point they are exported from
the nucleus and cleaved by the Dicer complex to yield the mature miRNA (Winter, Jung, Keller, Gregory, & Diederichs, 2009). Thus, it is possible that earlier transcripts are present in increased levels, and may, therefore, be more illustrative of CFA36 genomic levels. Nevertheless, it is the mature miRNA form which is physiologically active, maintaining that miR-10b upregulation is likely not involved in the pathogenesis of invasive canine UC.

**BRAF\textsuperscript{V600E} in canine UC**

Previous studies of human UC have elucidated a major role of MAPK pathway dysregulation in tumorigenesis. In particular, MAPK activation leads to initiation of the epithelial-mesenchymal transition and tumor invasion (Tomlinson, Baxter, Loadman, Hull, & Knowles, 2012). A prevalent mechanism of MAPK activation in metastatic epithelial tumors is an activating mutation of the RAF kinase BRAF, most commonly at codon 600 (BRAF\textsuperscript{V600E}) and associated with tumor aggression, invasion, and metastasis (Affolter, Samowitz, Tripp, & Bronner, 2013; W. Liu et al., 2007; J. Lu et al., 2015). Previous whole-exome sequencing experiments in our lab, in addition to others, have elucidated the existence of activating BRAF mutations in canine UC (unpublished data, Decker et al., 2015). Thus, we hypothesized that in a larger cohort of canine UC tumors, the BRAF\textsuperscript{V600E} mutation would maintain prevalence and confirm a similar importance of MAPK dysregulation in UC.

We sequenced primary canine bladder UCs, canine UC cell lines, prostatic carcinomas, and healthy bladder and prostate to evaluate the prevalence of the canine equivalent of BRAF\textsuperscript{V600E}. Upon comparative interrogation of the canine and human genomes, the canine equivalent of BRAF\textsuperscript{V600E} (exon 15) was found to be located at codon 450 (BRAF\textsuperscript{V450E}). In our canine tumors, 67% of primary UCs, 76% of primary prostatic carcinomas, and 80% (four of five) UC cell lines demonstrated the characteristic BRAF\textsuperscript{V450E}
thymine to adenine transversion, resulting in a non-synonymous amino acid substitution of glutamic acid for valine. This introduction of an abnormal, positively-charged amino acid (glutamic acid) in the activation pocket of BRAF mimics phosphorylation, resulting in phosphorylation-independent activation of BRAF (Agell et al., 2011; Horbinski, 2013). Furthermore, BRAF, the most naturally potent of the RAF kinases, is ~500 times more potent when mutated than wild type, leading to increased MAPK pathway activation without altered levels of mRNA or protein (Cantwell-Dorris, O’Leary, & Sheils, 2011).

Many human BRAFV600E tumors are “oncogene addicted”—they require constitutive activation of BRAF to survive and proliferate (A. Hall et al., 2013). In addition to leading to increased cell proliferation, constitutive MAPK activation also upregulates extracellular matrix components, including fibronectin, facilitating ECM remodeling and neoplastic cell invasion (Mesa et al., 2006). Consequently, previous studies in carcinomas have shown knockdown or inhibition of BRAF in mutant tumors significantly reduces tumor proliferation, invasion, and metastasis in vitro and in vivo (Nucera, Lawler, & Parangi, 2011). Pharmacologic BRAF inhibitors have proven efficacious in the treatment of human BRAFV600E tumors. Thus, BRAF inhibitors may be similarly valuable in the treatment of BRAFV450E canine tumors.

Due to the prevalence of BRAFV450E in canine lower urogenital tumors and potential pathologic significance, we hypothesized that BRAF inhibition represents an efficacious treatment for canine UC. Specifically, reduction of BRAF mRNA in UC cell lines would reduce tumor proliferation and invasion and suggest efficacy of pharmacologic BRAF inhibitors. To test our hypothesis, we used Matrigel invasion chambers in conjunction with targeted DsiRNA knockdown of BRAF. Although marginal decreases in cell proliferation and number of cells invaded were seen, BRAF knockdown had no significant effect on any of the
three tested cell lines, regardless of \textit{BRAF} mutation status. Interestingly, the mutant cell line K9TCC-Pu-An showed a minimal decrease in proliferation but rather increased invasiveness with \textit{BRAF} knockdown (106\% of control). Although the remaining two cell lines showed a small decrease in invasiveness, one cell line was wild type (K9TCC-Pu-Sh), suggesting this effect is mutation-independent. Thus, we conclude that although \textit{BRAF} knockdown may have slight effects on cell proliferation and invasion, these results are not predictable and suggest limited and variable efficacy in \textit{BRAF}-targeted therapies. It cannot be ignored, however, that \textit{BRAF} knockdown was less than ideal (~80\%), leaving a significant portion of the highly active \textit{BRAF} mutant. Alternatively, previous studies of human prostatic carcinoma have shown \textit{BRAF}^{V600E} may be necessary to initiate, but not sustain, tumor invasion (Jeong et al., 2008). Thus, gene knockdown or targeting after tumor establishment has negligible efficacy in these human tumors and may be similar in canine urogenital tumors.

In accordance with our findings, numerous studies have reported limited efficacy of \textit{BRAF} inhibitors due to the rapid acquisition of therapeutic resistance, particularly in non-melanotic tumor types (Thakur & Stuart, 2014). Surprisingly, \textit{BRAF}^{V600E} has an inhibitory effect on the PI3K pathway via inhibition of mTORC2 and AKT (Sicard et al., 2013). Thus, inhibition of \textit{BRAF} removes negative feedback of the PI3K/AKT pathway known to be central to UC development (Perna et al., 2015). Studies of pharmacologic \textit{BRAF} inhibition by vemurafenib also demonstrate decreased sensitivity in cells demonstrating bFGF/FGFR3 hyperactivation, as is frequently observed in both human and canine UC (Wilson et al., 2012; Yadav et al., 2012). In light of the role of the PI3K/AKT pathway in UC, it is unsurprising that \textit{BRAF}^{V450E} inhibition seems to have little effect on these tumors.

The prevalence of \textit{BRAF}^{V450E} in canine urothelial tumor reaffirms a critical role of MAPK pathway dysregulation in UC development, as has been described previously.
(Dangle et al., 2009; Ewald, et al., 2013). Although a discrepancy in \textit{BRAF} mutation frequency exists between humans and canine UC, redundancy in affected pathways highlights a shared disease pathogenesis and, therefore, potential therapeutic target. Differences in \textbf{BRAF}^{V600E} frequencies may provide insight into why canine tumors are far more invasive and aggressive than human tumors and help clinicians identify aggressive phenotypes prior to treatment. Furthermore, differences in population demographics (sex, specific chemical exposures) may lead to differences in mutation susceptibilities. Nevertheless, to date no canine tumor has demonstrated frequent mutation of \textit{BRAF}, demonstrating the diagnostic utility of \textit{BRAF} mutations and espousing canine UC as a model for the improvement of targeted therapeutics.

**Conclusions**

In the current study, we have shown that miR-10b and \textbf{BRAF}^{V450E} do not appear to be critically involved in the invasiveness of canine UC. Nevertheless, they represent differential molecular characteristics between neoplastic and normal bladder and may, therefore, be involved in disease pathogenesis and of diagnostic relevance. Distinctions between human and canine UC, just as similarities, help us hone in on evolutionarily-conserved driver mutations leading to bladder carcinogenesis. Mutations with increased prevalence in dogs, such as the \textbf{BRAF}^{V600E}, should be given further attention due to the discrepancy in tumor invasiveness between the species. Together, man and man’s best friend may improve our knowledge of UC.
Acknowledgements

Many thanks to Hiroyuki Mochizuki for his assistance in helping determine the genomic location of the canine BRAF mutation and teaching me how to evaluate sequence data.

References


Horbinski, C. (2013). To BRAF or not to BRAF: is that even a question anymore? J Neuropathol Exp Neurol, 72(1), 2-7. doi: 10.1097/NEN.0b013e318279f3db


Table 5.1. *Patients analyzed in the miR-10b study and their respective miR-10b fold changes (qRT-PCR), CFA36 Log2 ratios (oaCGH), and HOXD10 fold changes (qRT-PCR).*

<table>
<thead>
<tr>
<th>Patient</th>
<th>miR-10b Fold Change</th>
<th>CFA36 Log2</th>
<th>HOXD10 Fold Change</th>
</tr>
</thead>
<tbody>
<tr>
<td>TCC27</td>
<td>-2.456606299</td>
<td>0.35</td>
<td></td>
</tr>
<tr>
<td>TCC29</td>
<td>-2.024797706</td>
<td>0</td>
<td>2.084044804</td>
</tr>
<tr>
<td>TCC31</td>
<td>-4.340248896</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>TCC33</td>
<td>-4.414414872</td>
<td>0.35</td>
<td>14.18063625</td>
</tr>
<tr>
<td>TCC34</td>
<td>-3.510476005</td>
<td>1.07</td>
<td>1.640716008</td>
</tr>
<tr>
<td>TCC35</td>
<td>-2.859089051</td>
<td>0.28</td>
<td>10.39020779</td>
</tr>
<tr>
<td>TCC38</td>
<td>-1.5</td>
<td>2.72</td>
<td>37.01601224</td>
</tr>
<tr>
<td>TCC39</td>
<td>-3.063115994</td>
<td>2.35</td>
<td>9.702774474</td>
</tr>
<tr>
<td>TCC40</td>
<td>-5</td>
<td>0</td>
<td>4.742688651</td>
</tr>
<tr>
<td>TCC41</td>
<td>-1.945309895</td>
<td>0</td>
<td>8.057978238</td>
</tr>
<tr>
<td>TCC42</td>
<td>-3.381768206</td>
<td>0.79</td>
<td>9.911709553</td>
</tr>
<tr>
<td>TCC43</td>
<td>-6.344715857</td>
<td>1.56</td>
<td>12.82308926</td>
</tr>
<tr>
<td>TCC44</td>
<td>-5.947247938</td>
<td>0.25</td>
<td>9.792487699</td>
</tr>
<tr>
<td>TCC46</td>
<td>-1.785919022</td>
<td>1.41</td>
<td>10.80318989</td>
</tr>
<tr>
<td>TCC47</td>
<td>-2.4</td>
<td>1.63</td>
<td>5.55398878</td>
</tr>
<tr>
<td>TCC48</td>
<td>-3.199347671</td>
<td>1.76</td>
<td>3.902977961</td>
</tr>
<tr>
<td>TCC49</td>
<td>-1.943812265</td>
<td>1.64</td>
<td>3.155264293</td>
</tr>
<tr>
<td>TCC50</td>
<td>-11.44516746</td>
<td>1.89</td>
<td>1.833481913</td>
</tr>
<tr>
<td>TCC51</td>
<td>-2.717391104</td>
<td>1.57</td>
<td>18.74042829</td>
</tr>
<tr>
<td>UC1</td>
<td>-8.193298887</td>
<td>-</td>
<td>10.46730411</td>
</tr>
</tbody>
</table>
Table 5.2. *The effects of BRAF knockdown on neoplastic cell invasion.*

Each UC cell line (BRAF\textsuperscript{V450E} or WT) was treated with either negative control (non-targeting) or BRAF-targeting DsiRNA. Subsequent effects on proliferation (total cell number), number of invaded cells (including standard deviation (S.D.) of counts (n=3)), and invasion indices (\(\% \text{ invasion}^{\text{KNOCKDOWN}}/\% \text{ invasion}^{\text{CONTROL}}\)) were calculated for each.

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>V450E Status</th>
<th>Total Cell #</th>
<th>% Proliferation</th>
<th>Avg. # Invaded Cells (±S.D.)</th>
<th>Avg. Invasion Index (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>K9TCC-Pu-An</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Negative Control</td>
<td>+</td>
<td>350000</td>
<td>2366.67 (±450.92)</td>
<td>290000</td>
<td>82.86</td>
</tr>
<tr>
<td>Knockdown</td>
<td>+</td>
<td>290000</td>
<td>106.16</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>2101.33 (±533.97)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>K9TCC-Pu-Mx</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Negative Control</td>
<td>+</td>
<td>240000</td>
<td>4400.00 (±400)</td>
<td>200000</td>
<td>83.33</td>
</tr>
<tr>
<td>Knockdown</td>
<td>+</td>
<td>300000</td>
<td>81.94</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>300000</td>
<td>3004.67 (±309.56)</td>
<td>86.67</td>
<td></td>
</tr>
<tr>
<td>K9TCC-Pu-Sh</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Negative Control</td>
<td>-</td>
<td>360000</td>
<td>1545.33 (±50.65)</td>
<td>300000</td>
<td>83.33</td>
</tr>
<tr>
<td>Knockdown</td>
<td>-</td>
<td>300000</td>
<td>86.67</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Each UC cell line (BRAF\textsuperscript{V450E} or WT) was treated with either negative control (non-targeting) or BRAF-targeting DsiRNA. Subsequent effects on proliferation (total cell number), number of invaded cells (including standard deviation (S.D.) of counts (n=3)), and invasion indices (\(\% \text{ invasion}^{\text{KNOCKDOWN}}/\% \text{ invasion}^{\text{CONTROL}}\)) were calculated for each.
Figure 5.1. miR-148b/-874/-181b provides the most stable miRNA reference gene value.

Three reference genes previously evaluated in human UC were similarly evaluated in canine UC using NormFinder software to determine highest stability. Single miRNAs provided the least stable reference gene values (left), while combinations were generally favorable (right). The combination of all three reference genes was the most stable (right on X-axis) according to NormFinder stability values (Y-axis) and was used throughout further analysis.
Figure 5.2. *miR-10b* levels are significantly decreased in neoplastic urothelium compared to control.

$\Delta C_{\text{miR-10b-reference}}$ values were significantly elevated in UC biopsies compared to normal bladders (****p<0.0001), implying decreased levels of mature miR-10b (A). Accordingly, miR-10b fold changes were significantly (****p<0.0001) lower in UC patients than normal bladders, calculated by fold change=$2^{-\Delta\Delta C_{\text{tumor-control}}}$ (B). miR-10b normally targets the anti-invasion factor *HOXD10*, resulting in mRNA degradation and reduced levels of cellular *HOXD10*. In UC patients, however, *HOXD10* levels were significantly increased (average fold change=+9.7), further suggesting absence of the targeting miRNA. Thus despite being in the amplified region of CFA36, miR-10b levels are decreased in UC, and miR-10b likely does not contribute to UC invasiveness. Points represent individual patients, while error bars represent one standard error about the mean.
A simple linear regression model was run using CFA36 Log2 ratio values obtained from previous oaCGH analysis and miR-10b Log2 fold change values calculated from TaqMan analysis (JMP Pro 11). Results showed that, in addition to miR-10b levels being low in UC patients, levels did not vary with levels of the miR-10b genomic locus on CFA36 ($R^2<1$). Each point represents a single UC patient (n=19).
The canine equivalent of the human BRAF<sup>V600E</sup> mutation is evident in canine UC and prostatic carcinoma.

Electropherograms obtained from Sanger sequencing of canine UC biopsies (n=45), prostatic carcinoma biopsies (n=17), canine UC cell lines (n=5), and normal bladder and prostate (n=9) were analyzed for evidence of the canine equivalent of the BRAF<sup>V600E</sup> mutation—BRAF<sup>V450E</sup>. Results demonstrated that 100% of normal tissues were wild type (WT) (A), while 67% of UCs, 76% of prostatic carcinomas, and 80% of cell lines were BRAF<sup>V450E</sup> + (B,C). In addition, it was noted that all positive cases were heterozygous for the mutant allele, resulting in a dual peak at BRAF<sup>450</sup>. Often, the mutant allele was less prevalent than the wild type (B), indicative of tumor heterogeneity.
Figure 5.5. *DsiRNA knockdown of BRAF resulted in significantly reduced levels of cellular BRAF in BRAF*<sup>V450E</sup>* and wild type patients.*

*BRAF* RT-PCR of control (transfected with non-targeting DsiRNA, “X-“) and knockdown (*BRAF*-targeting DsiRNA, “X+”) showed efficient (~80%) knockdown of *BRAF* in targeted cell lines versus non-targeting. The reference gene *RPL32* was included to account for differences in total mRNA or off-target effects of knockdown on global gene expression. K9TCC-Pu-An (An, A), K9TCC-Pu-Mx (Mx, B), and K9TCC-Pu-Sh (Sh, A) were analyzed alongside no template controls containing only PCR reaction mix and water (NTC). K9TCC-Pu-Sh knockdown was the least effective.
Figure 5.6. *BRAF* knockdown marginally reduced cell number and invasiveness in UC cell lines.

K9TCC-Pu-An (A), K9TCC-Pu-Mx (M), and K9TCC-Pu-Sh (S) were transfected with non-targeting (“X−”) and *BRAF*-targeting (“X+”) DsiRNA. Compared to non-targeting control, knockdown minimally, yet insignificantly, reduced cell proliferation in cell lines (p=0.40) (A). The total number of cells invaded through the Matrigel membrane was decreased in all cases, although the difference did not reach statistical significance in any cell line (p=0.08-0.66) (B). When corrected for differences in proliferation (invasion index=\%invaded_{knockdown}/\%invaded_{negcontrol}), knockdown marginally reduced the invasive ability in two of three UC cell lines, while marginally increasing the invasive ability of one cell line, K9TCC-Pu-An (C). These effects appeared to be non-V450E specific, as K9TCC-Pu-Sh was BRAF<sup>WT</sup> while the other two were BRAF<sup>V450E+</sup>. Thus, *BRAF* mRNA reduction does not appear to be efficacious in the prevention of UC proliferation and invasion. Error bars represent the standard error about the mean.
Figure 5.7. The miR-10b locus is located in a region of CFA36 amplified in >80% of primary canine UCs and cell lines.

Previous studies of primary canine UCs (n=31) and UC cell lines (n=5) highlight the prevalence of CFA 36 amplification (A, arrow). Upon closer inspection of the amplified region of CFA36, a single microRNA—miR-10b—was revealed in the region of highest copy number gain (B, arrow, dark blue). When analyzing the cell lines and primary tumors separately (C), maintenance of the amplified miR-10b locus on CFA36 is evident, promoting the value to this region for tumor maintenance, in vivo and in vitro. We thus hypothesized that amplified regions of the miR-10b locus would lead to increased levels of mature miR-10b.
CHAPTER 6: CONCLUSIONS AND FUTURE DIRECTIONS

In the previous pages, we have begun to decipher the molecular mysteries of canine UC and evaluate their clinical translatability. We successfully identified a signature of chromosomal aberrations indicative of UC and detectable by fluorescence in situ hybridization (FISH) with >99% sensitivity and specificity (Chapter 2). In addition, we were able to validate the utility of the assay not only on tumor biopsies, but also on more clinically relevant free-catch urine samples. Thus, the genomic signature of UC provides a non-invasive means of cancer diagnosis. In the future, the ease at which urine diagnostic samples may be collected, at home or any clinic, facilitates the use of the assay as a screening technique for susceptible geriatric patients, including Scottish terriers, beagles, and Shetland sheepdogs. Genomic markers, including those elucidated in our study, may further provide therapeutic and prognostic knowledge, expanding precision medicine in veterinary oncology.

By incorporating human genomic data into our canine analyses and using chromosome synteny to enable simultaneous evaluation, we were able to, for the first time, document karyotypic similarities between human and canine UC (Chapter 2). Most notably, gains of HSA8/CFA13 underscore the evolutionarily conserved importance of these copy number aberrations in bladder tumor development. The increased frequency at which gains of HSA8 are seen in invasive tumors compared to superficial supports the idea that canine tumors, in which 97% of dogs gain CFA13, reflect characteristics of invasive human UC, as has previously been validated at a more clinical level. A narrow region of the HSA8 gain is shared with the CFA13 gain, suggesting this particular region is the most likely involved in a shared pathogenesis and, therefore, worthy of further investigation. Our findings not only provide evidence to support continued use of the canine as a model for human UC, but also
an invaluable research companion for determining crucial “driver” genomic alterations in invasive UC.

Due to the prevalence of CFA36 amplifications in and exclusive to canine UC, we sought to investigate its implications and potential role in neoplasia. The most highly amplified region of CFA36 contains the genomic locus of an oncogenic microRNA—miR-10b (Chapter 5). The known association of copy number and miRNA expression led us to hypothesize that genomic amplification of CFA36 results in increased expression of miR-10b, a well-known oncoMIR involved in human tumor proliferation and metastasis (Baffa et al., 2009; Y. Lu & Cheng, 2014; L. Zhang et al., 2006). Nevertheless, our results rather suggested that miR-10b is underexpressed in canine UC relative to normal urothelium. These results should be further investigated for biological relevance, including evaluation of miR-10b levels in metastatic lesions. Studies of human UC have shown that, while miR-10b is underexpressed in the primary tumor, metastatic lesions have increased miR-10b levels, suggesting miR-10b may be essential for metastasis or establishment of metastatic lesions (Xiao et al., 2014; A. Zaravinos et al., 2012). Moreover, miR-10b dysregulation may occur later in the development of the epithelial-mesenchymal transition, as well as in a minority of tumor cells, diluting perceptible differences within the primary tumor. Alternatively, invasive yet benign uterine tissues show decreased levels of miR-10b, suggesting that, via hyperactivation of AKT, decreased miR-10b in canine UC may actually promote invasion (Guo et al., 2015). Further studies should confirm expression levels of miR-10b in primary bladder tumors and metastases of dogs and humans and elucidate their potential pathologic relevance by utilizing miR-10b mimics to restore levels in invasive neoplastic cells.

In addition to further miR-10b studies, a more encompassing analysis of gene regulation and gene expression of the amplified region of CFA36 should be performed.
Epigenetic alterations, including methylation, acetylation, and potential chromosomal rearrangements, could result in lowered expression of the affected region despite amplification. Such epigenetic modifications hold valuable therapeutic consequences; hypermethylation or hypoacetylation result in decreased expression and are broad, yet precise, targets. Histone deacetylase inhibitors (Vorinostat, Romidepsin) and demethylation agents (5-azacitidine) are FDA approved and thus readily available for clinical integration. Interestingly, hypermethylation is a biological means of compensation for genomic amplification and is thus a logical explanation of unexpected miR-10b levels (Kurscheid et al., 2015). Since the HOXD cluster is also found in the amplified region and is associated with adult bladder function and cell differentiation, epigenetic disruptions may have tumorigenic consequences on this array of essential genes. Future studies should focus on connecting the genomic markers of canine UC, including possible epigenetic alterations, to more functional roles. The prevalence of highlighted genomic aberrations, including CFA13, 19, and 36, suggests these aberrations are not occurring by chance, but rather are either the cause or a direct, predictable consequence of another molecular alteration and warrant thorough investigation.

On an even more minute genomic level, the high prevalence of BRAF V450E suggests it plays a role in urothelial tumorigenesis (Chapter 5). Our results were unable to deduce the role of that mutation or why it is prevalent in canine UC and prostatic carcinoma, among other human tumors. However, the equivalent BRAF V600E is rare in human UC, despite a set of risk factors, pathogenesis, molecular aberrations, and clinical outcomes similar to those of dogs. Thus, it should be determined if the mutation is a passenger of tumorigenesis rather than a driver, and the significance, if any, of frequency discordance between the species. When considering the differences in the prevalence of invasive, aggressive tumors in dogs
and humans, a focus on such distinctions between the species may shed light on the
development of aggressive phenotypes in humans more similar to dogs.

In chapter 3, we evaluated five canine UC cell lines at both genome and mRNA
levels. Using oaCGH and FISH, we affirmed that the cell lines maintain genomic aberrations
of the primary tumor, increasing their value for genomic studies of the disease. In addition,
expression profiling highlighted massive gene dysregulation in pathways and gene
categories presumably at the crux of UC development. Deficiencies in lipid metabolism
(cyclic compounds) and xenobiotic metabolism, as well as increases in cell cycle regulation
and DNA replication, recombination, and repair, were highlighted in canine UC cell lines.
Future studies should supplement these deficient metabolic genes in carcinogen-exposed
urothelium to deduce their potential role in tumorigenesis. If indeed metabolic insufficiencies
promote carcinogenesis in UC development, the cell lines, which preserve this dysfunction,
may prove invaluable in furthering our understanding of UC development as it relates to
target metabolic pathways. Similarly, the metabolic capacity of predisposed breeds should
be evaluated both with and without UC to capitalize on the uniquely homogenous breed
structure of the canine population and better hone in on early drivers of disease.

Carcinogen studies should evaluate how UC-associated chemical compounds affect
chromosome structure and are involved in UC development, particularly by urinary
exposure. Numerous studies in humans, dogs, and rodent models have implicated common
chemicals as urothelial carcinogens, particularly aromatic hydrocarbons and amines
(Bartsch et al., 1990; Bartsch, Malaveille, Friesen, Kadlubar, & Vineis, 1993; Boada et al.,
2015; Glickman, Raghavan, Knapp, Bonney, & Dawson, 2004; Yoon, Kim, Tommasi, &
Besaratinia, 2012; Jian Zhang et al., 2012). Despite speculation of how these chemicals
may interact with the genome and inflict damage, we still have limited knowledge of by what
exposure and physiologic means damage is incurred. One intriguing hypothesis is that these aneuploidogenic exogenous compounds, and potentially endogenous compounds such as estrogen, instigate chromothripsis. Chromothripsis, the phenomenon of chromosome shattering observed in UC cell lines and primary tumors (chapters 2, 3), represents a means by which massive chromosome amplifications and deletions may occur simultaneously (Stephens et al., 2011). One of the most commonly afflicted chromosomes, CFA16, harbors the BRAF gene, making chromothripsis of potential relevance to the high rate of mutation observed in that gene as is reported with other mutational hotspots (Rausch et al., 2012). Further studies should evaluate the ability of suspected urothelial carcinogens to induce such chromosomal alterations. A more thorough understanding of these chemicals, the route of exposure, and their effects would help owners better understand chemical dangers and how to avoid them. The more we know about a disease, from its causes, through physiologic mechanisms, to clinical symptoms, predictions, and outcomes, the more efficiently we are able to approach treatment efficacies and expectations.

The clinical predictive value of canine UC cell lines as they relate to therapeutic response remains to be thoroughly evaluated. Our studies, combined with previous, have demonstrated molecular and clinical similarities of primary UC and cell lines (Chapter 3, Chapter 4) (K. Rathore & M. Cekanova, 2014). Although previous studies have tested the efficacy of chemotherapeutics on UC cell lines, comprehensive assessment of how similarly in vivo disease reacts remains elusive, impeding translational advancements (de Brito Galvao et al., 2012). Future therapeutic studies should focus on the in vitro model and the in vivo model in parallel, determining efficacy correlations and using the cell lines to further our understanding of the molecular mechanisms of canine UC.
We evaluated, at quantitative, functional, and therapeutic levels, a newly discovered molecular target in canine UC—PTTG1 (Chapter 4). PTTG1 has only been characterized in human UC at a quantitative level and never studied in veterinary medicine, to our knowledge (Lai et al., 2010). Our results elucidated that 100% of canine UC biopsies and cell lines show increased levels of PTTG1 mRNA. We further found, as is seen in other human carcinomas, that PTTG1 increases tumor proliferation and invasiveness in vitro. Hence, PTTG1-targeting therapeutics, including vorinostat, may prove highly valuable to the clinical management of canine UC, as well as their evolutionarily-similar human counterpart.

Our studies affirmed the ability of vorinostat to reduce levels of PTTG1 in the dog as in humans. Thus, we promote utilizing the FDA-approved HDACi vorinostat to curtail tumor growth and invasion in UC. In addition, vorinostat and low levels of PTTG1 show radiosensitizing effects, revealing a valuable therapeutic combination capable of reducing tumor volume and preventing further proliferation and invasion (Chen et al., 2010; Folkvord et al., 2009; Saelen et al., 2012). More practically, vorinostat is available in an oral form, which may be attractive to clients wary of IV chemotherapeutic treatment. Alternatively, intravesical treatment has been tested in human bladder cancer and proven efficacious for chemosensitization and prevention of tumor progression (D. Wang et al., 2013). The previously described efficacy of vorinostat in human bladder cancer, combined with quantitative PTTG1 results, suggests securin may play a similar role in human UC as canine. In order for such findings to remain relevant, however, subsequent in vivo experiments should ensue. Experiments should include quantification of PTTG1 levels in non-neoplastic bladder lesions and patient urine samples for rapid prognostic identification.

Our findings regarding overexpression of PTTG1 are further intriguing in the context of massive genomic amplification observed in canine UC (Chapter 2). Securin is responsible
for holding sister chromatids together prior to anaphase by binding and inactivating separase, allowing maintenance of the cohesion ring around sister chromatids (Solomon & Burton, 2008). Thus, increased levels of securin may lead to prolonged association of sister chromatids, lagging sister chromatids subjected to micronucleus inclusion and chromothripsis, and resultant aneuploidy (Haber, 2014). Research indicates 79% of all canine bladder tumors and at least 65% of all human tumors are aneuploid, suggesting proteins involved in chromosome segregation and mitosis may be oncogenic agents (Holmberg V Fau - Wahren, Wahren B Fau - Esposti, & Esposti, 1984; Zhou, 2013). The high frequency of chromothripsis in canine UC further implicates errors in chromosome segregation (Shapiro, 2014). Future studies should evaluate how altered levels of PTTG1 in canine UC may affect chromosome number and structure.

Throughout all of our work, one recurrent molecular theme has remained—potential overactivation of the PI3K/AKT pathway in UC. Relevant roles of PTTG1, miR-10b, and BRAFV600E in pathway activation reinforce the idea that, similar to humans, dysregulation of the PI3K/AKT pathway may be vital to UC development and should be thoroughly characterized. Future studies should investigate this pathway in depth in dogs, including stepwise comparison to humans. Due to the therapeutic implications associated with precision medicine, identification of molecular targets within this pathway central to UC and other tumors may have far reaching translational and comparative applications.

Altogether, our results reaffirm previous notions of the dog as a valuable weapon in the fight against human UC. Not only were we able to confirm that such similarities exist at the genomic level, but we successfully used the canine genome to narrow genomic regions of interest in UC development and simultaneously develop a non-invasive diagnostic assay for canine UC; not only were we able to reaffirm similar molecular and gene expression

203
alterations between humans and dogs, but we successfully utilized canine tumors and their cell lines to further our understanding of frequent molecular alterations, their functional consequences, and their therapeutic potential. As human cancer treatments improve, so, too, should those of man’s best friend. In the fight against cancer, sometimes those who are unable to say anything are able to tell us the most. It is our hope that our work will help improve the welfare of canine UC patients, from keeping our pets happy and healthy at diagnosis, through therapeutic selection and treatment, and ultimately improving prognosis.

References


Appendix A: Chapter 3 Supplementary Table

Supplementary Table 1. *Regions implicated in GISTC analysis of primary tumors.*

GISTIC analysis was performed on oaCGH data from 31 previously analyzed primary tumors (Shapiro et al., 2015). Regions are shown indicating the called aberration (copy number (CN) loss or gain), Q-bound, and G-score. The G-score reflects the frequency as well as magnitude of the aberration in the cohort, while the Q-bound indicated significance (α=0.05). Italicized regions are those shared with the cell lines according to individual cohort GISTIC analysis.

<table>
<thead>
<tr>
<th>Genomic Region</th>
<th>Aberration Type</th>
<th>Q-Bound</th>
<th>G-Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>chr1:122,513,882-123,822,611</td>
<td>CN Loss</td>
<td>0.043807452</td>
<td>5.386684491</td>
</tr>
<tr>
<td>chr1:124,581,235-124,760,612</td>
<td>CN Gain</td>
<td>0.011994128</td>
<td>7.204288788</td>
</tr>
<tr>
<td>chr2:38,781,469-38,820,189</td>
<td>CN Gain</td>
<td>4.55E-17</td>
<td>21.8615804</td>
</tr>
<tr>
<td>chr2:78,762,568-78,799,453</td>
<td>CN Gain</td>
<td>0.019891928</td>
<td>6.882582169</td>
</tr>
<tr>
<td>chr2:86,801,393-86,827,776</td>
<td>CN Loss</td>
<td>0.001018735</td>
<td>7.840821179</td>
</tr>
<tr>
<td>chr2:86,827,776-86,968,017</td>
<td>CN Gain</td>
<td>0.012382096</td>
<td>7.181094997</td>
</tr>
<tr>
<td>chr3:94,582,749-94,671,155</td>
<td>CN Gain</td>
<td>0.010435217</td>
<td>7.290444937</td>
</tr>
<tr>
<td>chr3:10,461,352-10,522,266</td>
<td>CN Gain</td>
<td>0.001278155</td>
<td>7.720319778</td>
</tr>
<tr>
<td>chr3:11,200,757-11,263,177</td>
<td>CN Gain</td>
<td>0.043633713</td>
<td>6.50262168</td>
</tr>
<tr>
<td>chr3:13,643,989-14,572,441</td>
<td>CN Gain</td>
<td>4.11E-05</td>
<td>10.48502939</td>
</tr>
<tr>
<td>chr3:13,462,713-13,514,935</td>
<td>CN Gain</td>
<td>0.029070462</td>
<td>6.610639871</td>
</tr>
<tr>
<td>chr3:14,453,553-14,587,903</td>
<td>CN Gain</td>
<td>0.05557067</td>
<td>6.88934624</td>
</tr>
<tr>
<td>chr3:14,319,194-14,669,423</td>
<td>CN Gain</td>
<td>4.31E-10</td>
<td>16.84270396</td>
</tr>
<tr>
<td>chr3:14,453,553-14,587,903</td>
<td>CN Gain</td>
<td>1.45E-04</td>
<td>9.786086038</td>
</tr>
<tr>
<td>chr5:3,419,036-3,601,704</td>
<td>CN Gain</td>
<td>0.046904935</td>
<td>6.28770883</td>
</tr>
</tbody>
</table>
Supplementary Table 1 Continued.

<table>
<thead>
<tr>
<th>Chromosome</th>
<th>Start</th>
<th>End</th>
<th>Type</th>
<th>CN Change</th>
<th>Log_{10} P-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>chr16:17,297,364-18,167,022</td>
<td>CN Gain</td>
<td>0.017028731</td>
<td>6.976852141</td>
<td></td>
<td></td>
</tr>
<tr>
<td>chr16:62,203,097-62,515,898</td>
<td>CN Loss</td>
<td>2.66E-06</td>
<td>10.67014528</td>
<td></td>
<td></td>
</tr>
<tr>
<td>chr17:3,598,461-4,134,138</td>
<td>CN Gain</td>
<td>0.015096509</td>
<td>7.054194419</td>
<td></td>
<td></td>
</tr>
<tr>
<td>chr17:59,759,776-59,862,260</td>
<td>CN Loss</td>
<td>1.42E-04</td>
<td>8.823776963</td>
<td></td>
<td></td>
</tr>
<tr>
<td>chr18:33,384,051-36,701,280</td>
<td>CN Loss</td>
<td>2.66E-06</td>
<td>10.67014528</td>
<td></td>
<td></td>
</tr>
<tr>
<td>chr18:58,769,360-58,847,127</td>
<td>CN Gain</td>
<td>0.007693817</td>
<td>7.48639562</td>
<td></td>
<td></td>
</tr>
<tr>
<td>chr19:23,338,201-26,186,292</td>
<td>CN Loss</td>
<td>5.06E-08</td>
<td>13.89351758</td>
<td></td>
<td></td>
</tr>
<tr>
<td>chr20:60,570,985-61,154,912</td>
<td>CN Gain</td>
<td>1.81E-06</td>
<td>12.1159728</td>
<td></td>
<td></td>
</tr>
<tr>
<td>chr21:51,479,490-53,031,193</td>
<td>CN Loss</td>
<td>0.0441719</td>
<td>5.187900687</td>
<td></td>
<td></td>
</tr>
<tr>
<td>chr24:49,661,910-49,817,217</td>
<td>CN Gain</td>
<td>0.011125345</td>
<td>7.255449779</td>
<td></td>
<td></td>
</tr>
<tr>
<td>chr25:53,370,311-54,184,739</td>
<td>CN Gain</td>
<td>0.004214336</td>
<td>7.859845437</td>
<td></td>
<td></td>
</tr>
<tr>
<td>chr26:30,241,704-30,306,343</td>
<td>CN Loss</td>
<td>5.06E-08</td>
<td>14.33353767</td>
<td></td>
<td></td>
</tr>
<tr>
<td>chr26:4,080,115-4,206,619</td>
<td>CN Gain</td>
<td>0.011125345</td>
<td>7.255449779</td>
<td></td>
<td></td>
</tr>
<tr>
<td>chr27:24,467,658-24,490,479</td>
<td>CN Gain</td>
<td>0.032259803</td>
<td>6.542205149</td>
<td></td>
<td></td>
</tr>
<tr>
<td>chr28:41,778,738-42,415,754</td>
<td>CN Gain</td>
<td>0.004214336</td>
<td>7.859845437</td>
<td></td>
<td></td>
</tr>
<tr>
<td>chr30:38,193,697-38,488,912</td>
<td>CN Loss</td>
<td>0.011638422</td>
<td>6.438774973</td>
<td></td>
<td></td>
</tr>
<tr>
<td>chr31:38,193,697-38,488,912</td>
<td>CN Loss</td>
<td>0.011638422</td>
<td>6.438774973</td>
<td></td>
<td></td>
</tr>
<tr>
<td>chr31:41,069,045-41,387,787</td>
<td>CN Gain</td>
<td>6.81E-04</td>
<td>8.920188876</td>
<td></td>
<td></td>
</tr>
<tr>
<td>chr32:22,604,639-23,600,574</td>
<td>CN Gain</td>
<td>4.55E-17</td>
<td>35.62825418</td>
<td></td>
<td></td>
</tr>
<tr>
<td>chr33:6,316,412-7,080,681</td>
<td>CN Gain</td>
<td>0.001346915</td>
<td>8.562169582</td>
<td></td>
<td></td>
</tr>
<tr>
<td>chr34:3,874,100-4,310,910</td>
<td>CN Gain</td>
<td>7.73E-07</td>
<td>12.5439271</td>
<td></td>
<td></td>
</tr>
<tr>
<td>chrX:117,895-143,538</td>
<td>CN Gain</td>
<td>0.009308034</td>
<td>7.384361204</td>
<td></td>
<td></td>
</tr>
<tr>
<td>chrX:125,169,722-125,367,697</td>
<td>CN Gain</td>
<td>8.92E-06</td>
<td>11.29069566</td>
<td></td>
<td></td>
</tr>
<tr>
<td>chrX:20,490,410-20,519,961</td>
<td>CN Gain</td>
<td>4.55E-17</td>
<td>21.24676534</td>
<td></td>
<td></td>
</tr>
<tr>
<td>chrX:27,926,779-32,983,769</td>
<td>CN Loss</td>
<td>0.007186505</td>
<td>6.728428748</td>
<td></td>
<td></td>
</tr>
<tr>
<td>chrX:74,807,177-74,854,890</td>
<td>CN Loss</td>
<td>6.68E-13</td>
<td>21.92065465</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>