P53R245W MUTATION ELICITS METASTATIC PHENOTYPE IN PTEN DEFICIENT PROSTATE CANCER

Ky Pham

Follow this and additional works at: https://digitalcommons.library.tmc.edu/utgsbs_dissertations

Part of the Bioinformatics Commons, Genetics Commons, Molecular Genetics Commons, and the Translational Medical Research Commons

Recommended Citation
https://digitalcommons.library.tmc.edu/utgsbs_dissertations/973

This Thesis (MS) is brought to you for free and open access by the Graduate School of Biomedical Sciences at DigitalCommons@TMC. It has been accepted for inclusion in UT GSBS Dissertations and Theses (Open Access) by an authorized administrator of DigitalCommons@TMC. For more information, please contact nha.huynh@library.tmc.edu.
P53R245W MUTATION ELICITS METASTATIC PHENOTYPE IN PTEN DEFICIENT PROSTATE CANCER

by

Ky Pham, M.D.

APPROVED:

M. James You, M.D., Ph.D., Advisory Professor

Guillermina Lozano, Ph.D.

Sean Post, Ph.D.

Bin Wang, Ph.D.

George A. Calin, M.D., Ph.D.

APPROVED:

Dean, The University of Texas MD Anderson Cancer Center UTHealth Graduate School of Biomedical Sciences
P53R245W MUTATION ELICITS METASTATIC PHENOTYPE IN PTEN DEFICIENT PROSTATE CANCER

A

THESIS

Presented to the Faculty of

The University of Texas

MD Anderson Cancer Center UTHealth

Graduate School of Biomedical Sciences

in Partial Fulfillment

of the Requirements

for the Degree of

MASTER OF SCIENCE

by

Ky Pham, M.D

Houston, Texas

August 2019
Acknowledgements

I would like to thank Dr. James You for his mentorship over the past two years. I would also like to thank my other committee members, especially Dr. Gigi Lozano who not only shared her $Trp53^{wtm-R245W}$ allele, but also gave much valuable feedback in the write up of this thesis, and Dr. Sean Post who shared his experience on bioinformatics. Next, I would like to thank the members in Dr. You’s laboratory, specifically Hua He who supervised me on the relevant lab techniques, and Wei Gao who helped with the invasion assay. I would also like to thank two members in Dr. George Calin’s laboratory, Simone Anfossi and Barbara Pardini, both of whom helped with the exosome assay. Lastly, I would like to thank Dr. Manu Sebastian and my mentor Dr. James You, for lending their expertise in the diagnosis of the prostate cancer samples and in the identification of micrometastasis. On a final note, I would like to thank my alma mater, LSUHSC School of Medicine in New Orleans, and my former professors, who taught me the importance of continuing medical education.
Trp53 mutations are the most frequent genetic alterations in prostate cancer and are associated with more aggressive disease and worse overall survival. The majority of Trp53 mutations in prostate cancer are missense mutations, resulting in amino acid substitutions with profound effect. In addition to the loss of wild type function, missense mutations in Trp53 result in a gain-of-function (GOF) phenotype. This GOF phenotype confers biologic advantages to the tumor cells, enabling them to metastasize and invade distant organs. In this study, we generated mice carrying a conditional prostate-specific p53R245W mutant and Pten deletion to access the role of this common p53 mutant in the pathogenesis and metastasis of prostate cancer. We found that Trp53R245W/Pten−/− mice have invasive prostate cancer with complete penetrance and showed stable expression of the mutant p53. In addition, we found metastasis in Trp53R245W/Pten−/− mice (3/15), which was absent in Trp53+/− Pten−/− mice (0/14). Analysis of metastasis also showed stable expression of the mutant p53. Taken together, these results indicate that p53R245W mutation has GOF in Pten-deficient prostate cancer.
Contents

Approval Signatures ........................................................................................................... i
Title Page ........................................................................................................................... ii
Acknowledgements ........................................................................................................... iii
Abstract ............................................................................................................................ iv
Table of Contents ............................................................................................................... v
List of Figures ..................................................................................................................... vi
List of Tables ...................................................................................................................... vii
Keywords ........................................................................................................................... viii
Abbreviations .................................................................................................................... ix

Chapter 1: Introduction ...................................................................................................... 1
Chapter 2: Generation of prostate specific \textit{Trp53}\textsuperscript{R245W/- Pten\textsuperscript{+/-} and Trp53\textsuperscript{-/-} Pten\textsuperscript{-/-}} mice .................................................................................................................................. 12
Chapter 3: Prostate specific \textit{Trp53}\textsuperscript{R245W/- Pten\textsuperscript{+/-}} alterations elicit adenocarcinoma with complete penetrance ................................................................................................................. 18
Chapter 4: Metastatic potential of \textit{Trp53}\textsuperscript{R245W/- Pten\textsuperscript{+/-}} mice ......................................................................................................................... 30
Chapter 5: Discussion and future directions .................................................................... 36
Chapter 6: Materials and Methods .................................................................................. 40
Bibliography ....................................................................................................................... 44
Vita ........................................................................................................................................ 49
List of Figures

Figure 1. Overview of Pten and p53 roles in prostate cancer ..............................................4
Figure 2. Human p53 protein domain structure and common sites of missense mutations in PCa ..................................................................................................................7
Figure 3. Prevalence of Trp53 alterations in PCa ...................................................................9-10
Figure 4. Cre-mediated Trp53<sub>wm-R245W</sub> allele recombination is specific to the prostate.15
Figure 5. Cre-mediated Pten deletion is specific to the prostate ............................................16
Figure 6. Fluorescence imaging of TdT<sup>+</sup> and TdT<sup>-</sup> PCa .............................................21
Figure 7. Survival analysis curve of PCa ..................................................................................22
Figure 8. Gross characteristics of PCa in Trp5<sub>3R245W</sub>/ Pten<sup>-/-</sup> and Trp53<sup>-/-</sup> Pten<sup>-/-</sup> mice.22
Figure 9: Histology of PCa in Trp53<sub>R245W</sub>/ Pten<sup>-/-</sup> mice .............................................23-24
Figure 10. Accumulation of p19<sup>Arf</sup> leads to stabilization of mutant p53 in Pten deficient PCa .........................................................................................................................26-27
Figure 11. AR is downregulated in Pten deficient PCa .............................................................28
Figure 12. Metastasis of Trp53<sub>R245W</sub>/ Pten<sup>-/-</sup> mice shows stable expression of the p53R245W mutant protein .................................................................32
Figure 13. Invasion assays of Trp53<sup>-/-</sup> Pten<sup>-/-</sup> and Trp53<sub>R245W</sub>/ Pten<sup>-/-</sup> PCa cells ........34
List of Tables

Table 1. Prostate histologic diagnosis and IHC results of Trp53<sup>+/−</sup> Pten<sup>+/−</sup> and Trp53<sup>R245W</sup>-Pten<sup>/−</sup> mice .......................................................... 20

Table 2. Metastasis of PCa in Trp53<sup>R245W</sup>-Pten<sup>/−</sup> and Trp53<sup>/−</sup> Pten<sup>/−</sup> mice cohorts……31
Keywords

Prostate cancer, Pten, p53, PbCre, missense mutations, gain of function, invasion, metastasis, p53 stability, genetic heterogeneity
Abbreviations

PCa = prostate cancer
PIN = prostatic intraepithelial neoplasia
ADT = androgen deprivation therapy
CRPC = castration resistant prostate cancer
GOF = gain of function
AR = androgen receptor
Pb-Cre 4 = probasin Cre 4
TdT = Rosa26-LSL-tdTomato
WT = wild type
Chapter 1: Introduction
Prostate cancer (PCa) is the most common cancer among men in the Western world, and is the second leading cause of cancer related death among men. More than 95% of all PCa are adenocarcinoma. These tumors follow a sequential progression, from normal epithelium to prostatic intraepithelial neoplasia (PIN), then to PCa. The development of metastatic PCa may occur if the tumor gains additional growth advantages. Each step in this progression can be thought of as a result of genetic alteration(s), with each gene playing different role(s). The progression to metastasis deserves special attention due to its importance on morbidity and mortality. Metastasis then is defined as the spread of cancer cell from its primary site to a distant site via lymphatic or hematogenous route. This is in contrast to local invasion, where the tumor cell only invades neighboring tissue via direct extension. In humans, the three main sites of PCa metastasis are lower vertebra-proximal femur, lungs, and liver.

PCa displays significant intratumoral morphological heterogeneity, with areas of PIN, various grades of PCa, and necrosis. Another feature of PCa is its genetic heterogeneity, containing multiple independent foci of cancer that are often genetically distinct\(^1\). These characteristics of PCa carry clinical significance. A tumor focus with high metastatic potential might metastasize early and cause significant morbidity and mortality even after surgical removal of the primary site whereas a nearby focus with low metastatic potential might be locally invasive throughout its lifetime and would only require surgical removal and/or close observation. A better understanding of the molecular/genetic mechanisms underlying these differences is critical to improve the current management strategies for PCa.

Phosphatase and tensin homolog deleted on chromosome 10 (Pten), Androgen Receptor (AR), and Trp53 are among the most frequently altered genes in prostate cancer and are associated with more aggressive diseases and worse overall survival\(^2;3\). Pten is a dual
phosphatase that, through its lipid phosphatase activity, serves to oppose PI3K activity. Inactivation of Pten leads to uncontrolled PI3K/AKT/mTOR signaling cascades, resulting in increased cell growth, cell survival, and migration, and decreased apoptosis and cell cycle arrest. AR is a nuclear hormone receptor that plays important roles in both normal prostate development and PCa growth. The expression of AR varies in PCa\(^1\). A recent study shows that Pten deletion can suppress AR transcription output, and that this suppression plays a role in the development of castration resistant growth, regardless of the actual AR expression level\(^4\). p53 is a transcription factor that functions as a tetramer. It has low expression in normal tissue. Upon cellular stress, p53 is upregulated and stimulates the expression of its myriad of target genes. One such target is p21, which binds cyclin-CDK complexes and inhibits their activity, arresting the cell cycle to allow time for proper cellular repair\(^5\). Cellular stress also upregulates p14\(^\text{Arf}\), which decreases MDM2 activity. This will inhibit MDM2-mediated degradation of p53, allowing the damaged cell to undergo apoptosis\(^6\).

Inactivation of Pten is identified in \(~30\%\) primary PCa, and as high as \(60\%\) in metastasis\(^7\). Many studies have investigated the mechanism of Pten inactivation, and although the rates vary depending on the grade and stage of the tumors and the assays that were used, all seem to agree that genomic deletions account for the majority, with 15-20% deletion seen in primary PCa and \(~40\%\) deletion seen in metastasis. Other mechanisms of Pten inactivation make up the minority and include genomic rearrangement, DNA methylation, truncating mutations, and a few missense mutations\(^8\). Another important feature of Pten is its haploinsufficiency. This characteristic is supported by observations that monoallelic \(\texttt{Pten} \) loss in mice is sufficient to induce PIN, a precursor to PCa, but these mice never progress to PCa. In contrast, hypomorphic models, in which the mice contain only one copy of the \(\texttt{Pten} \) allele
(Pten\textsuperscript{hypo/-}) and that protein expression from this allele is below that of wild type allele, exhibited PCa with aggressiveness corresponding inversely to the Pten protein level\textsuperscript{9}. Finally, homozygous deletion of Pten results in early embryonic lethality\textsuperscript{10}.

It is evident that Pten plays a role both in prostate tumorigenesis and in tumor progression (Figure 1). Indeed, while monoallelic Pten loss is sufficient to induce PIN in mice at 12-16 months of age\textsuperscript{11}, biallelic Pten loss results in a much more rapid development of PIN, usually within 9 weeks. This is then followed by a long latency period (4-6 months) before development of PCa. Chen et al. (2005) showed that this latency period represents cellular senescence, where the tumor cell undergoes permanent cell cycle arrest, and is mediated by the presence of wild type p53. This senescence phenotype is ablated by deletion of p53, resulting in a dose-dependent acceleration of carcinoma development in Pten null prostate. In this sense, p53 also displays a characteristic of haploinsufficiency.

Figure 1. Overview of Pten and p53 roles in prostate cancer. Pten\textsuperscript{+/-} results in PIN, but not PCa. Pten\textsuperscript{-/-} results in rapid (6-9 weeks) development of PIN and after a long latency (4-6
months), in PCa. p53 deletion, on the other hand, does not play a role in the initiation of PCa, but has significant role in the progression of PCa. As shown in the diagram, it allows cells in PIN stage to bypass senescence and progress to advanced PCa, the aggressiveness of which depends on the dosage of p53 (This figure was obtained from Shen, M.M., and Abate-Shen, C. (2010). Molecular genetics of prostate cancer: new prospects for old challenges. Genes & development 24, 1967-2000. It was modified and printed under Creative Commons Attribution-NonCommercial 4.0 International Public License.)

Although p53 has a limited role in the initiation of PCa, it plays a significant role in the progression of PCa (Figure 1). Homozygous deletion of Trp53 in mice, without other genetic alteration, does not lead to PCa. Chen et al. showed that prostates from these mice were indistinguishable from those of wild type mice, even after 18 months follow up. p53 does, however, accelerate the progression of PCa initiated by other genetic events, including Pten inactivation. Homozygous double deletion of Pten and Trp53 in mouse prostate epithelial cells leads to invasive PCa with key features of human castration-resistant prostate cancer (CRPC). However, despite the aggressive nature of PCa in mice from homozygous double deletion models, there has been no evidence of metastasis, even after extended follow up.

p53 missense mutations, particularly in the hotspot region between codons 125 and 300, exhibit gain of function (GOF) phenotypes, specifically metastasis to many tissues. Besides the classic Li-Fraumeni Syndrome models harboring germline missense mutations in p53, there have been more recent models harboring somatic missense mutations in p53 that resulted in high frequency of metastasis. The GOF mechanism that has been described in these recent models involves the interaction of mutant p53 with other transcription factors, affecting expressions of target genes that favor tumor growth. There are two prerequisites for this
mechanism to be effective: 1) stabilization of the mutant p53 and 2) availability/accessibility of the interacting transcription factors. When these prerequisites are met, the downstream effects can be profound. For example, the R248W mutant can interact with either p63 or p73 tumor suppressor, neither of which binds to wild type p53. These novel interactions inhibit the expression of target genes that are important for tumor suppression downstream. The R248W mutant can also bind Ets2 and Sp1, forming new complexes that stimulate the aberrant expression of other target genes that are important for GOF properties. By interacting with multiple transcription factors and affecting changes in the expression of multiple target genes, the R248W mutant can amplify its GOF activities severalfold.

Since the above mechanisms require stable mutant p53 and availability/accessibility of interacting transcription factors, any condition that changes this dynamic can prevent mutant p53 from manifesting its GOF properties. On the one hand, mutant p53, similar to its wild type counterpart, is inherently unstable. Tumor specific signals, including DNA damage, reactive oxygen species, activation of oncogenes, and inactivation of another tumor suppressor, are required for the stabilization of mutant p53. On the other hand, cellular and tumor microenvironment signals, such as hypoxia, changes in pH, inflammation, and interactions with stromal cells, can affect the availability/accessibility of interacting transcription factors. In addition, any of these conditions can change with disease, cell type, and even within the same tumor owing to heterogeneous mutant p53 stabilization.

The site within the hotspot region at which missense mutation occurs also plays a potentially important role in the manifestation of GOF properties. Missense mutation in the hotspot region can result in either a structural p53 mutant, where the DNA-binding domain structure is altered, or a contact mutant, where an arginine residue that normally interacts with
target gene DNA is substituted with another amino acid\textsuperscript{24}. Figure 2 shows the structure of p53 protein and some of the most common structural and contact mutants in PCa. Zhang et al (2018) showed that both the p53R172H structural mutant and p53R245W contact mutant drive metastasis in breast cancer, but that the contact mutant exhibited a significantly higher incidence of metastasis than the structural mutant counterpart\textsuperscript{20}. Although the mechanism for this difference was not specifically defined, there are a couple of plausible reasons for this difference. First, the two mutants might bind different interacting transcription factors hence affect expression of different target genes. Secondly, the two mutants might bind the same interactors and affect the expression of the same target genes, but that the contact mutant binds to its target genes with a higher affinity hence affect the expression of those target genes to a greater degree than the structural mutant\textsuperscript{21,24}.

**Figure 2.** Human p53 protein domain structure and common sites of missense mutations in PCa. Human p53 is composed of 393 amino acid residues. The majority of mutations in p53
occurs between codons 125 and 300. Mutations at the four amino acids highlighted here are the most common in PCa. NLS = nuclear localization sequence; NES = nuclear export sequence. (This Figure was obtained from Tanaka, T., Watanabe, M., and Yamashita, K. (2018). Potential therapeutic targets of TP53 gene in the context of its classically canonical functions and its latest non-canonical functions in human cancer. Oncotarget 9, 16234-16247. It was modified and printed under Creative Commons Attribution License 3.0 (CC BY 3.0).)

In regards to PCa, the latest data from cbioportal.org includes a total of 4850 PCa samples, of which 930 (20%) have alterations in Trp53 (Figure 3a). Although the alteration frequency is different among the studies, possibly due to sample size, differences in grade/stage of the profiled samples, and/or sequencing methods that were used, all seem to agree that mutation accounts for the majority of p53 alterations in PCa. Specifically, 581 out of these 930 alterations are missense mutations, and 307 of these 930 alterations are truncating mutations (Figure 3). Most of the mutations are congregated in the hotspot region, with mutations at the 248 and the 273 codons being the two most common.

To better understand the role of p53 missense mutation in the pathogenesis and metastasis of PCa, we generated mice carrying conditional prostate-specific p53R245W mutant and Pten deletion. Missense mutation at R245 results in a contact mutant that substitutes arginine with another amino acid, and in the case of R245W, with tryptophan. Considering the GOF roles of R248 mutation (R245 in mice) in other tissues and the relative prevalence of R248 mutation in prostate cancer metastasis, we predict that our Trp53R245W/Pten−/− model will yield a metastatic phenotype.
Figure 3. Prevalence of Trp53 mutations in PCa.

a) Alteration frequency of Trp53 in PCa across 18 different studies obtained from cbioportal.org. Notice the majority are mutations (green bars).

b) Mutations mapped to the p53 protein structure, obtained from cbioportal.org.

c) Zoom-in view of the five most common amino acids that are mutated in PCa. The height of the bars represents the number of PCa samples with the particular mutation, per 4850 total PCa samples profiled. The blue bars represent the number of all positive PCa samples with the particular mutation (R175H = 24/4850, G245S = 21/4850, R248Q = 47/4850, R248W = 11/4850, R273C = 43/4850, R273L = 10/4850, R273H = 14/4850, R282W = 21/4850). The orange bars represent the number of known metastatic PCa samples with the particular
mutation (R175H = 11/4850, G245S = 4/4850, R248Q = 18/4850, R248W = 3/4850, 
Chapter 2: Generation of prostate specific $Trp53^{R245W/-} Pten^{-/-}$ and $Trp53^{-/-} Pten^{-/-}$ mice
To determine if R245W mutant promotes formation of Pten deficient PCa, we generated compound mice using Probasin Cre4 (Pb-Cre4)\textsuperscript{26}. This model allows for prostate specific recombination and expression of the R245W mutant allele, prostate specific deletion of Pten, and prostate specific expression of Rosa26-LSL-tdTomato (TdT), a conditional allele that expresses tomato red fluorescent protein in response to Cre\textsuperscript{27}.

The p53R245W mutant mice were generated using the \textit{Trp53}\textsuperscript{wm-R245W} allele construct, which was produced by inserting partial wild-type p53 cDNA encoding exons 7-11 (with the poly A signal), flanked by loxP sites, into intron 6 of the mutant p53R245W allele (Fig 4a). In the absence of Cre recombinase, the \textit{Trp53}\textsuperscript{wm-R245W} allele expresses p53 protein that maintains wild type function\textsuperscript{20}. In prostate epithelial cells, Probasin driven Cre recombinase excises the inserted cDNA to generate the \textit{Trp53}\textsuperscript{R245W} mutant allele that expresses the p53R245W mutant protein.

To generate \textit{Trp53}\textsuperscript{-/-} \textit{Pten}\textsuperscript{-/-} mice, we made multiple crosses of male PB-Cre4 mice (C57BL/6 x DBA2 background) with female mice carrying either the \textit{Trp53}\textsuperscript{loxP} allele (mixed C57BL/6/129 S background) or the \textit{Pten}\textsuperscript{loxP} allele (mixed C57BL/6 FVB/N background). We then bred the subsequent \textit{Trp53}\textsuperscript{-/-} \textit{Pten}\textsuperscript{-/-} progeny with mice carrying the \textit{Trp53}\textsuperscript{wmR245W} allele (C57BL/6/129 S background) to get the desired \textit{Trp53}\textsuperscript{R245W/-} \textit{Pten}\textsuperscript{-/-} genotype. We also tried to incorporate TdT in our crossing, but this was not used in our inclusion criteria. In total, we were able to generate 14 mice for our \textit{Trp53}\textsuperscript{-/-} \textit{Pten}\textsuperscript{-/-} cohort, 15 mice for our \textit{Trp53}\textsuperscript{R245W/-} \textit{Pten}\textsuperscript{-/-} cohort, and 10 PbCre- mice for our wild type control. The mice were monitored routinely according to protocols set by M.D. Anderson Cancer Center Institutional Animal Care and Use Committee. We followed these mice over a period of 10 months for survival analysis. Prior to
the current project, our lab also generated 12 $Pten^\prime$ mice, which will be included in the survival analysis for comparison purposes.

While PB-Cre4 mice was documented as showing robust, tissue-specific, and post-natal expression of Cre in the prostate epithelium\textsuperscript{26}, there have been reports of scattered Cre expression in non-prostatic tissues, namely the seminal vesicles, testis, and ovary\textsuperscript{26, 28}. Although the limited expression of Cre in seminal vesicles and testis was not shown to be significant, the expression in ovary was shown to result in non-specific allele recombination in the developing embryo. This non-specific recombination only occurs if the transmission of Cre is through maternal carrier\textsuperscript{28}. For this reason, we limited the transmission of Cre through paternal carrier, sacrificing any female mice that showed positive PB-Cre4 allele in our genotyping. To test the specificity of our PB-Cre4 system, we demonstrated that Cre-mediated recombination of allele was restricted to the prostate (see Figures 4 and 5).
Figure 4. Cre-mediated Trp53<sup>wm-R245W</sup> allele recombination is specific to the prostate.

a) Schematic representation of Trp53<sup>wm-R245W</sup> allele. Red triangle loxP site; light blue rectangle Frt site left after removing the selection marker; A, Trp53 polyadenylation signaling sequence; * 733 C→T and 735 C→G mutations were introduced in exon 7 for the Trp53<sup>wm-R245W</sup> allele. (This figure was obtained from Zhang, Y., Xiong, S., Liu, B., Pant, V., Celii, F., Chau, G., Elizondo-Fraire, A.C., Yang, P., You, M.J., El-Naggar, A.K., et al. (2018). Somatic Trp53 mutations differentially drive breast cancer and evolution of metastases. Nature communications 9, 3953<sup>20</sup>. It was printed with permission from Nature Publishing Group under license number 4590980970297.)

b) PCR results for the Trp53<sup>wm-R245W</sup> allele prior to recombination (left) and after recombination (right). The 500 bp mark represents the Trp53<sup>wm-R245W</sup> allele prior to recombination, as detected by using primers surrounding the 5’ loxp site. The 427 bp mark
represents the $Trp53^{R245W}$ allele after recombination, as detected by using primers surrounding the mutated site in exon 7. Notice the presence of the R245W recombinant allele in the prostate, but absence of the allele in other tissues.

**Figure 5. Cre-mediated Pten deletion is specific to the prostate.**

a) Schematic of Pten genomic structure and recombinant alleles. The transcript from the Pten null allele eliminates exon 5, resulting in a translational frameshift. B, BgIII; S, SacI; E, EcoRI; X, XhoI; A neomycin-resistance cassette was flanked by Flp recombination target (FRT) sites (white bars), and a Cre recognition LoxP site immediately upstream of the 5’ FRT
was placed 5’ of exon 5 (arrow). A second LoxP site is placed downstream of exon 5 (arrow).

(This figure was obtained from You, M.J. (2010). Mouse Models of Lymphoma and Lymphoid Leukemia In Neoplastic Hematopathology, D. Jones, ed. (Humana Press), p 593. It was printed with permission from Humana Press under license number 4613860057704.)

b) PCR results for $Pten^{loxp}$ allele and $Pten^{null}$ allele. Notice the presence of the $Pten^{null}$ allele (210 bp) in the prostate, and absence in other tissues. The 974 bp mark represents the $Pten^{loxp}$ allele prior to Cre-mediated deletion.
Chapter 3: Prostate specific $Trp53^{R245W-}$ $Pten^{/-}$ alterations elicit adenocarcinoma with complete penetrance
Considering the aggressiveness of tumors with R245W mutants in other tissues\(^{20}\), we suspected that PCa in our \(T\text{r}p53^{R245W/-} Pten^{-/-}\) mice to also exhibit aggressive characteristics. Indeed, of the mice in our \(T\text{r}p53^{R245W/-} Pten^{-/-}\) cohort, all (15/15) developed adenocarcinoma of the prostate (see Table 1). Fluorescence imaging of TdT+ mice showed positive signs of tumor starting at \(~20-21\) weeks of age (Figure 6). By 24 weeks of age, most mice had visibly enlarged abdomen, and by 28-30 weeks of age, almost all mice had enlarged abdomen to > 2cm, at which time they were marked for euthanasia. Survival is defined at the time the mice were marked for euthanasia and/or at the time of actual death. Kaplan-Meier survival curve of \(T\text{r}p53^{R245W/-} Pten^{-/-}\) cohort shows no statistically significant difference compared to \(T\text{r}p53^{-/-} Pten^{-/-}\) cohort (Figure 7). It is worth to mention that tumor onset and progression, as accessed by fluorescence imaging and daily inspection, of the \(T\text{r}p53^{R245W/-} Pten^{-/-}\) mice cohort is very similar to that of the \(T\text{r}p53^{-/-} Pten^{-/-}\) mice cohort (data not shown). Finally, the average weight of the primary tumor in \(T\text{r}pp53^{R245W/-} Pten^{-/-}\) mice is also similar to that of \(T\text{r}p53^{-/-} Pten^{-/-}\) mice (Figure 8).
<table>
<thead>
<tr>
<th>Mouse ID</th>
<th>Genotype</th>
<th>Prostate Histology Diagnosis</th>
<th>IHC Results</th>
<th>Have Cell Lines?</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>PTEN p19Arf</td>
<td>p53 p21 AR Ki67</td>
</tr>
<tr>
<td>290</td>
<td>Pten&lt;sup&gt;-/-&lt;/sup&gt; p53&lt;sup&gt;R245W/-&lt;/sup&gt; Tdt&lt;sup&gt;Cre/Cre&lt;/sup&gt;</td>
<td>PCA with extensive necrosis</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>935</td>
<td>Pten&lt;sup&gt;-/-&lt;/sup&gt; p53&lt;sup&gt;R245W/-&lt;/sup&gt; Tdt&lt;sup&gt;Cre/Cre&lt;/sup&gt;</td>
<td>PCA with focal necrosis</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>829</td>
<td>Pten&lt;sup&gt;-/-&lt;/sup&gt; p53&lt;sup&gt;R245W/-&lt;/sup&gt; Tdt&lt;sup&gt;Cre/Cre&lt;/sup&gt;</td>
<td>PCA</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>801</td>
<td>Pten&lt;sup&gt;-/-&lt;/sup&gt; p53&lt;sup&gt;R245W/-&lt;/sup&gt; Tdt&lt;sup&gt;Cre/Cre&lt;/sup&gt;</td>
<td>PCA</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>939</td>
<td>Pten&lt;sup&gt;-/-&lt;/sup&gt; p53&lt;sup&gt;R245W/-&lt;/sup&gt; Tdt&lt;sup&gt;Cre/Cre&lt;/sup&gt;</td>
<td>PCA with focal necrosis, PIN presence</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>810</td>
<td>Pten&lt;sup&gt;-/-&lt;/sup&gt; p53&lt;sup&gt;R245W/-&lt;/sup&gt; Tdt&lt;sup&gt;Cre/Cre&lt;/sup&gt;</td>
<td>PCA</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>201</td>
<td>Pten&lt;sup&gt;-/-&lt;/sup&gt; p53&lt;sup&gt;R245W/-&lt;/sup&gt; Tdt&lt;sup&gt;Cre/Cre&lt;/sup&gt;</td>
<td>PCA with extensive necrosis</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>218</td>
<td>Pten&lt;sup&gt;-/-&lt;/sup&gt; p53&lt;sup&gt;R245W/-&lt;/sup&gt; Tdt&lt;sup&gt;Cre/Cre&lt;/sup&gt;</td>
<td>PCA with focal necrosis</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>216</td>
<td>Pten&lt;sup&gt;-/-&lt;/sup&gt; p53&lt;sup&gt;R245W/-&lt;/sup&gt; Tdt&lt;sup&gt;Cre/Cre&lt;/sup&gt;</td>
<td>PCA</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>916</td>
<td>Pten&lt;sup&gt;-/-&lt;/sup&gt; p53&lt;sup&gt;R245W/-&lt;/sup&gt; Tdt&lt;sup&gt;Cre/Cre&lt;/sup&gt;</td>
<td>PCA</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>922</td>
<td>Pten&lt;sup&gt;-/-&lt;/sup&gt; p53&lt;sup&gt;R245W/-&lt;/sup&gt; Tdt&lt;sup&gt;Cre/Cre&lt;/sup&gt;</td>
<td>PCA</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>923</td>
<td>Pten&lt;sup&gt;-/-&lt;/sup&gt; p53&lt;sup&gt;R245W/-&lt;/sup&gt; Tdt&lt;sup&gt;Cre/Cre&lt;/sup&gt;</td>
<td>PCA</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>932</td>
<td>Pten&lt;sup&gt;-/-&lt;/sup&gt; p53&lt;sup&gt;R245W/-&lt;/sup&gt; Tdt&lt;sup&gt;Cre/Cre&lt;/sup&gt;</td>
<td>PCA</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>703</td>
<td>Pten&lt;sup&gt;-/-&lt;/sup&gt; p53&lt;sup&gt;R245W/-&lt;/sup&gt; Tdt&lt;sup&gt;Cre/Cre&lt;/sup&gt;</td>
<td>PCA</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>728</td>
<td>Pten&lt;sup&gt;-/-&lt;/sup&gt; p53&lt;sup&gt;R245W/-&lt;/sup&gt; Tdt&lt;sup&gt;Cre/Cre&lt;/sup&gt;</td>
<td>PCA</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>949</td>
<td>Pten&lt;sup&gt;-/-&lt;/sup&gt; p53&lt;sup&gt;-/-&lt;/sup&gt; Tdt&lt;sup&gt;Cre/Cre&lt;/sup&gt;</td>
<td>PCA with extensive necrosis</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>753</td>
<td>Pten&lt;sup&gt;-/-&lt;/sup&gt; p53&lt;sup&gt;-/-&lt;/sup&gt; Tdt&lt;sup&gt;Cre/Cre&lt;/sup&gt;</td>
<td>PCA and PIN</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>209</td>
<td>Pten&lt;sup&gt;-/-&lt;/sup&gt; p53&lt;sup&gt;-/-&lt;/sup&gt; Tdt&lt;sup&gt;Cre/Cre&lt;/sup&gt;</td>
<td>PCA with extensive necrosis</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>226</td>
<td>Pten&lt;sup&gt;-/-&lt;/sup&gt; p53&lt;sup&gt;-/-&lt;/sup&gt; Tdt&lt;sup&gt;Cre/Cre&lt;/sup&gt;</td>
<td>PCA</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>921</td>
<td>Pten&lt;sup&gt;-/-&lt;/sup&gt; p53&lt;sup&gt;-/-&lt;/sup&gt; Tdt&lt;sup&gt;Cre/Cre&lt;/sup&gt;</td>
<td>PCA</td>
<td>-</td>
<td>+</td>
</tr>
</tbody>
</table>

Table 1. Prostate histologic diagnosis and IHC results of Trp53<sup>R245W/-</sup> Pten<sup>-/-</sup> and Trp53<sup>-/-</sup>

**Pten<sup>-/-</sup> mice.** IHC results: for Pten, negative results indicate that > 80% of cells are negative; for p19Arf, positive results indicate that > 50% of cells are positive; for p53, positive results indicate that > 35-40% of cells are positive; for p21, negative results indicate that > 80% of cells are negative; for AR, negative results indicate that > 80% of cells are negative. IHC results for p19Arf and AR were also quantified using imageJ (see Materials and Methods section).
Figure 6. Fluorescence imaging of TdT\(^+\) and TdT\(^-\) PCa.
Figure 7. Survival analysis curve of PCa.

Kaplan-Meier plot shows no statistical difference in survival between $Trp53^{R245W/-} Pten^{-/-}$ cohort and $Trp53^{-/-} Pten^{-/-}$ cohort; p value = 0.3007.

Figure 8. Gross characteristics of PCa in $Trp53^{R245W/-} Pten^{-/-}$ and $Trp53^{-/-} Pten^{-/-}$ mice.

Gross images of PCa in a) $Trp53^{R245W/-} Pten^{-/-}$ mice at 28 weeks and b) $Trp53^{-/-} Pten^{-/-}$ mice at 28.7 weeks. c) Average weight of PCa in $Trp53^{R245W/-} Pten^{-/-}$ mice (n=7) vs $Trp53^{-/-} Pten^{-/-}$ mice (n=5). P value = 0.3830.
On a microscopic level, the PCa in *Trp53^{R245W-} Pten^{-/-}* mice show poorly differentiated adenocarcinoma with many other aggressive features. As seen in Figure 9, these PCa cells are largely abnormal, with nuclear atypia, prominent nucleoli, and visible mitoses. Necrosis and angiogenesis, two features marking aggressive growth of tumors, can be seen adjacent to areas of carcinoma. PIN, a precursor to carcinoma, can also be seen within one low power field (10X). This juxtaposition of high-grade adenocarcinoma, necrosis, and angiogenesis with PIN highlights the morphological heterogeneity of PCa. Lastly, IHC stain for Ki67 show that PCa from these mice are highly proliferative, with all PCa samples showing > 20% Ki67 positive cells. These aggressive features are also seen in PCa from *Trp53^{-/-} Pten^{-/-}* mice (data not shown).
Figure 9: Histology of PCa in Trp53<sup>R245W</sup>- Pten<sup>−/−</sup> mice.

H&E stain of PCa in Trp53<sup>R245W</sup>- Pten<sup>−/−</sup> mice show morphological heterogeneity containing
areas of PCa, angiogenesis a), necrosis b), and PIN b).

c) IHC stain shows > 50% of Ki67 positive cells.

IHC stain for p53 shows that Trp53R245W/ Pten−/− prostates express abundantly stable mutant p53, but only in areas with carcinoma and not in normal areas or PIN (Figure 10a). To determine the mechanism of mutant p53 stabilization in Pten deficient PCa, we first performed IHC stain of Pten to confirm that it is deleted (Figure 10b). Next we looked at the level of p19Arf, which has been shown to stabilize mutant p5322; 23, and also been shown to accumulate in response to Pten loss13; 30. We found that the level of p19Arf is indeed increased in Pten deficient PCa relative to that of age matched PbCre- control (Figure 10c). Finally, to determine that the positive stain of p53 is of mutant and not of wild type protein, we showed that the expression of p21 is not upregulated relative to PbCre- control (Fig 10d).
Figure 10. Accumulation of p19Arf leads to stabilization of mutant p53 in Pten deficient PCa.

IHC stain for p53 shows that a) Trp53R245W/ Pten-/ prostate expresses abundantly stable mutant p53, but only in areas with carcinoma and not PIN.

b) IHC stain for Pten in Trp53R245W/ Pten-/ PCa (top) and in PbCre- control (bottom).

c) IHC stain for p19Arf in Trp53R245W/ Pten-/ PCa (top) and in PbCre- control (bottom).

d) IHC stain for p21 in positive control (top), in Trp53R245W/ Pten-/ PCa (middle), and in PbCre- control (bottom).

e) number of p19Arf positive cells per one low power field (100x) in Trp53R245W/ Pten-/ PCa and PbCre- control; p value = 0.0059.

Considering the documented literature linking the development of CRPC and the suppression of AR transcription output in Pten deficient PCa⁴, we tested for the IHC level of
AR in PCa from both our *Trp53<sup>R245W</sup>* *Pten<sup>−/−</sup>* and our *Trp53<sup>−/−</sup> Pten<sup>−/−</sup>* cohorts. We found that the expression of AR is downregulated in Pten deficient PCa relative to PbCre- control (Fig 11), supporting the study that Pten deletion suppresses AR transcription output.

Figure 11. AR is downregulated in Pten deficient PCa.

a) IHC stain of Pten in *Trp53<sup>−/−</sup> Pten<sup>−/−</sup>* PCa (top), *Trp53<sup>R245W</sup>* *Pten<sup>−/−</sup>* PCa (middle), and in PbCre- control (bottom).
b) IHC stain of AR in Trp53\(^{-/-}\) Pten\(^{-/-}\) PCa (top), Trp53\(^{R245W/-}\) Pten\(^{-/-}\) PCa (middle), and in PbCre- control (bottom).

c) Number of AR positive cells per one low power field (100x) in Trp53\(^{-/-}\) Pten\(^{-/-}\) PCa, Trp53\(^{R245W/-}\) Pten\(^{-/-}\) PCa, and PbCre- control.

Although significant differences between the Trp53\(^{R245W/-}\) Pten\(^{-/-}\) cohort and the Trp53\(^{-/-}\) Pten\(^{-/-}\) cohort do not exist based on phenotypic evaluation of primary PCa, a few conclusions can reasonably be drawn from our results. First, Trp53\(^{R245W/-}\), similar to Trp53\(^{-/-}\), promotes the formation of Pten deficient PCa. These PCa show complete penetrance and exhibit many signs of aggressive phenotype. The morphology of these PCa mimics that of human: adenocarcinoma and intratumoral morphological heterogeneity. Secondly, the p53R245W mutant is stable in the majority of our Trp53\(^{R245W/-}\) Pten\(^{-/-}\) cohort (13/15 or 87%). Finally, although we have not found significant differences in tumor onset between the two cohorts based on macroscopic evaluation, by no means can we assume there were no early histological differences in the initiation of PCa between these two cohorts.
Chapter 4: Metastatic potential of $Trp53^{R245W/-} Pten^{-/-}$ mice
To determine the metastatic potential of R245W mutant mice, we performed gross dissections of all visible lymph nodes. Out of 15 mice from our Trp53<sup>R245W</sup>*/<sup>Pten</sup>−/− cohort, we were able to isolate five mesenteric lymph nodes, and upon H&E stain, three were shown to be positive for adenocarcinoma and two were normal lymphoid tissues (Table 2). We then performed IHC stain for cytokeratin AE1/AE3 on the positive lymph nodes to confirm that the adenocarcinoma is of prostate origin (Figure 12g). By the same manner, out of 14 mice from our Trp53<sup>−/−</sup> Pten<sup>−/−</sup> cohort, we were able to isolate four mesenteric lymph nodes, all of which were shown to be normal lymphoid tissues upon H&E stain. To evaluate for mutant p53 stability in metastasis, we performed IHC stain for p53 on the positive lymph nodes and found that they are indeed stable (Figure 12e).

<table>
<thead>
<tr>
<th></th>
<th>Trp53&lt;sup&gt;R245W&lt;/sup&gt;*, Pten&lt;sup&gt;−/−&lt;/sup&gt;</th>
<th>Trp53&lt;sup&gt;−/−&lt;/sup&gt;, Pten&lt;sup&gt;−/−&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lymph nodes isolated</td>
<td>5</td>
<td>4</td>
</tr>
<tr>
<td>Presence of PCa in lymph nodes</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>Lungs examined</td>
<td>14</td>
<td>5</td>
</tr>
<tr>
<td>Presence of PCa in lungs&lt;sup&gt;1&lt;/sup&gt;</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Livers examined</td>
<td>14</td>
<td>5</td>
</tr>
<tr>
<td>Presence of PCa in livers&lt;sup&gt;1&lt;/sup&gt;</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Bones examined</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>Presence of PCa in bones&lt;sup&gt;2&lt;/sup&gt;</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Table 2. Metastasis of PCa in Trp53<sup>R245W</sup>*/Pten<sup>−/−</sup> and Trp53<sup>−/−</sup> Pten<sup>−/−</sup> mice cohorts.

<sup>1</sup>Ten micron-interval sections of liver and bilateral lungs were examined with H&E.

<sup>2</sup>Ten serial sections of lower vertebra-proximal femur were analyzed with H&E.
Figure 12. Metastasis of $Trp53^{R245W}$/ $Pten^{-/-}$ PCa shows stable expression of the p53R245W mutant protein.

a) Gross image showing metastasis to mesenteric lymph node.

H&E stain of b) primary PCa and d) metastasis. Notice that in d), metastatic carcinoma had completely replaced the normal lymphoid tissue.

p53 stain of c) primary PCa and e) metastasis.

f) and g) IHC stains for cytokeratin AE1/AE3 in primary tumor and metastasis are positive,
confirming metastasis is of prostate origin.

Besides lymph nodes in the \( \text{Trp53}^{R245W/-\text{Pten}^{-/-}} \) cohort, there were no overt metastasis in other tissues/organs identified in either the \( \text{Trp53}^{R245W/-\text{Pten}^{-/-}} \) or the \( \text{Trp53}^{-/-\text{Pten}^{-/-}} \) cohort. Considering the three main sites of prostate cancer metastasis in humans are lower vertebra-proximal femur, lungs, and liver, we also looked at these organs for possible micrometastasis. Specifically, ten micron-interval sections of liver and bilateral lungs were analyzed with H&E, the results of which were negative. Ten serial sections of lower vertebra-proximal femur were also analyzed with H&E, the results of which were also negative (see Table 2).

To compare the metastatic potential of PCa cells in \( \text{Trp53}^{R245W/-\text{Pten}^{-/-}} \) mice relative to that of \( \text{Trp53}^{-/-\text{Pten}^{-/-}} \) mice, we performed invasion assays of PCa cell lines from the two groups (Figure 13). As can be seen, invasion is higher in \( \text{Trp53}^{R245W/-\text{Pten}^{-/-}} \) PCa cell lines vs that of \( \text{Trp53}^{-/-\text{Pten}^{-/-}} \) PCa cell lines, supporting the higher metastatic potential of \( \text{Trp53}^{R245W/-\text{Pten}^{-/-}} \) PCa and hence the GOF phenotype of the R245W mutant.
Figure 13. Invasion assays of Trp53<sup>−/−</sup> Pten<sup>−/−</sup> and Trp53<sup>R245W−</sup> Pten<sup>−/−</sup> PCa cells.

a) Images of the invading cells.

b) Quantification of the invading cells from the Trp53<sup>−/−</sup> Pten<sup>−/−</sup> (n = 3) and the Trp53<sup>R245W−</sup> Pten<sup>−/−</sup> (n = 4) cohorts. P value = 0.0275.

In this section, we have shown that mice from Trp53<sup>R245W−</sup> Pten<sup>−/−</sup> cohort exhibited a metastatic phenotype, which was absent in the Trp53<sup>−/−</sup> Pten<sup>−/−</sup> cohort. Specifically, three out of
fifteen $Trp53^{R245W/-} \text{ Pten}^/-$ mice developed metastasis to lymph nodes remotely located from primary tumors. All of these lymph nodes showed stable expression of the mutant p53. Although the penetrance is low (20%), the lesions that we did identify are macroscopic (see Figure 12a), and the metastatic lesions completely replaced the normal lymphoid tissue (see Figure 12d). Furthermore, due to our limited number of sections, we cannot exclude the possibility of micrometastasis in bones, lungs, and liver. Regardless, a few conclusions can be drawn from our data. First, $Trp53^{R245W/-} \text{ Pten}^/-$ mice exhibited grossly identifiable metastasis (3/15), whereas $Trp53^/- \text{ Pten}^/-$ mice showed no metastasis (0/14). Secondly, the majority of $Trp53^{R245W/-} \text{ Pten}^/-$ mice show stable expression of the mutant p53, both in the primary tumor (13/15) and in the metastasis (3/3). Lastly, PCa cell lines from $Trp53^{R245W/-} \text{ Pten}^/-$ mice show increased invasion in culture compared to those from $Trp53^/- \text{ Pten}^/-$ mice. These three conclusions point to the p53R245W mutant as the likely driver for prostate cancer metastasis.
Chapter 5: Discussion and future directions
In this study, we have shown that \( Trp53^{R245W/-} \) promotes the formation of Pten deficient PCa. Primary PCa in \( Trp53^{R245W/-} Pten^{-/-} \) mice, similar to that of \( Trp53^{-/-} Pten^{-/-} \) mice, are poorly differentiated adenocarcinoma with complete penetrance and exhibit many other histological features of aggressive growth. Furthermore, we have also shown that the R245W mutant exhibit a GOF phenotype in Pten deficient PCa in the formation of metastasis. The fact that R245W mutant is stable in areas of carcinoma and in metastasis but not in normal area or PIN suggests that within the heterogeneous prostate, R245W mutant is only stable in certain groups of cells and promote cancer formation and metastasis from those groups of cells. When R245W mutant is not stable, cancer metastasis appears to be arrested in those groups of cells.

Regarding the mechanism of mutant p53 stabilization, our IHC results indicate that homozygous deletion of Pten might play a role in that it leads to increased accumulation of p19\(^{Arf}\), which has been shown to inhibit Mdm2-mediated degradation of p53\(^{22,23}\). The fact that there is strong positive staining of p53 but not of p21 suggests that the positive staining represents the R245W mutant cell and not of normal adjacent cell. Considering PCa from the majority of our \( Trp53^{R245W/-} Pten^{-/-} \) mice have stabilized mutant p53 (13/15), we would expect to find more metastasis. The fact that we only found three metastasis suggests that 1) the ten cross sections were not enough to thoroughly look at the aforementioned organs for micrometastasis; or 2) the primary PCa from our \( Trp53^{R245W/-} Pten^{-/-} \) mice reached the size limit for euthanasia prior to forming identifible metastasis.

Of the three PCa cell lines that we were able to secure from our \( Trp53^{R245W/-} Pten^{-/-} \) mice, all showed increased invasion relative to those from \( Trp53^{-/-} Pten^{-/-} \) mice. We would also like to perform migration assays of PCa cell lines from the two cohorts to further characterize the metastatic potential. In terms of downstream mechanisms of R245W driven metastasis, we
will perform RPPA analysis and RNA sequencing of the metastatic $Trp53^{R245W/-}$ $Pten^{-/-}$ PCa and of the nonmetastatic $Trp53^{-/-}$ $Pten^{-/-}$ PCa. We will then perform pathway analysis and validate our results with functional studies.

A notable difference in prostate anatomy in mice versus that in human is that in mice, the prostate is a multi-lobular structure consisting of the ventral, lateral, dorsal, and anterior lobes. In human, the prostate is arranged in a zonal architecture consisting of the central, periurethral, and peripheral zones. The peripheral zone occupies the most volume and is the site of most common occurrence of prostate cancer. Analysis of gene expression profiling supports the notion that the dorsolateral lobe in mouse prostate is most analogous to the peripheral zone in human prostate\textsuperscript{31}. The pattern of Cre expression, being highest in the lateral lobe, followed by the ventral, and then the dorsal and anterior lobes, in PB-Cre4 system accurately mimics this preferential site for carcinoma formation in human\textsuperscript{26}.

While our current model supports the GOF property of R245W mutant in PCa in the formation of metastasis, it might not completely recapitulate the \textit{in vivo} process of PCa formation in human. PCa formation in human is a slow and insidious process, requiring cumulative effects of genetic and environmental factors. Although the formation of PCa generally follows PIN, and the formation of metastatic PCa may occur once PCa is formed, these progressions are very subtle. For this reason, a heterozygous model ($Trp53^{R245W/+}$), one that is predicted to have a more gradual onset of cancer formation, progression, and metastasis than the $Trp53^{R245W/-}$ model, would more closely recapitulate prostate cancer pathogenesis in human.

Despite some of the limitations of our model, if our study can define the role of R245W mutant in driving metastasis in PCa, this would have a significant impact in the current clinical
management of the disease. While surgical prostatectomy is usually performed once a
diagnosis of high-grade prostate cancer is confirmed, androgen deprivation therapy (ADT) will
also be initiated for those that present with metastasis. However, after a short period of
responsiveness, patients will develop resistance to ADT, at which stage, treatment is very
limited. Considering the prevalence of R248 and other missense mutations in prostate cancer
metastasis, understanding the mechanism that drives metastasis in these mutations will
potentially give clinicians novel therapeutic targets to either prevent metastasis or treat the
existing metastatic disease. Specifically, for cases with p53 mutation and Pten deletion, one
such strategy would be to inhibit p14^Arf in order to prevent mutant p53 stabilization and
accumulation, thereby suppressing its GOF activities.
Chapter 6: Materials and Methods
Generation of Mice

We obtained PB-Cre4 mice (C57BL/6 x DBA2 background) from Jackson lab\textsuperscript{26}; mice carrying p53\textsuperscript{loxP} allele and p53\textsuperscript{wmR245W} allele (both mixed C57BL/6/129 S background) from our collaborator Dr. Gigi Lozano\textsuperscript{19, 20}; and mice carrying PTEN\textsuperscript{loxP} allele (mixed C57BL/6 FVB/N background) is maintained in our lab\textsuperscript{32}. Mice were crossed as described in Chapter 2. They were monitored routinely and sacrificed in compliance with US Public Health Service Policy on Humane Care and Use of Laboratory Animals. All study protocols were approved by M.D. Anderson Cancer Center Institutional Animal Care and Use Committee.

DNA preparation and PCR analysis of \textit{Tp53} alleles

DNA was isolated from various mouse tissues by overnight lysis at 55°C in a buffer containing proteinase K, followed by extraction and isopropanol precipitation. For each PCR reaction, 50-100ng of genomic DNA was used. PCR program was set at: Initialization 95°C 5 minutes; Denaturation 94°C 1 minute; Annealing 60°C 45 seconds; Extension 72°C 1 minute; Final elongation 72°C 6 minutes; 30 cycles.

The primers used for \textit{Trp53\textsuperscript{wm}}\textsuperscript{R245W} allele were forward: 5’-ACCTTATGAGCCACCCGA-3’; reverse: 5’-GGAAGACACACGGATCCAGGT-3’.

Primers for Cre-mediated recombinant \textit{Trp53\textsuperscript{R245W}} allele were forward: 5’-ATCCAGGCCGGGAAATAGAG-3’; reverse: 3’-AGCGTATGGGCAATCATTGGTGA-5’

Primers for \textit{Trp53\textsuperscript{flo}} allele were forward: 5’-AAGGGGTATGAGGGACAAGG-3’; reverse: 3’-GAAGACACAAAGGGGGAGGG-5’

Primers for Cre-mediated \textit{Trp53} floxed out allele were forward: 5’-CAC AAA AAC AGG TTA AAC CCA G-3’; reverse: 5’- GAA GAC AGA AAA GGG GAG GAG -3’
Primers for $Pten^{\text{flx}}$ allele were forward: 5’- CTTCGGAGCATGTCTGGCAATGC -3’; reverse: 3’- AAGGAAGAGGGTGAGGATAC-5’

Primers for PbCre were forward: 5’-TCTGCACCTTGTCACTGAGG-3’, reverse: 5’-ATGTTCAGCTGGCCCAAATG-3’

**Histology and Immunohistochemical (IHC)**

Tissues were fixed in 10% v/v formalin and embedded in paraffin. Sections were stained with haematoxylin and eosin (H&E) by the MDACC Research Histology Core Laboratory, The University of Texas MD Anderson Cancer Center, Houston, TX.

Tissues were also IHC stained by MDACC Science Park Research Histology, Pathology & Imaging Core, Smithville, TX; using these antibodies:

- p53 (CM5), Novacastra, #NCL-p53-CM5p, 1:250 at room temperature for 1.5 hours; Buffalo Grove, IL.
- Pten, Cell Signaling, #9559, 1:100 at 4° overnight; Danvers, MA.
- p19$^{\text{Arf}}$, Abcam, #ab80, 1:50 at 4° overnight; Cambridge, UK
- p21, Santa Cruz, #sc-6246, 1:50 at 4° overnight; Dallas, TX
- AR, Abcam, #ab74272, 1:200 at room temperature for 1 hour; Cambridge, UK
- Ki67, Bethyl Labs, #IHC-00375-1, 1:250 at room temperature for 2 hours; Montgomery, TX

IHC results for p19$^{\text{Arf}}$ and AR were quantified using imageJ$^{33, 34}$, and were graphed using GraphPad Prism 8.

**Primary cell culture**
Tumor was dissected and placed into a 10 cm petri dish with 5-10 mL of DMEM culture medium. Tumor tissues were minced into small fragments (1-3 mm³) using razor blades then followed by trypsin treatment to obtain dissociated single cells from the tumor fragments. The cell solution was mixed with 10 ml pipette, then filtered through a 70-100 µm sterile mesh to remove any large and undigested tumor fragment. The filtered solution was then pelleted by centrifugation at 1200 rpm for 10 min. The cell pellet was then re-suspended with DMEM culture medium containing 10% fetal bovine serum with 5% CO₂ and kept incubated at 37 °C.

**Invasion Assay**

For *in vitro* Transwell invasion assay, the upper chamber (BD Bioscience) was pre-coated with 0.1ml of Corning matrigel, which was diluted 1:10 with serum free DMEM. Cells were cultured in 10 cm dishes with 70%-80% confluence prior to sub-culturing into the invasion chamber. A suspension of 1*10⁵ cells was added into the upper chamber of each well, and 0.5 ml of DMEM culture medium containing 10% fetal bovine serum was added to the lower portion of each well. After 10hrs incubation, a cotton swab moistened with DMEM medium was inserted into the top of the matrigel coated chambers to remove the non-invading cells. The chambers were then placed into the methanol at room temperature for 10 min to allow for cell fixation, followed by 10 min staining with 0.2% crystal violet. The chambers were washed with PBS to remove the crystal violet. The invading cells were evaluated under a microscope (Olympus) and images were captured with the built-in camera. The invaded cells were counted using Image J and results were analyzed and graphed using GraphPad Prism 8.
Bibliography


Vita

Ky D. Pham was born in Dongnai, Vietnam on July 21st, 1983. His father served as a Lieutenant in the Republic of Vietnam Marine Division during the Vietnam War. Ky and his parents along with two sisters moved to the US in 1992. He spent his early childhood in San Diego, CA where he attended elementary and middle school. He and his family moved to Baton Rouge, LA in 1997 to be closer to relatives. He enrolled in college at LSU-Baton Rouge in 2001 with a full scholarship and to be close to family. Ky majored in Biochemistry and after graduating summa cum laude in 2005, he spent time helping his parents with the family-owned business and traveled across Europe, before starting medical school in 2007. Ky received the LSUHSC Alumni Award Scholarship as part of his acceptance to LSUHSC School of Medicine in New Orleans. He completed his MD degree in 2012, did an intern year in Internal Medicine and 1.5-year residency training in Anesthesiology at LSU New Orleans. Dr. Pham then moved back to Baton Rouge in 2015 to work at Dr. Dao Xuong’s primary care clinic. After working in primary care for more than one year, Dr. Pham started pursuing his research interest, first by helping Dr. Argyrios Stampas at TIRR Memorial Hermann on spinal cord injury research. Dr. Pham then began a formal research program at the University of Texas Graduate School of Biomedical Sciences in 2017 under the mentorship of Dr. M. James You. He will complete his research program by August 2019, and has already accepted a research position with the Department of Hematopathology at MD Anderson Cancer Center.