Initiation of prostate cancer in mice by $T{p}^{53}{R}^{270H}$: evidence for an alternative molecular progression

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**SUMMARY**

Tp53 mutations are common in human prostate cancer (CaP), occurring with a frequency of ~30% and ~70% in localized and metastatic disease, respectively. In vitro studies have determined several common mutations of Tp53 that have specific gain-of-function properties in addition to loss of function, including the ability to promote castration-resistant (CR) growth of CaP cells in some contexts. To date, a lack of suitable mouse models has prohibited investigation of the role played by Tp53 mutations in mediating CaP progression in vivo. Here, we describe the effects of conditional expression of a mutant Tp53 (Tp53$^{R270H}$), equivalent to the human hotspot mutant R273H in the prostate epithelium of mice. Heterozygous $T{p}^{53}{R}^{270H}$/+ (129S4[Tp53tm3Tyj]) and “Nkx3.1-Cre” (129S5[Nkx3-1tm3(cre)Mms]) mice with prostate-specific expression of the Tp53$^{R270H}$ mutation (p53$^{R270H}$/+ Nkx3.1-Cre mice) were bred onto an FVB/N background via speed congenesis to produce strain FVB.129S4[Tp53tm3Tyj/wt], FVB.129S5[Nkx3-1tm3(cre)Mms/wt] and littermate genotype negative control mice. These mutant mice had significantly increased incidences of prostatic intraepithelial neoplasia (PIN) lesions, and these appeared earlier, compared with the Nkx3.1 haploinsufficient (Nkx3.1-Cre het) littermate mice, which did not express the Tp53 mutation. PIN lesions in these mice showed consistent progression and some developed into invasive adenocarcinoma with a high grade, sarcomatoid or epithelial-mesenchymal transition (EMT) phenotype. PIN lesions were similar to those seen in Pten conditional knockout mice, with evidence of Akt activation concomitant with neoplastic proliferation. However, the invasive tumor phenotype is rarely seen in previously described mouse models of prostatic neoplasia. These data indicate that the Tp53$^{R270H}$ mutation plays a role in CaP initiation. This finding has not previously been reported. Further characterization of this model, particularly in a setting of androgen deprivation, should allow further insight into the mechanisms by which the Tp53$^{R270H}$ mutation mediates CaP progression.

**INTRODUCTION**

Prostate cancer (CaP) is the leading cancer diagnosis in men in the United States, with new cases for 2012 estimated at 241,740 and over 28,000 estimated annual deaths from the disease (http://www.cancer.gov/cancertopics/types/prostate). Clinical cures are achieved in approximately 80% of patients presenting with localized disease; however, once metastasis occurs, response to second-line therapy, usually treatment with an androgen receptor agonist such as bicalutamide, is only 25%, with the response duration lasting for only a few months. Although multiple studies have documented a link between mutations in Tp53 and disease progression in individuals with CaP, the exact mechanism by which these Tp53 mutations, in particular gain-of-function (GOF) Tp53 mutations, mediate disease progression remains to be fully elucidated (Tomkova et al., 2008).

Tp53-null mice do not develop prostatic intraepithelial neoplasia (PIN) or CaP; however, acceleration and advancement of tumorigenesis is observed in Tp53-null compound models (combining additional oncogenic genetic manipulations), supporting the current dogma that mutations in Tp53 drive late-stage CaP progression (Navone et al., 1993). Somewhat surprisingly, very few genetically engineered mouse models (GEM) that address the contribution of mutant Tp53 to CaP progression exist. Transgenic mice expressing Tp53$^{R270H}$ (equivalent to the human hotspot mutant R273H) under control of the probasin promoter did develop PIN at 1 year of age, but targeted ‘knock in’ of Tp53$^{R270H}$ did not result in any observed prostate pathology (Elgavish et al., 2004; Olive et al., 2004).

Owing to the high frequency with which Tp53 is mutated in human CaP, we aimed to develop and characterize GEM with prostate-specific expression of mutant Tp53. Our mouse model challenges the current dogma that mutation in Tp53 is only important in promoting disease progression in late-stage CaP by demonstrating that Tp53 can act as an initiating factor. Here we describe the generation and characterization of the Tp53$^{R270H}$/+ Nkx3.1-Cre mouse model. This model, which has been bred onto a fully congenic FVB/NJ strain background, conditionally expresses the R270H Tp53 GOF mutation in the prostate epithelium of mice, resulting in the development of both PIN and CaP lesions.

**RESULTS**

Validation, congenesis and characterization of Tp53$^{R270H}$/+ Nkx3.1-Cre mice

Fig. 1A shows a schematic representation of the targeting vector and the floxed allele following excision of the STOP cassette by...
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R270H mutation is located in exon 8 (marked as a star). (B) allele following excision of the STOP cassette by Nkx3.1 Cre recombinase. The mouse. (C) onto FVB/NJ was performed to ensure a uniform strain background. Speed mouse.

(A)

Fig. 1. Validation and characterization of the Tp53R270H/+ Nkx3.1-Cre mouse. (A) Schematic representation of the targeting vector and the floxed allele following excision of the STOP cassette by Nkx3.1 Cre recombinase. The R270H mutation is located in exon 8 (marked as a star). (B) Congenic backcross onto FVB/NJ was performed to ensure a uniform strain background. Speed congenics (N3 through N5) produced a close to 100% FVB/NJ congenic mouse. (C) RT-PCR and sequencing analysis of laser capture microdissected PIN lesions confirmed that Nkx3.1-Cre-mediated deletion of the floxed STOP cassette resulted in prostate-specific expression of the p53 R270H mutation. (D) Well-developed PIN lesions were observed in Tp53R270H/+ Nkx3.1-Cre mice as early as 4 months and more than 60% of mice examined between 5 and 60 weeks developed grade 2 or higher PIN (n=16). Nkx3.1 haploinsufficient littermate mice (Nkx3.1-Cre; n=6), which did not express the Tp53 mutation, did not develop PIN even at 60 weeks (see Fig. 2).

Cre recombinase. Nkx3.1-Cre mice were used to induce Cre-mediated deletion of the floxed STOP cassette specifically in the prostate gland. Nkx3.1 is a transcription factor that is expressed in the prostate epithelium and is one of the earliest markers for prostate development (Bhatia-Gaur et al., 1999). Speed congenic backcross with medium density single-nucleotide polymorphism (SNP) analysis of strain background contribution with FVB/NJ target was performed to ensure a uniform strain background (Fig. 1B) resulting in the fully congenic strain FVB.129S4(Tp53R270H/+);FVB.129S(Nkx3.1-CreMm1(cre)Mm1) (hereafter abbreviated to Tp53R270H/+; Nkx3.1-Cre). Laser-capture microdissection followed by RNA extraction, reverse-transcriptase PCR (RT-PCR) and sequencing confirmed the expression of the Tp53R270H mutation in PIN lesions (Fig. 1C).

Prostate-specific expression of the Tp53 mutant initiates early PIN lesions in Nkx3.1-Cre heterozygous mice

Heterozygous Tp53R270H/+ Nkx3.1-Cre mice with prostate-specific expression of the Tp53R270H mutation (n=16) had a high incidence of atypia or PIN grade 1 (PIN 1) with progression to PIN 2 and PIN 3 as described (Park et al., 2002), which, in homozygous Tp53R270H/+ Nkx3.1-Cre/+ mice, could be observed as early as 5 weeks of age (Fig. 1D, Fig. 2D-F) and, in heterozygous Tp53R270H/+ Nkx3.1-Cre/+ mice, was seen by 6 months of age. Most lesions were in the dorsolateral prostate and coagulating gland and less so in the ventral prostate. No changes were observed in the seminal vesicles or the periurethral glands.

Nkx3.1 haploinsufficient littermate mice (Nkx3.1-Cre+/-; n=6) that did not express the Tp53 mutation did not develop PIN of grade 2 or higher but did have some focal atypia after 12 months of age (Fig. 2A-C). Tp53 mutant mice (Tp53R270H/+; Nkx3.1-Cre+/-) younger than 6 months did not have PIN lesions of grade 2 or higher (Fig. 3A,B) but some had focal atypia seen as enlarged and hyperchromatic nuclei in small clusters with loss of cell polarity and foci of PIN grade 1, although the distinction of focal atypia and PIN 1 was subjective. Mice with homozygous Tp53R270H/+ Nkx3.1-Cre+/- had PIN 2 lesions as early as 4 months of age (Fig. 1D) and animals with PIN lesions of higher grades almost always had areas of lower grades of PIN as well. In one mouse, there was a single lobe/gland that demonstrated grades 1-4 of PIN (Fig. 3B). Qualitatively, Ki67 staining confirmed higher proliferation in the PIN lesions, with 40% of PIN lesion cells being positive for Ki67 (data not shown). Non-atypical adjacent or control areas not recognized as PIN on hematoxylin and eosin (H&E) stains, indicating that mutant p53 is expressed by these cells (Fig. 3B, lower panel). Occasional atypical cells with p53 stabilization, as determined by immunohistochemistry (IHC) detection, were seen in areas of CaP (Fig. 4A, Fig. 5B,C) and PIN (Fig. 5A). The intensity of the AR expression was decreased in many of the PIN lesions, and was undetectable in some (Fig. 2G, Fig. 5A). Levels of phosphorylated AKT (pAKT) were elevated in PIN lesions, indicating possible direct activation of the AKT pathway by expression of the Tp53R270H mutation (equivalent to the human Tp53R273H hotspot mutation) can mediate initiation of CaP, at least in the context of Nkx3.1 haploinsufficiency and on a congenic FVB/NJ strain background. This has not previously been reported.

Determination of Tp53 stabilization and expression of AR and pAKT in PIN lesions

Immunohistochemical analysis determined that Tp53 is stabilized in a variable percent of cells (ranging from 10-70%) in the PIN lesions, indicating that mutant p53 is expressed by these cells (Fig. 3B, lower left panel). Occasional atypical cells with p53 stabilization, as determined by immunohistochemistry (IHC) detection, were seen in areas not recognized as PIN on hematoxylin and eosin (H&E) stains, probably representing small neoplastic initiation foci. These data indicate that the expression of the Tp53R270H mutant is clonal. Future studies will focus on elucidating why this is. Rare non-atypical nuclei also had p53 positivity, but diffuse stabilization of p53 in all or most prostate epithelial cells was not seen. Androgen receptor (AR) expression was present throughout the prostate epithelium and included areas of CaP (Fig. 4A, Fig. 5B,C) and PIN (Fig. 5A). The intensity of the AR expression was decreased in many of the PIN lesions, and was undetectable in some (Fig. 2G, Fig. 5A). Levels of phosphorylated AKT (pAKT) were elevated in PIN lesions, indicating possible direct activation of the AKT pathway by expression of the Tp53R270H allele (Fig. 2F, Fig. 3B, lower right panel). In careful analyses of the earliest lesions (as illustrated in Fig. 2D-F), p53 mutation, as
detected by IHC positivity, consistent with protein stabilization, seemed to precede AKT activation/phosphorylation, and AR expression was maintained in early lesions. Reduced AR expression was seen later in more well-developed PIN lesions (Fig. 2I). The invasive CaP showed a distinct sarcomatoid or epithelial-mesenchymal transition (EMT) phenotype. A similar EMT phenotype has been observed with an inducible FGFR1 mouse model; however, the involvement of p53 in that model was not reported (Acevedo et al., 2007). Tumor cells were highly atypical, and mitoses were numerous. Invasive tumor grew in solid nests and sheets, with only focal evidence of glandular or microacinar differentiation. Gleason grade for such a tumor in the human prostate would be high, numerical pattern '5' or a score of '5+5'.

**Prostate-specific expression of the Tp53 mutant can mediate progression to CaP**

Heterozygous \(\text{Tp53}^{R270H/+}\) \(\text{Nkx3.1-Cre}\) mice developed invasive CaP by 30 weeks of age, with incomplete penetrance. Although the observed PIN lesions were typical of GEM prostate models, including the \(\text{PTEN}\) conditional models, the invasive tumor phenotype was not (Fig. 4).

The invasive CaP showed a distinct sarcomatoid or epithelial-mesenchymal transition (EMT) phenotype. A similar EMT phenotype has been observed with an inducible FGFR1 mouse model; however, the involvement of p53 in that model was not reported (Acevedo et al., 2007). Tumor cells were highly atypical, and mitoses were numerous. Invasive tumor grew in solid nests and sheets, with only focal evidence of glandular or microacinar differentiation. Gleason grade for such a tumor in the human prostate would be high, numerical pattern '5' or a score of '5+5'. Cells comprising the tumor were both epithelioid and spindled, and invaded around normal glands. IHC of the CaP lesions showed p53 staining but AR expression was lost in an increasing percentage of cells and decreased in intensity even in positive cells (Fig. 5B-D). In addition, markers of EMT, including coexpression of cytokeratins 8/18 and vimentin, verify the impression that the tumor has both epithelial (CK8/18) and mesenchymal (vimentin) differentiation (Fig. 4C,E). Although p53 expression and pAKT were seen in the adjacent PIN or atypia (Fig. 2B,C, bottom left) no vimentin expression was seen in the in situ lesions. Neuroendocrine differentiation, a common feature in some mouse models with Tp53 inactivation through SV40 large T antigen expression (Masumori et al., 2001; Chiaverotti et al., 2008), was not observed in the in situ or invasive carcinomas in these mice (Fig. 2F). No metastases to other sites were observed.

**DISCUSSION**

\(\text{Tp53}^{R270H/+}\) \(\text{Nkx3.1-Cre}\) mice develop foci of atypia or PIN 1 as early as 5 weeks, then well-developed neoplastic foci (PIN 2 or higher) as early as 4 months of age. In early lesions, Tp53 mutation, as detected by IHC positivity and confirmed by laser capture microdissection sequencing, precedes the earliest foci of AKT hyperphosphorylation, and these foci seem to give rise to the larger PIN lesions with progression to invasive carcinomas. This alternative progression model (Fig. 6) therefore challenges the conventional concept that loss of Tp53 function is exclusively a late event in CaP progression and that aberrations in the PTEN-AKT pathway occur prior to Tp53 mutation (Gonzalgo and Isaacs, 2003; Shen and Abate-Shen, 2010). The data suggest that Tp53 mutation can be an initiating event in prostate neoplasia, and suggest that early Tp53 mutation might be associated with progression towards higher grade carcinomas with tumor virulence features including EMT.

High Tp53 mutation frequencies have indeed been detected in metastatic and CR CaP, with 52-89% of tumors expressing Tp53 protein by IHC (Olivier et al., 2009). However, in support of our finding, our group and others have documented the occurrence of Tp53 mutations in ~30% of localized human CaP (Chi et al., 1994; Heidenberg et al., 1995; Hughes et al., 1995; Bauer et al., 1996; Moul et al., 1996; Prendergast et al., 1996; Byrne et al., 1997; Meyers et al., 1998; Schlechte et al., 1998; Shi et al., 2002; Downing et al., 2003). The difference in rates of Tp53 mutation reported by these studies is likely to be due in part to the different methodologies used to assess the presence of p53 mutations (for a review, see Robles and Harris, 2010). The presence of Tp53 mutations has also been documented in human PIN lesions (Downing et al., 2001).

Although the role of mutant Tp53 in promoting the initiation of CaP remains controversial, mutant Tp53 has been shown to...
facilitate initiation in other cancer types, for example breast, lung and esophageal cancers (Olivier et al., 2009). Several models have been proposed for defining how Tp53 mutations infer oncogenicity (Soussi, 2007). Current data suggest that genomic instability is the most likely mechanism of action that mediates initiation of Tp53R270H-driven breast carcinomas (Wijnhoven et al., 2005), whereas GOF activity is important in the initiation of Tp53R270H-driven lung carcinomas (Olive et al., 2004). Further analysis and manipulation of our Tp53R270H/+ Nkx3.1-Cre model will allow for elucidation of the mechanism(s) by which Tp53R270H drives initiation and progression of CaP.

Similar to Tp53 mutation, inactivation of PTEN is also a frequent occurrence in individuals with CaP (Shen and Abate-Shen, 2010). Increased AKT activity suggests that PTEN inactivation might occur in our model, although PTEN status remains to be determined. The current dogma is that p53 acts as a ‘failsafe’ protein after loss of PTEN function. Although it is well known that combined inactivation of PTEN and Tp53 in compound models of CaP greatly accelerates tumor development (Jeet et al., 2010), in terms of disease progression the importance of whether p53 or PTEN loss of function occurs first remains to be determined.

It should be noted that, although Nkx3.1-null mice do not develop PIN until 1-2 years of age, it is possible that Nkx3.1 haploinsufficiency is required for the phenotype observed in our Tp53R270H/+ Nkx3.1-Cre conditional knockout mice (B, upper right panel and lower 2 panels). Immunohistochemical analysis determined that p53 is stabilized in 10-70% of the cells within PIN lesions (B, lower left), further validating the expression of the p53 R270H mutation in these lesions. Levels of pAKT were elevated in almost all PIN lesions (B, lower right panel).
METHODS

Generation of conditionally inactive mutant Tp53 mice

Mice containing a Cre-activatable Tp53^{R270H} knock-in allele were obtained from the Tyler Jacks Lab (Olive et al., 2004). The loxP-flanked conditional STOP cassette (Tuveson et al., 2004) was cloned into the XhoI site in intron 1 of the murine Tp53 locus. The R270H missense mutation was generated as described previously (de Vries et al., 2002). In our colony, the presence of the loxP–STOP-cassette–loxP (LSL) cassette in intron 1 of Tp53 genomic DNA is detected using the following primers to generate a wild-type band of 170 bp and a mutant band of 270 bp: wt F- 5’-TTACACATCCAGCCTCTGTGG-3’; mutant F- 5’-AGCTAGCCACCATGGCTTGAGTAAGTCTGCA-3’; R- 3’-CTTGGA-GACATAGCCACACTG-3’. To determine the presence of the recombined alleles of mutant Tp53, genomic DNA was amplified using the following primers flanking the integration site of the remaining loxP site in Tp53 intron 1: F- 5’-AGCCTGCC-TAGCTTCCTCAGG-3’; R- 5’-CTTGGAGACATAGCCACACACTG-3’. The Tp53^{R270H} (amino acid 270 in mice Tp53 corresponds to 273 in human Tp53) mutant has a wild-type Tp53 conformation but is defective in DNA binding, because R270H is in the DNA-binding domain of Tp53. The Nkx3.1-Cre mouse is a knock-out/knock-in mouse in which the Nkx3.1 coding region is replaced with the Cre recombinase coding sequence to result in prostate-specific expression of Cre, but also an Nkx3.1-null allele (Lin et al., 2007; Thomsen et al., 2008). The presence of wild-type

Fig. 5. Heterogeneity of staining for AR in a PIN lesion and invasive carcinoma. In some PIN lesions (A), AR staining of the nuclei has become faint or is even lost (arrowheads) compared with nuclei of normal prostate epithelium (arrows). In invasive carcinoma, progressive loss of AR is seen with areas of strong nuclear staining in less than 50% of tumor cells (B), weak nuclear staining in less than 20% of cells (C; arrowheads) and areas of negative nuclear AR staining (D).

Fig. 6. Schematic diagram of alternative molecular progression in prostate cancer compared with conventional model. CA, carcinoma; PIA, proliferative inflammatory atrophy.
p53 mutation initiates PIN

Microdissection, PCR and sequencing
RNA isolated from microdissected PIN lesion was amplified with the following primers to validate expression of *Tp53* R270H mRNA: Forward primer 5’-TTCTGGGACGGACGCTTTTGG-3’, Reverse primer 5’-AAAAATTCCCCATCAAGTGT-3’. PCR products were purified using Takara recochips (Clontech, Madison, WI) and sequenced in both directions at the UC Davis Department of Biological Sciences DNA sequencing core facility.

Histopathology and immunohistochemistry
Standard histopathology and IHC was performed as described previously (Park et al., 2002). The presence and number of PIN lesions was assessed by pathological analysis of H&E-stained sections. PIN lesion grades for GEM are described elsewhere (Park et al., 2002). Primary antibodies used were anti-Ki67 Ab-4 (1:500; Neomarker, Fremont, CA), anti-pAKT (1:2000; Cell Signaling, Danvers, MA), anti-CK8/18 (1:2000; Fitzgerald, Acton, MA), anti-AR (Millipore, Billerica, MA), anti-vimentin (Epitomics, Burlingame, CA), anti-p53 (Santa Cruz Biotech, Santa Cruz, CA) and anti-synaptophysin (Invitrogen, Carlsbad, CA).

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COMPETING INTERESTS
The authors declare that they do not have any competing or financial interests.

AUTHOR CONTRIBUTIONS

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