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Inactivation of *Tp53* and *Pten* drives rapid development of pleural and peritoneal malignant mesotheliomas

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Abstract

Malignant mesothelioma (MM) is a therapy-resistant cancer arising primarily from the lining of the pleural and peritoneal cavities. The most frequently altered genes in human MM are *CDKN2A*, which encodes components of the p53 (p14ARF) and RB (p16INK4A) pathways, *BAP1*, and *NF2*. Furthermore, the p53 gene (*TP53*) itself is mutated in ~15% of MMs. In many MMs, the PI3K-PTEN-AKT-mTOR signaling node is hyperactivated, which contributes to tumor cell survival and therapeutic resistance. Here, we demonstrate that inactivation of both *Tp53* and *Pten* in the mouse mesothelium is sufficient to rapidly drive aggressive MMs. *Pten*^{L/L};*Tp53*^{L/L} mice injected intraperitoneally or intrapleurally with adenovirus expressing Cre recombinase developed high rates of peritoneal and pleural MMs (92% of mice with median latency of 9.4 weeks, and 56% of mice with median latency of 19.3 weeks, respectively). MM cells from these mice showed consistent activation of Akt-mTOR signaling, chromosome breakage/aneuploidy, and upregulation of Myc; occasional downregulation of Bap1 was also observed. Collectively, the findings suggest that when *Pten* and *Tp53* are lost in combination in mesothelial cells, DNA damage is not adequately repaired and genomic instability is widespread, while Akt activation due to *Pten* loss protects genomically damaged cells from apoptosis, thereby increasing the likelihood of tumor formation. Additionally, mining of an online TCGA dataset revealed co-deletions of *PTEN* and *TP53* and/or *CDKN2A/p14ARF* in ~25% of human MMs, indicating that cooperative losses of these genes contribute to the development of a significant proportion of these aggressive neoplasms and suggesting key target pathways for therapeutic intervention.

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Conflicts Of Interest

The authors declare no conflict of interest in relation to this study.

Keywords

mesothelioma; p53; Pten; Bap1; genomic instability

1 | INTRODUCTION

Malignant mesothelioma (MM) is a therapy-resistant neoplasm of the serosal lining primarily of the pleural and peritoneal cavities, causally associated with asbestos exposure. At the cellular level, MM is driven by tumor suppressor gene (TSG) inactivation, occurring in various combinations in a given neoplasm, suggesting that a multi-step process drives MM pathogenesis (Murthy and Testa, 1999). Studies carried out as early as the mid-1990s identified two TSGs frequently mutated or deleted in MM: *CDKN2A* (Altomare et al., 2005a; Cheng et al., 1994; Xio et al., 1995) and *NF2* (Bianchi et al., 1995; Sekido et al., 1995). More recently, *BAP1* was identified as a critical TSG mutated in both sporadic (Bott et al., 2011; Testa et al., 2011) and familial MM (Testa et al., 2011). In addition to confirming the frequent involvement of these three TSGs in sporadic MM, recent next generation sequencing studies have uncovered recurrent alterations of other genes, including *CUL1* (Guo et al., 2015), *TP53*, *SETD2* and *DDX3X* (Bueno et al., 2016).

The two most frequently altered driver genes in human MM are *CDKN2A*, which encodes components of both the p53 (p14ARF) and RB (p16INK4A) pathways, and *BAP1*. Deletions of *CDKN2A* have been reported in 75–90% of primary MMs and cell lines (Altomare et al., 2005a; Cheng et al., 1994; Xio et al., 1995). As early as 1991, Cote et al. reported the mutation of one *TP53* allele and the loss of the wild type allele, i.e., biallelic inactivation, in 2 of 4 human MMs they studied (Cote et al., 1991). In the COSMIC and The Cancer Genome Atlas (TCGA) databases, 12% and 16% of pleural MM cases, respectively, have mutations of *TP53*. We reported *TP53* mutations in 3 of 20 (15%) MMs, and notably, two of these three tumors did not show a homozygous deletion of *CDKN2A/p14ARF* (Altomare et al., 2005a), suggesting that alterations of the p53 pathway in MM can occur in connection with defects in either gene, thereby providing further support for a critical role of this pathway in MM pathogenesis.

The tumor suppressor PTEN is a negative regulator of the PI3K-AKT-mTOR pathway, and its loss is pro-tumorigenic, contributing to tumor aggressiveness in part by mediating cell survival and reducing sensitivity to chemotherapy (Song et al., 2012). While mutations of *PTEN* are uncommon in human MM, immunohistochemical analyses have revealed absence of PTEN expression in 16%–62% of cases in different series (Agarwal et al., 2013; Garland et al., 2007; Opitz et al., 2008). Moreover, receptor tyrosine kinases are frequently upregulated and/or activated in MM, which can concomitantly activate pro-tumorigenic survival and proliferative signals through the PI3K-AKT-mTOR pathway in a high percentage of MMs (Kanteti et al., 2014; Menges et al., 2014; Ou et al., 2011; Perrone et al., 2010). Phospho (P)-AKT staining, indicative of AKT activation, has been reported in 65%–84% of human MMs (Altomare et al., 2005b; Cedres et al., 2016; Garland et al., 2007), and Akt phosphorylation was consistently observed in MMs from asbestos-treated mice (Altomare et al., 2005b). One of nine human MM cell lines that we tested had elevated AKT

activity under serum-starvation conditions, which was associated with a homozygous deletion of *PTEN*, and treatment of this line with an mTOR inhibitor resulted in growth arrest (Altomare et al., 2005b). Inhibition of this pathway at multiple steps of the signaling cascade has shown promise in preclinical MM cell culture experiments (Barbone et al., 2008; Indovina et al., 2012; Yamaji et al., 2017).

Here, we demonstrate that combined inactivation of both *Pten* and *Tp53* is capable of inducing rapid, aggressive MMs at high penetrance in both pleural and peritoneal cavities of *Pten^{L/L};Tp53^{L/L}* mice following the administration of adenovirus expressing Cre recombinase (adeno-Cre). Primary MMs and MM cell lines derived from these mice showed hyperactivated Akt-mTOR signaling, and the MM cells displayed genomic instability. Furthermore, analysis of TCGA data revealed co-deletion of *PTEN* and *TP53* and/or *CDKN2A/p14ARF* in ~25% of human MMs, suggesting that cooperative losses of these genes contribute to the development of a significant proportion of these aggressive neoplasms, for which PI3K-AKT-mTOR and p53/DNA repair pathways may serve as critical targets for therapeutic intervention.

2 | MATERIALS AND METHODS

2.1 | Animal husbandry and ethics approval

Pten^{L/L} and *Tp53^{L/L}* mice, both in a 129S6/svEv background, were provided by Dr. Antonio Di Cristofano (Albert Einstein College of Medicine). Mice were crossed to generate *Pten^{L/L};Tp53^{L/L}* mice. All mouse work was carried out in the Laboratory Animal Facility of Fox Chase Cancer Center, which is fully accredited by AAALAC International, is in full compliance with USDA, and is OLAW-approved and assured. Studies were performed according to NIH's Guide for the Care and Use of Laboratory Animals, under protocol 18-03 approved by the Committee on the Ethics of Animal Experiments of Fox Chase Cancer Center.

2.2 | Adenovirus expressing Cre recombinase or LacZ, and intrapleural and intraperitoneal injections of mice

Adenovirus expressing Cre recombinase (Adeno-Cre) or LacZ (Adeno-LacZ) was purchased from the Viral Vector Core of the University of Iowa. Approximately $\sim 4 \times 10^{10}$ PFU adeno-Cre or adeno-LacZ (Viral Vector Core, University of Iowa) in 50 μ L were injected intrathoracically (i.t.) (Jongsma et al., 2008), more specifically intrapleurally, or intraperitoneally (i.p.) in 10–12-week-old mice of both genders. Mice were monitored daily and were euthanized upon visible signs of distress, including extreme fatigue, labored breathing, or when mice exhibited a 10% change in body weight. Tissues of all organs of the pleural and peritoneal cavities were collected, and tumor specimens were subjected to histopathological assessment. Tumor tissue, ascites, and peritoneal cavity lavage were isolated for RNA and protein analyses.

2.3 | Histopathology and immunohistochemistry

For immunohistochemistry (IHC), slides of formalin-fixed, paraffin-embedded MM samples were incubated with antibodies, and signals were detected via peroxidase activity. IHC was

performed with DAB (3, 3'-diaminobenzidine) and counterstained with hematoxylin. Cytokeratin 8 was localized using TROMA-I antibody (DSHB, Cat# TROMA-I, RRID: AB_531826), and mesothelin was localized with antibody D-16 (Santa Cruz Biotechnology, Cat# sc-27702, RRID: AB_649060). For WT1, we used antibody C-19 (Santa Cruz Biotechnology, Cat# sc-192, RRID: AB_632611), whereas cell proliferation was assessed with an anti-Ki67 antibody (Dako, Cat# M7249, RRID: AB_2250503). Phosphorylated (activated) Akt and S6 ribosomal protein staining were visualized with anti-P-Akt (Ser473) antibody (Cell Signaling Technology, Cat# 4060, RRID: AB_2315049) and anti-P-S6 (Ser235/236) rabbit 91B2 monoclonal antibody (Cell Signaling Technology, Cat# 4857, RRID: AB_2181035), respectively.

2.4 | Primary MM cell cultures

Primary MM cells (passages 6–8) from adeno-Cre-injected *Pten^{L/L};Tp53^{L/L}* mice were isolated from ascites and/or peritoneal lavage, and their mesothelial tumor origin was confirmed by RT-PCR with markers for MM, including N-cadherin, cytokeratin 18/19 and WT1, and control Gapdh was used to assess template integrity. RT-PCR for mesothelin used primers 5'-ATCAAGACATTCCTGGGTGGG-3' and 5'-CGGTTAAAGCTGGGAGCAGAG-3'. Primers for other markers have been previously described (Altomare et al., 2005b). Representative RT-PCR marker results of MM cells from *Pten^{L/L};Tp53^{L/L}* mice are shown in Supplemental Figure 1.

2.5 | Immunoblot analysis

Immunoblots were prepared with 30–50 µg of protein lysate/sample, as previously described (Tan et al., 2016). Antibodies used for immunoblotting included a pan-Akt antibody (Cell Signaling Technology, Cat# 9272, RRID: AB_329827); anti-P-Akt (Ser473) antibody (Cell Signaling Technology, Cat# 4060, RRID: AB_2315049); rabbit anti-c-Myc XP monoclonal antibody, unconjugated, clone D84C12 (Cell Signaling Technology, Cat# 5605S, RRID: AB_1903938); p53 mouse 1C12 monoclonal antibody (Cell Signaling Technology, Cat# 2524, RRID: AB_331743); rabbit anti-PTEN monoclonal antibody, unconjugated, clone 138G6 (Cell Signaling Technology, Cat# 9559, RRID: AB_390810); anti-P-S6 ribosomal protein (Ser235/236) (91B2) rabbit monoclonal antibody (Cell Signaling Technology, Cat# 4857, RRID: AB_2181035); mouse anti-Gapdh monoclonal antibody, unconjugated, clone 6c5 (Santa Cruz Biotechnology, Cat# sc-32233, RRID: AB_627679); BAP1 antibody (Bethyl Cat# A302-243A, RRID: AB_1731059); NF2/merlin (H-260) antibody (Santa Cruz Biotechnology, Cat# sc-28247, RRID: AB_2149822).

2.6 | Cytogenetic and array-based comparative genomic hybridization analyses

Cytogenetic studies were carried out on cell cultures derived from MMs of adeno-Cre-injected *Pten^{L/L};Tp53^{L/L}* mice. Preparation of metaphase spreads and Giemsa-banding were performed essentially as described previously (Testa et al., 1994). Array-based comparative genomic hybridization (aCGH) analysis was performed using 244K genomic DNA arrays from Agilent (Santa Clara, CA), as previously described (Timakhov et al., 2009).

2.7 | Mining of co-deletions of *PTEN* and *TP53* and/or *CDKN2A/p14ARF* in TCGA dataset

Gene copy number losses of *PTEN*, *TP53*, and *CDKN2A/p14ARF* in a TCGA dataset of 87 MMs were determined based on the GISTIC 2.0 algorithm, GISTIC, RRID: SCR_000151 (Mermel et al., 2011). The gene level discrete amplification and deletion indicators were obtained from Broad Institute Firehose portal (<http://gdac.broadinstitute.org/>) (TCGA data version 2016_01_28 for MESO). To depict copy number indicator and mutation data, OncoPrinter (v.1.0.1), available at cBioPortal, RRID: SCR_014555 (<http://www.cbioportal.org/>), was used. Mutation data for MM samples were obtained from Genomic Data Commons Data Portal (GDC Data Portal), RRID: SCR_014514 (<https://portal.gdc.cancer.gov/>).

3 | RESULTS

3.1 | *Pten* inactivation alone is generally not sufficient for MM formation but can cooperate with *Tp53* inactivation to rapidly drive pleural and peritoneal MMs

We and others have previously demonstrated frequent activation of the PI3K-AKT pathway in human and murine MM samples, occasionally via inactivation of *PTEN* (Altomare et al., 2005b). To determine if loss of *Pten* and consequent Pi3k-Akt activation is sufficient to induce murine MMs, a pilot study was performed. Fifteen *Pten*^{L/L} mice were injected i.t. with adeno-Cre, as described (Jongsma et al., 2008), and monitored for tumor formation. Only 1 of 15 (7%) mice developed MM (12 months post injection). Since several laboratories have demonstrated that deletion of *Tp53* in combination with *Pten* can lead to tissue-specific cancer development in the prostate, bladder and brain in conditional knockout mice (e.g., Nardella et al., 2010; Puzio-Kuter et al., 2009), and since p53/DNA repair/BAP1 and PI3K-PTEN-AKT-mTOR pathways have been implicated as critical pathways in MM (Lo Iacono et al., 2015), we crossed *Pten*^{L/L} mice to *Tp53*^{L/L} mice to assess cooperativity of these TSGs in MM pathogenesis.

A summary of the mouse experiments performed using i.t. or i.p. injections of adenovirus, along with the overall number of resulting tumors, is presented in Table 1. Nearly all (23/25, 92%) *Pten*^{L/L};*Tp53*^{L/L} mice injected i.p. with adeno-Cre developed MM, with a median latency of 9.4 weeks; in contrast, none of the eight *Pten*^{L/L};*Tp53*^{L/L} mice injected i.p. with adeno-LacZ developed MM by the completion of this experiment, i.e., 33 weeks post-injection (Figure 1a). Additionally, none of the 12 *Tp53*^{L/L} mice injected i.p. with adeno-Cre developed tumors by 14 weeks post-injection. Among *Pten*^{L/L};*Tp53*^{L/L} mice injected i.t. with adeno-Cre, 19/34 (56%) developed pleural MM, with a median latency of 19.3 weeks (Figure 1b). Some *Pten*^{L/L};*Tp53*^{L/L} mice injected with adeno-Cre died of other tumor types (Figure 2a,b), particularly among the mice injected i.t. The histology of most pleural (54%) and peritoneal (67%) MMs was sarcomatoid, with the remainder being biphasic, i.e., mixed sarcomatoid and epithelioid (Figure 2a,b). The i.p.-injected mice often had ascites, which was used to establish primary MM cell lines for subsequent studies reported here.

Histopathological and immunohistochemical characteristics of a representative mouse MM is shown in Figure 1c. It should be noted that 100% of the evaluated MMs were mesothelin positive (range: 10–60% of cells positive); 80% were positive for WT1 (range: 10–60% cells

positive); and 40% were cytokeratin positive (range: 4–30% cells positive). Based on Ki67 immunohistochemistry, approximately half of the MMs were highly proliferative (range: >30–45% positive cells), whereas the remaining tumors were mildly to moderately proliferative (range: 10–30% positive).

3.2 | MM tumors and cell lines established from *Pten*^{L/L};*Tp53*^{L/L} mice show activated PI3K-Akt-mTor signaling

The critical oncogenic pathway controlled by PTEN is the PI3K-AKT-mTOR signaling cascade (Nardella et al., 2010). Protein lysates of primary MM cell lines derived from i.p.-injected *Pten*^{L/L};*Tp53*^{L/L} mice were immunoblotted, and all 12 lines tested showed loss of Pten and p53 and elevated P-Akt and P-S6 expression compared to normal mesothelial cells from control *Pten*^{L/L};*Tp53*^{L/L} mice in which *Pten* and *Tp53* were not excised (Figure 3a). Myc upregulation was also consistently observed in the MM lines, whereas Nf2 expression was unchanged in MM cells versus normal mesothelial cells, and Bap1 expression was downregulated in a subset (4/12, 33%) of the MM lines analyzed (Figure 3a). IHC analysis of peritoneal and pleural MMs from adeno-Cre-injected *Pten*^{L/L};*Tp53*^{L/L} mice revealed positive staining for P-Akt and P-S6, consistent with Pten loss and consequent PI3K-Akt-mTor activation (representative staining shown in Figure 3b).

3.3 | MM cell lines established from *Pten*^{L/L};*Tp53*^{L/L} mice show genomic instability and numerous chromosomal breaks

Since both p53 and Pten are considered guardians of the genome and their loss can result in chromosomal damage (Shen et al., 2007; Williams and Schumacher, 2016), we decided to assess genomic stability of primary MM cell lines from adeno-Cre-injected *Pten*^{L/L};*Tp53*^{L/L} mice. Conventional cytogenetic analysis was carried out on five MM lines derived from peritoneal MMs. All five lines showed evidence of genomic instability, including aneuploidy, small markers, centromeric fragments, double minutes, and chromatid and/or chromosome breaks (Figure 4a–c). Occasional triradial and quadriradial chromosomes (Figure 4d), or metaphases showing widespread centromeric separation, were observed in several cell lines.

In two MM lines, 829 and 833, Giemsa-banding permitted the identification of clonal alterations, with both lines showing gains of chromosomes 4 and 15. The chromosome quality of the remaining cell lines (805, 806, 808) was suboptimal for detailed analysis, but aCGH analysis permitted a precise assessment of the genomic imbalances in these samples. Although cell line 805 showed marked genomic instability, including apparently random trisomies, in the karyotypic analysis, no genomic imbalances were evident by aCGH, indicating that none of these chromosomal gains were representative of a major clonal population. Cell line 806 exhibited multiple DNA copy number alterations consistent with the findings of the conventional cytogenetic analysis, including gains of chromosomes 6, 8, 10, 11, 13, 14, 15, and 18. MM cells 808 had multiple genomic imbalances including gains of 1A4B, 8, 9, 14, 15, 17, 18 and 19 and losses of 4, 7, 10, 12, 13, and X. Overall, whole chromosome gains predominated, with losses of chromosomes documented only in cell line 808. Trisomy 15 was the most common recurring change, occurring in 4 of 5 MM lines. The overall chromosomal findings are summarized in Figure 4e.

3.4 | *PTEN* and *TP53* loss/inactivation in human MM specimens

In order to estimate the prevalence of concomitant loss of *PTEN* and *TP53* in human MM cases, we surveyed somatic mutation and copy number data for an MM cohort (n=87) available in TCGA (Figure 4). Analysis of gene level discrete amplification and deletion indicators derived from GISTIC 2.0 algorithm indicated that 32% and 31% of MMs had hemizygous or, in a few cases, homozygous deletions of *TP53* and *PTEN*, respectively. Among these, 13/87 (15%) had co-deletions of *PTEN* and *TP53* or, in one case, a *PTEN* deletion and a *TP53* truncating mutation. Although no damaging mutations were observed in *PTEN*, 14/87 cases (16%) had predicted *TP53* loss-of-function mutations, eight of which were accompanied by deletion of the remaining allele. Deletions of *CDKN2A* were observed in 60% of cases, 46% being deep, putative homozygous deletions. Among these, 19 of 87 (22%) had co-deletions of *PTEN* and *CDKN2A/p14ARF*, 10 of which did not show losses/mutations of *TP53*. Altogether, co-deletion/inactivation of *PTEN* and the p53 pathway TSGs *TP53* and *p14ARF* were observed in 23 of 87 (26%) MMs (Figure 5).

4 | DISCUSSION

The fact that *Pten* inactivation alone is generally not sufficient to drive MM formation is not unexpected. Prior work with conditional knockout mice revealed that i.t.-induced excision of a single TSG resulted in few or no MMs, but instead MM formation required inactivation of multiple TSGs (Jongsma et al., 2008). That same report showed that *Tp53^{L/L}* mice injected i.t. with adeno-Cre developed lymphomas, osteosarcomas or leiomyosarcomas, but no MMs (Jongsma et al., 2008). Our findings demonstrate that combined inactivation of *Pten* and *Tp53* can synergize to drive the rapid development of a high incidence of MMs.

The inherent genomic instability and pro-survival signaling afforded by the combined loss of p53 and Pten appear to be fundamental to the high penetrance and aggressive nature of the MMs observed in *Pten^{L/L};Tp53^{L/L}* mice. p53 regulates a myriad of pathways involved in cell proliferation, survival and migration—all hallmarks of cancer (Hanahan and Weinberg, 2000). A key tumor suppressor function of p53 is to respond to DNA damage by inducing cell cycle arrest until the damage is repaired or to initiate programmed cell death to remove genomically unstable cells bearing excessive or unrepairable DNA damage (Matsumoto et al., 1999). However, when *TP53* is inactivated, DNA damage can accumulate and mutations may occur that drive tumorigenesis. Inactivation of *PTEN* can also lead to genomic instability through various mechanisms (Harper and Elledge, 2007).

In addition to genomic errors that may be caused during DNA replication, abnormal chromosome segregation during mitosis is a cause of chromosome instability, and loss of PTEN has been associated with aneuploidy in cancer patients, while *Pten* loss in mice has been shown to drive genomic instability and tumor formation (Hou et al., 2017). In addition to its lipid phosphatase function that inactivates the PI3K/AKT pathway, PTEN was shown about a decade ago to have a nuclear role in controlling chromosomal integrity (Shen et al., 2007). Loss of *Pten* in MEFs resulted in extensive centromere breakage and was found to localize to centromeres, where it physically associated with CENP-C, a component of the kinetochore, and led to the accumulation of structural and numerical chromosome alterations (Shen et al., 2007).

When both *Pten* and *p53* are lost, not only does DNA damage not trigger a cell cycle arrest checkpoint, but *Pten*'s role as a guardian of the genome is compromised, and downstream activation of Akt promotes cell survival. Consequently, chromosomal alterations may accumulate at an elevated rate, which may help to explain why the combination of these two genetic drivers leads to a very high incidence of MMs in *Pten*^{L/L};*Tp53*^{L/L} mice, whereas MM formation generally does not occur with excision of *Pten* or *Tp53* genes alone. A similar situation has been reported for *Brca2* and *Tp53*, which were found to act synergistically in double-mutant mice to promote genomic instability and deregulation of T-cell apoptosis (Cheung et al., 2002).

The *in vivo* data presented here provide compelling genetic evidence that combined loss/inactivation of *Tp53* and *Pten* is sufficient to drive aggressive pleural and peritoneal MMs with high penetrance and short latency (Figure 1a, b. Analysis of primary MMs and tumor-derived cell lines verified activation of the Akt-mTor signaling cascade. Chromosomal analyses revealed aneuploidy, small markers, centromeric fragments, chromatid/chromosome breaks, and occasional double minutes in all cell lines, and radial chromosomes, such as the type formed by DNA crosslinkers (Matsumoto et al., 1999), were observed in some lines, as well. All cell lines had at least some metaphases with centromere fragments or with unusually large gaps separating the centromere from the remainder of the chromosome. Such centromeric fragments have been previously reported in *Pten*^{-/-} MEFs (Shen et al., 2007).

It is noteworthy, however, that in one of our MM cell lines (805), no clonal genomic imbalances were obvious by either conventional cytogenetics or aCGH, suggesting that combined inactivation of *Pten* and *Tp53* is sufficient to drive MM without the need to acquire clonal somatic copy number imbalances. With regard to the clonal genomic imbalances that were observed in our series of MM cell lines, several recurrent alterations were identified, including gains of 4, 8, 14, 15, 18, and 19, with gain of 15 being observed in 4 of 5 MM lines tested (Figure 4e). Notably, several of these chromosomal gains (+8, +15, +19) have been reported previously in cell lines derived from mouse MMs induced by carcinogenic fibers inoculated into the peritoneal cavity of wild type and *Nf2*^{+/-} mice (Jean et al., 2011). The recurrent gains of chromosome 15 seen in our series is notable, because it harbors the *Myc* locus, and *Myc* was consistently overexpressed in our MM cell lines (Figure 3a). Interestingly, in human MM, FISH analysis has revealed trisomy of *MYC* in 15% of cases and 4 copies of *MYC* in another 15% of cases (Riquelme et al., 2014). In mice, *Pten* inactivation in hematopoietic stem cells has been reported to lead to a myeloproliferative disorder, followed by acute T-lymphoblastic leukemia (T-ALL), and transformation to T-ALL was connected with the acquisition of a recurring translocation involving chromosomes 14 and 15 that resulted in overexpression of *Myc* (Guo et al., 2008). In this mouse model, the authors concluded that *Pten* deletion acted as an initial "hit" that hyperactivates Akt signaling to promote cell survival and genomic instability, which leads to chromosome alterations—in this case, translocation-mediated upregulation of *Myc*—that may confer a proliferative advantage and consequent clonal expansion and ultimately malignancy.

Collectively, our findings have potential translational implications. Co-deletion of *PTEN* and *TP53* occurs in a subset of human MMs (Figure 5), and our *in vivo* evidence indicates that cooperative losses of these genes result; in the formation of a highly aggressive form of MM. Moreover, somatic mutations in human MM have been proposed to cluster in the p53/DNA repair/BAP1 and PI3K-PTEN-AKT-mTOR pathways (Lo Iacono et al., 2015), further suggesting that they may serve as critical therapeutic targets. While mutations of *PTEN* and *TP53* are relatively uncommon in human MM, haploinsufficiency for these genes appears to be underappreciated, with each occurring in more than 30% of MMs in one large series, including compound haploinsufficiency in 15% of the cases (Figure 5). Moreover, loss of another p53 pathway-related gene, *CDKN2A/p14ARF*, was deleted in 60% of the cases in this series. Altogether, 23 of 87 (26%) MMs had losses of *PTEN* with *TP53* and/or *p14ARF*. Admittedly, deletions are biallelic in the MMs from *Pten^{L/L};Tp53^{L/L}* mice, whereas deletions of *PTEN* and *TP53* (but not *CDKN2A/p14ARF*) are generally monoallelic in human MMs, but even partial inactivation of tumor suppressors has been shown to contribute critically to tumorigenesis (Berger et al., 2011).

Finally, since conditional *Pten^{L/L};Tp53^{L/L}* mice develop highly penetrant, rapid pleural and peritoneal MMs without the need for carcinogenic asbestos, they may serve as a useful preclinical model for testing the efficacy of novel small molecule inhibitors. However, our *in vivo* model has limitations for testing the efficacy of new compounds aimed to reactivate the normal function of Pten and Tp53. For example, since this model has biallelic loss of *Tp53*, this fact would exclude small molecules that work by reactivating p53, e.g., RITA (reactivation of p53 and induction of tumor cell apoptosis), which activates p53 function in tumors that retain expression of mutant or wild type p53 (Di Marzo et al., 2014; Zhao et al., 2010). One way to circumvent this issue with a drug such as RITA would be to only inactivate one *Tp53* allele, i.e., by using *Pten^{L/L};Tp53^{+/-}* mice, although tumor formation would likely take much longer than in *Pten^{L/L};Tp53^{L/L}* mice, based on studies of tumor latency in mice with heterozygously- versus homozygously-floxed alleles of other TSGs in conditional mouse models for MM (Jongsma et al., 2008). Another limitation with regard to the translational implications of the *Pten^{L/L};Tp53^{L/L}* model is that, unlike MMs developing in *Nf2^{+/-}* mice exposed to asbestos, where somatic mutations of other genes such as *Cdkn2a/b* are acquired (Altomare et al., 2005a), somatic alterations of other TSGs commonly involved in the pathogenesis of human MM are not recapitulated in the *Pten^{L/L};Tp53^{L/L}* model. However, the fact that the MMs in *Pten^{L/L};Tp53^{L/L}* mice arise in a short time frame and consistently exhibit aggressive sarcomatoid or biphasic MMs with high penetrance and short latency, especially in mice injected i.p. with adeno-Cre, may provide advantages for some preclinical applications.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations pertaining to labeling of figures

aCGH	array-based comparative genomic hybridization
adeno-Cre	adenovirus expressing Cre-recombinase
IHC	immunohistochemistry
i.p.	intraperitoneally
i.t.	intrathoracically
MM	malignant mesothelioma
P-Akt	phosphorylated (phospho)-Akt
P-S6	phospho-S6
TCGA	The Cancer Genome Atlas

FIGURE 1.

Development of MM in *Pten*^{L/L};*Tp53*^{L/L} mice induced by adeno-Cre. (a) Kaplan-Meier survival curves of *Pten*^{L/L};*Tp53*^{L/L} mice injected i.p. with adeno-Cre versus *Pten*^{L/L};*Tp53*^{L/L} mice injected i.p. with control adeno-LacZ. Deaths shown were due to MM, except one mouse injected with adeno-LacZ that died due to a biliary tract obstruction. (b) Dot plot depicting age of deaths due to pleural MM in *Pten*^{L/L};*Tp53*^{L/L} mice injected i.t. with adeno-Cre. Median survivals of mice injected i.p. and i.t. with adeno-Cre were 9.4 and 19.3 weeks, respectively. (c) Histopathologic assessment of representative MM from *Pten*^{L/L};*Tp53*^{L/L} mouse. Serial tumor sections depicting H&E staining and IHC for MM markers mesothelin

and WT1. Ki67 staining indicates active cellular proliferation of the MM cells. All original images are at 400× magnification. Scale bar = 50 μ M.

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Spectrum of tumors in *Tp53^{L/L};Pten^{L/L}* mice injected intrapleurally (i.t.) with adeno-Cre

Pathological diagnosis	No. tumors	% of all tumors
MM	19*	58
Histiocytic sarcoma	10	30
Other tumors**	4	12
Total	33	100

* Sarcomatoid (10); biphasic (9)

** Other tumors include hepatocholangiocarcinoma (1), angiosarcoma (1), lymphoma (1), and spindle cell tumor (1).

Spectrum of tumors in *Tp53^{L/L};Pten^{L/L}* mice injected intraperitoneally (i.p.) with adeno-Cre

Pathological diagnosis	No. tumors	% of all tumors
MM	23*	88
Other tumors**	3	12
Total	26	100

* Sarcomatoid (15); biphasic (8)

** Other tumors include lymphoma (1), osteosarcoma (1), and spindle cell tumor (1).

FIGURE 2.

Spectrum of tumors observed in *Tp53^{L/L};Pten^{L/L}* mice injected intrapleurally or intraperitoneally with adenovirus expressing Cre recombinase. (a) Types and incidence of various tumors seen in animals injected intrapleurally with adeno-Cre. Of 33 tumors observed, 19 (58%) were MMs. (b) Tumors identified in *Tp53^{L/L};Pten^{L/L}* mice injected intraperitoneally with adeno-Cre. Among 26 tumors identified, 23 (88%) were MMs. Among the total 42 MMs observed in mice injected i.t. and i.p. with adeno-Cre, 25 were sarcomatoid and 17 were biphasic tumors.

b **FIGURE 3.**

Activity of Pten-Akt-mTOR pathway and expression of p53 in primary MMs and MM cell lines derived from *Pten^{L/L};Tp53^{L/L}* mice injected i.p. with adeno-Cre. (a) Immunoblot analysis of primary MM cell lines (passages 6–8) from *Pten^{L/L};Tp53^{L/L}* mice was performed using antibodies against p53, P-Akt, total Akt, P-S6, total S6, Pten, Myc, Bap1, Nf2, and Gapdh. NM1 and NM2 are early passage (5) primary normal mesothelial cells derived from 8-week-old *Pten^{L/L};Tp53^{L/L}* mice not injected with adenovirus, isolated as described (Bot et al., 2003). (b) Immunohistochemical assessment of representative MM from *Pten^{L/L};Tp53^{L/L}* mouse. Adjacent tumor sections depicting P-Akt and P-S6 immunostainings.

eSummary of cytogenetic and aCGH findings in MM cells from *Tp53*^{-/-};*Pten*^{-/-} mice

MM line	Modal no.	Metaphases with mar, min ^a	Metaphases with breaks ^b	Genomic imbalances ^c
805	43-44	10/14	6/14	(None)
806	48	6/12	8/12	+6, +8 ,+10,+11,+13, +14 , +15 , +18
808	61-88	10/14	5/14	-X,+1A4B,-4,-7, +8 ,+9,-10,-12,-13, +14 , +15 ,+17, +18 , +19
829	40-41	7/18	3/18	+4 , +15 , +19
833	42	5/14	2/14	+4 , +15

^a Here, min includes small mar, centromeric fragments, and dmin.

^b Includes chromatid and chromosome breaks.

^c Genomic imbalances shown for cell lines 805, 806, and 808 are from aCGH and conventional cytogenetic analysis, whereas those for lines 829 and 833 are from conventional cytogenetic analysis only. Recurrent imbalances are highlighted in bold.

FIGURE 4.

Cytogenetic analysis of early passage (3–6) MM cell lines derived from *Pten*^{L/L};*Tp53*^{L/L} mice injected i.p. with adeno-Cre. (a–c) Partial metaphase spreads showing examples of centromeric fragments (long arrows). (d) Partial metaphase exhibiting severe chromosomal damage, including radials (open arrows), chromosome breaks/acentric fragments (short arrows), and ring (r). (e) Summary of cytogenetic and aCGH findings in five MM cell lines from *Pten*^{L/L};*Tp53*^{L/L} mice.

FIGURE 5.

Co-deletion/inactivation of *PTEN* and *TP53* in 87 human MM specimens from TCGA. Percentages at left indicate proportions of cases with gene copy number loss based on GISTIC 2.0 algorithm (Mermel et al., 2011). Boxes indicate concomitant somatic losses/truncating mutations of *PTEN* and *TP53* (13 cases: red boxes) or *PTEN* and *CDKN2A/p14ARF* (10 cases: green boxes) in human MM cases; 10 of these 23 cases had losses of both *TP53* and *CDKN2A/p14ARF*. Note missense mutations in *TP53*, but not *PTEN*, were predicted to be damaging.

TABLE 1

Number of mice injected with adenovirus expressing Cre recombinase and tumor incidence

Genotype	Adeno-Cre injected mice		Mice with tumors*	
	Peritoneal	Pleural	Peritoneal	Pleural
<i>Tp53^{L/L}</i>	12	0	0	0
<i>Pten^{L/L}</i>	0	15	0	1
<i>Pten^{L/L};Tp53^{L/L}**</i>	25	34	25 24 (1 tumor) 1 (2 tumors)	25 17 (1 tumor) 8 (2 tumors)

^{*}, some *Pten^{L/L};Tp53^{L/L}* mice developed more than one type of tumor, as indicated below the total number of mice that developed tumors;

^{**}, in addition to injections with adeno-Cre, 8 *Pten^{L/L};Tp53^{L/L}* mice injected i.p. with adeno-LacZ, and no tumors were observed in these animals.