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Mutant p53 Promotes Epithelial Ovarian Cancer by Regulating Tumor Differentiation, Metastasis, and Responsiveness to Steroid Hormones

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Abstract

Mutations in the tumor protein p53 (TP53) are the most frequently occurring genetic events in high-grade ovarian cancers, especially the prevalence of the Trp53R172H, mutant allele. In this study, we investigated the impact of the Trp53R172H, mutant allele on epithelial ovarian cancer (EOC) in vivo. We used the Pten/KrasG12D, mutant mouse strain that develops serous EOC with 100% penetrance to introduce the mutant Trp53R172H allele (homolog for human TP53R172H). We demonstrate that the Trp53R172H mutation promoted EOC but had differential effects on disease features and progression depending on the presence or absence of the wild-type (WT) TP53 allele. Heterozygous WT/Trp53R172H alleles facilitated invasion into the ovarian stroma, accelerated intraperitoneal metastasis, and reduced TP53 transactivation activity but retained responsiveness to nutlin-3a, an activator of WT TP53. Moreover, high levels of estrogen receptor α in these tumors enhanced the growth of both primary and metastatic tumors in response to estradiol. Ovarian tumors homozygous for Trp53R172H mutation were undifferentiated and highly metastatic, exhibited minimal TP53 transactivation activity, and expressed genes with potential regulatory functions in EOC development. Notably, heterozygous WT/Trp53R172H mice also presented mucinous cystadenocarcinomas at 12 weeks of age, recapitulating human mucinous ovarian tumors, which also exhibit heterozygous TP53 mutations (~50%-60%) and KRAS mutations. Therefore, we present the first mouse model of mucinous tumor formation from ovarian cells and supporting evidence that mutant TP53 is a key regulator of EOC progression, differentiation, and responsiveness to steroid hormones.

Introduction

As the fifth leading cause of death in women, ovarian cancer is a devastating disease without effective detection and therapeutic management. The majority (>90%) of human ovarian cancers are of epithelial cell origin (1, 2) and based on their histologic features are categorized as serous, endometrioid, mucinous, and clear cell type (3). These subtypes differ significantly in their potential sites of origin, molecular signature, prognosis, and response to treatments, making epithelial ovarian cancer (EOC) not a single disease, but multiple diseases (4).

Mutations in the tumor protein p53 (TP53) are among the most common and frequent events in EOC, especially in the high-grade serous subtype [>90%; The Cancer Genome Atlas Research Network (TCGA; ref. 5)]. Furthermore, the R175H mutation of TP53 is one of the most frequently occurring (3.8%) in serous EOC (TCGA; ref. 6). When the Trp53R172H mutation (mouse homolog for the R175H mutation in human) is expressed in cells depleted of Apc and Pten or in cells with inactivated Rb and Brca1/2, it promotes epithelial ovarian tumor progression and metastasis (7, 8). Moreover, a recent examination of TCGA database reveals that p53 gain-of-function (GOF) mutations, including the R175H mutation, in ovarian tumors of human patients are associated with a higher incidence of platinum resistance, local recurrence, and distant metastasis (9). Intriguingly and importantly, recently published studies show that TP53 mutations in human EOC tumor samples are frequently present as heterozygous (10, 11), indicating that it is critical to understand not only the biology of tumors homozygous for wild-type (WT) or mutant alleles of p53, but also the interaction between WT and mutant alleles in a tumor type-specific context.

Oncogenic alterations are necessary for ovarian cancer development, but strong evidence indicates that steroid hormones, such as estradiol, also play critical roles in promoting the timing of onset, progression and metastasis of EOC (11–13). A majority of EOC cases occur during the peri and postmenopausal period (peak at ages 65–75), when the
tumor-promoting effects of estradiol are unopposed by progesterone (1). Several recent epidemiologic and clinical studies suggest that the expression of steroid hormone receptors is linked to ovarian cancer subtypes, progression, prognosis, and response to hormone treatment (14–18). Thus, how oncogenic factors, TP53, and steroid hormones interact to impact EOC development is becoming an emerging area of research that is highly relevant for developing and revising clinical therapeutic strategies.

Our laboratory previously generated mice in which the Pten gene was conditionally deleted and Kras mutant mice were generated by Cre recombinase driven by Amhr2 promoter (Pten/Kras–/– mutant mice; refs. 19, 20). These mice developed ovarian surface epithelial (OSE) cell–derived, low-grade serous adenocarcinomas with 100% penetrance. When Trp53 was deleted in the OSE cells of the Pten/Kras–/– mutant mice, tumor growth was markedly reduced, but the expression of estrogen receptor α (ESR1) was elevated (21), and when exposed to estradiol, the primary ovarian lesions grew rapidly and metastasized throughout the intraperitoneal cavity. These studies documented clearly and for the first time that the functional status of TRP53 can determine steroid hormone responsiveness in EOC cells.

On the basis of the foregoing considerations, we hypothesized that (i) specific Trp53 mutations would not only alter tumor growth and metastasis but also the responsiveness of EOC cells to steroid hormones, (ii) the effect of any given mutation would also depend on whether a WT allele was present or absent, and (iii) each condition would exhibit a distinct molecular signature. Specifically, because the human TP53R175H allele is frequently associated with high-grade ovarian cancer, we introduced a germline Trp53 R172H–/– (Pten/Kras–/–) strain to generate OSE cells either heterozygous or homozygous for the Trp53 R172H–/– allele. Our findings indicate that the expression of heterozygous Trp53– mutant alleles drives a tumor phenotype that is distinct from that of WT Trp53 or homozygous Trp53– mutant alleles in determining ovarian tumor behavior and outcome.

Materials and Methods

Animal studies

All animals were housed under a 14-hour light/10-hour dark schedule in the Center for Comparative Medicine at Baylor College of Medicine (Houston, TX) and provided food and water ad libitum. All animals were maintained according to the NIH Guide for the Care and Use of Laboratory Animals and approved by the Animal Care and Use Committee at Baylor college of Medicine. Mice carrying the germline Trp53 R172H–/– mutation were generated by Dr. Guillermina Lozano (MD Anderson Cancer Center, Houston, TX; ref. 22). These mice were bred to Pten/–/–, Amhr2-Cre (PK) mice described previously to obtain PK mice expressing WT Trp53 (PKP53 WT) and either heterozygous (PKP53 H/–) or homozygous mutant Trp53 R172H– (PKP53 H/H, ref. 19).

Laser capture microdissection and sequencing of human mucinous tumor samples

Laser capture microdissection of human mucinous tumor samples is described in detail in Supplementary Materials and Methods. DNA was extracted from microdissected tumor cells using a QIAamp DNA Micro Kit (Qiagen) and quantified using a NanoDrop spectrometer (NanoDrop). Mutation analysis of TP53 (exons 5–8) was performed by Sanger Sequencing using PCR primers as described previously (23).

Infection of tumors into syngenic mice and hormone pellet implants

Isolation, culturing, and injection of OSE cells from genetically engineered mice are described in detail in Supplementary Materials and Methods. Genotype-confirmed mice of WT and mutant Trp53 status were implanted with one half of a 0.36-mg estradiol pellet (0.36 mg) or with progesterone pellet (25 mg) one day prior to giving estradiol pellet (SE-121 for 17β-estradiol and P-131 for progesterone, Innovative Research of America), and tumor progression was monitored at 1, 2, or 4 weeks after pellet implanting.

RNA extraction, real-time RT-PCR, and microarray analyses

DNA expression vectors (0.20 µg DNA; empty vector or vector expressing mutant Trp53 R172H–/–) were transfected into cells using Attractene Transfection Reagent (Qiagen) according to the manufacturer’s instructions and lysed for RNA extraction 24 hours following transfection. Total RNA extraction, reverse transcription, real-time PCR, and microarray analyses were described in Supplementary Materials and Methods. The microarray data have been deposited to GEO with the accession number GSE65206.

Cell adhesion, proliferation, and viability assays

To assess cell attachment to the culture dish, PKP53 WT, PKP53 H/–, and PKP53 H/H OSE cells at similar passages were plated at 1 × 105 cells per well in a 12-well plate. After culturing for 2 hours, the plates were gently swirled and pipetted three times. Unattached cells were removed; attached cells that remained on the dish were trypsinized and counted with a hemocytometer. To measure the rate of proliferation, OSE cells at similar passages were plated at 1 × 105 cells per well in each 12-well plate. After 36 hours of culture, when cells of all genotypes had attached well and were proliferating but not confluent, cells were trypsinized and counted using a hemocytometer. Cell viability was measured using the WST-1 assay according to the manufacturer’s instructions following 72 hours of nutlin-3a treatment. The WST-1 reagent was incubated with the cells for 4 hours before its absorbance was read at 450 nm (Roche).

Western blot analyses

Whole-cell lysates were collected using methods described previously (24). Proteins were separated using a 10% Bis-Tris gel and transferred to an Immobilon-P Membrane (Millipore). Primary antibodies used included ESR1 (sc-542; 1:1,000), PR (sc-7208; 1:1,000), and p53 (sc-6243; 1:1,000; Santa Cruz Biotechnology) and signals were detected using the Peirce ECL Western Blotting Substrate (Thermo Scientific).

TP53 luciferase assay

OSE cells were transfected according to the manufacturer’s instructions (P53 Cignal Reporter Assay Kit, Qiagen Sciences). Nutlin-3a (generously provided by Dr. Robert Bast, MD Anderson...
Cancer Center, Houston, TX) or DMSO vehicle control were added to the cultures after overnight transfection, and luciferase assays were performed after 24 hours of treatment using the Dual-Glo Luciferase Assay System (Promega).

Statistical analysis
Data are represented as mean ± SEM. Comparisons between experimental groups with a single variance were analyzed using unpaired Student t test, and a two-tailed P < 0.05 was considered statistically significant. Comparison between experimental groups with more than one variance was analyzed using randomized two-way ANOVA. When ANOVA indicated overall significance, Student–Newman–Keuls test was used to compare individual means.

Results
Trp53R172H mutation promotes epithelial ovarian tumor progression
Before 12 weeks of age, ovarian epithelial tumors in the PKP53+/− and PKP53+/+ mice exhibited similar serous, papillary-like structures on the surface of the ovary that stained positively for cytokeratin 8 (KRT8), a marker for OSE cells (Fig. 1A). However, the tumors in the PKP53+/− mice appeared more locally invasive into the ovarian stroma and more metastatic to the omentum than those in the PKP53+/− mice of the same age (Fig. 1A). Furthermore, the PKP53+/− mice died consistently between 12 and 13 weeks of age (n = 8), whereas most PKP53+/− mice died between 16 and 40 weeks of age (Fig. 1B and C; ref. 19), indicative of a more aggressive tumor progression.

Epithelial ovarian tumors expressing homozygous Trp53R172H mutation have early onset transformation and metastasis
Papillary-like epithelial ovarian lesions also developed in homozygous PKP53+/+ mice as early as 4 weeks of age and appeared to be in a more advanced stage compared with those in PKP53+/− mice of the same age (Fig. 2A, top, red arrows). Notably, a significant number of KRT8-positive metastatic tumor cells were present on the omentum of PKP53+/+ mice at 4 weeks of age, whereas only minimal metastasis to the omentum was observed in PKP53−/− mice (Fig. 2A, bottom panels of KRT8 staining). The PKP53+/+ tumor cells that metastasized to the omentum were highly proliferative as shown by KRT8 and phosphohistone H3 (pH3) costaining (Fig. 2A, bottom). Unfortunately, the PKP53+/+ mice died rapidly at 5 weeks from as yet unknown causes (Fig. 1B and C).

To further investigate the behavior of PKP53+/+OSE cells in vivo, we generated OSE cell lines from PKP53+/+ and injected these cells into the ovaries of syngenic hosts. Within 2 weeks, large, undifferentiated tumors were rampant in the ovaries injected with PKP53−/− cells (Fig. 2B, top). Consistent with metastatic tumors seen in PKP53+/+ mice, these injected tumor cells also grew rapidly at sites throughout the intraperitoneal cavity, such as the bowel mesentery, the pancreas, and the omentum (Fig. 2B, bottom). In the pancreas, these tumor cells appeared invasive.

Epithelial ovarian tumors expressing heterozygous Trp53R172H develop mucinous-like structures
A unique feature of the PKP53+/− mice is that they developed large cystic-like structures within the ovary between 8 and 12 weeks of age (8/10 females; Figs. 1B and 3A, top); these structures contained serous as well as mucinous-secretory-like cells that were not observed in PKP53+/+ or PKP53−/− mice at any age, and morphologically, they resemble mucinous ovarian EOC in human patients (Fig. 3B, top). KRT8 and hematoxylin and eosin (H&E) staining showed that the PKP53+/− OSE cells (red arrows) invaded the ovarian stroma and presented various stages of differentiation into secretory mucinous-like structures (Fig. 3A, bottom, black arrows). A previously characterized human ovarian cancer cell line (RMUG-L) that was obtained from a human mucinous ovarian carcinoma, carries heterozygous WT and mutant TP53 (c.614A>G) alleles as confirmed by us recently (23), exhibited a similar morphologic and secretory phenotype when inoculated into the intraperitoneal cavity of the SCID mice (Fig. 3C; refs. 25, 26). To confirm the mucinous tumor phenotype in the PKP53+/− ovary, we performed immunohistochemical analyses using an antibody detecting Mucin 5AC (MUC5AC), a marker of the human ovarian mucinous-like cancer subtype (Fig. 3D; refs. 27, 28). Indeed, the secretory structures in the mouse tumors are immunopositive for MUC5AC (Fig. 3C and KRT8 (Fig. 3E), but not immunonegative for FOXL2 (a marker for granulosa cells) or PAX8 (a marker for fallopian tube secretory cells, Fig. 3E; refs. 29, 30), suggesting that mucinous-like cells arise from the ovarian surface epithelial cells and not from granulosa cells or fallopian tube epithelial cells. In some cystic structures, mucinous cells that were KRT8 and MUC5AC positive were contiguous with KRT8-positive and MUC5AC-negative cells, providing further evidence that MUC5AC-positive secretory cells and KRT8-positive epithelial cells may share a common precursor (Fig. 3F).

To determine the mutant status of TP53 in human mucinous EOCs, we have sequenced laser capture microdissected mucinous tumor cells from 12 human patient samples. Of these, 4 samples (33%) had mutations in exons 5–8 of the TP53 gene, and in all 4 cases, the WT allele is still expressed (Fig. 3B, bottom, and Supplementary Fig. S1). These results support recent studies indicating that a significant number of human mucinous tumors express heterozygous mutant and WT alleles, including the R175H mutation (Supplementary Table S1; refs. 10, 31). Specifically, Supplementary Data provided in the study by Mackenzie and colleagues (10) show that in a dataset composed of 37 laser capture microdissected human mucinous ovarian tumor samples, 63% had mutations in KRAS, 56.8% had mutations in TP53, and 36% of tumors with KRAS mutations also had mutations in TP53. Importantly, 68% of TP53 mutations in these patient samples are heterozygous, with the coexistence of a WT and mutant allele. This observation is also consistent with the studies by Reichstein and colleagues (31), which demonstrated that at least half (57%) of human mucinous ovarian tumor samples express mutations in KRAS, and 37% of these mutations are coexpressed with mutations in TP53. To our knowledge, the PKP53+/−-mutant mouse strain is the first model that presents a mucinous-like tumor phenotype that is not derived from cells of the Müllerian duct or gut (2, 32).
Figure 1. Mutant Trp53R172H allele promotes epithelial ovarian tumor progression. A, representative images of tissue sections stained with H&E or KRT8 of ovaries and omentum from PKP53+/+ and PKP53H+/+ mice at 12 weeks of age. Regions in frame are shown at higher magnification below. Black and red dashed lines, surface of the ovary; black arrowheads, ovarian bursa. Scale bar, 400 µm (top row) and 80 µm (bottom two rows). B, survival ages and phenotypic characteristics of mutant mice with different p53 status. *, data presented here on PKP53+/+ mice are from previously published studies and new information collected from this study (19). **, PKP53H+/+ mice die rapidly from unknown causes. ***, female progenies with homozygous Trp53R172H/R172H mutation are born at lower than Mendelian ratio due to female-specific exencephaly, thus making it harder to obtain large sample size for the cohort. C, Kaplan-Meier analysis shows that Trp53R172H/R172H homozygous mutation is associated with the shortest term of survival, and Trp53R172H/+ heterozygous mutation is associated with significantly reduced term of survival compared with mice expressing WT Trp53. (P < 0.0002 for comparison between any two groups, log-rank test).
Epithelial ovarian tumors expressing heterozygous Trp53<sup>R172H</sup> have elevated expression of ESR1.

To determine how expression of the Trp53<sup>R172H</sup>-mutant allele affects OSE tumor responses to steroid hormones, we examined the expression and localization of ESR1 and progesterone receptor (PR) in tumor cells of PKP53<sup>+/+</sup>, PKP53<sup>H/+</sup>, and PKP53<sup>H/H</sup> mice using in vivo and in vitro approaches. At 8 weeks of age (Fig. 4A and Supplementary Fig. S2), the majority of in vivo ovarian tumor cells positive for KRT8 in the PKP53<sup>+/+</sup> mice were not ESR1 positive, whereas the majority of the KRT8-positive tumor cells in PKP53<sup>H/+</sup> mice exhibited intense ESR1 staining. KRT8-positive tumor cells in the PKP53<sup>H/H</sup> mice exhibited low ESR1 staining. Consistent with these observations, the levels of Esr1 and Pgr mRNA are elevated in the PKP53<sup>H/+</sup> tumor cells compared with those of the other two genotypes, and this difference was statistically different for Esr1 but not Pgr (Fig. 4B). ESR1 protein levels were also analyzed in cell lines derived from OSE cells isolated from ovaries of mice of each genotype. Western blotting confirmed that ESR1 was elevated in cells derived from the PKP53<sup>H/+</sup> mice compared with the other two genotypes. Western blots for PR indicated that the protein levels of the PR-A isoform were higher than...
those of the PR-B isoform in cells of all three genotypes and that PR-A was higher in PKP53+/+ cells compared with the other two (Fig. 4C). When OSE cells were treated with nutlin-3a, a small molecule that stabilizes active TRP53 protein, it increased Esr1 mRNA expression in PKP53+/+ and PKP53H/+ cells, but not in PKP53H/H cells (Fig. 4D), suggesting that TRP53 regulates transcription of Esr1 gene in OSE cells, and the presence of WT and mutant TP53 alleles and exhibits secretory mucinous structures when inoculated into the intraperitoneal cavity of SCID mice. Scale bar, 25 µm. D, mucinous structures in PKP53+/+ mice express MUC5AC, a marker for human mucinous ovarian carcinoma. Adjacent sections without first antibody (Ab) were used as negative controls. Scale bar, 100 µm. E, mucinous structures in PKP53+/+ mice express KRT8 but do not express FOXL2 (nuclear staining) or PAX8 (nuclear staining). Red arrowheads, mucinous structures; red arrows: epithelial structures. Scale bar, 150 µm. F, some cystic structures in the ovary of PKP53+/+ mice contain contiguous regions that are positive for both MUC5AC and KRT8 (red arrowheads) or for KRT8 alone (red arrows; inset is higher magnification of regions in frames). Black arrowhead, ovarian bursa. Scale bar, 200 and 100 µm.

FIGURE 3.
Mucinous epithelial ovarian tumors develop in PKP53H/+ mice. A, at 12 weeks of age, 80% of PKP53H/+ mice develop mucinous structures, and some of them become cystic (top). Right, regions in frame are shown at higher magnification. Red arrows, OSE cells; black arrows, secretory mucinous structures. Scale bar, 900 µm, 180 µm, and 90 µm. B, representative image of H&E stained human mucinous EOC tumor (top) and the corresponding sequencing results of the TP53 gene in laser capture microdissected tumor cells from the same patient. Black arrows, secretory mucinous structures. Scale bar, 200 µm. C, the RMUG-L human cell line, derived from a mucinous ovarian carcinoma, carries heterozygous WT and mutant TP53 alleles and exhibits secretory mucinous structures when inoculated into the intraperitoneal cavity of SCID mice. Scale bar, 900 µm.
Estradiol promotes tumor growth on the ovary and omentum in PKP53$^{H+/+}$ mice

On the basis of the elevated expression of $Esr1$ mRNA and protein in tumors of the PKP53$^{H+/+}$ mice, we next investigated the in vivo response of tumors in these mice to estradiol. Four weeks of estradiol exposure induced rampant growth and local invasion of the primary tumors within the ovarian stroma and proliferation of metastatic cells present on the omentum (Fig. 5A and B). We also tested the effect of estradiol on PKP53$^{H+/+}$ tumor cells by subcutaneously injecting these cells into ovariectomized syngenic hosts (Fig. 5E). Estradiol significantly stimulated subcutaneous tumor growth of the PKP53$^{H+/+}$ tumor cells. Previously, we have demonstrated that progesterone blocks effects of estradiol on tumor growth and metastasis in the PKP53$^{+/+}$ mice (11). However, when progesterone was given one day before estradiol to the PKP53$^{H+/+}$ mice, it did not block estradiol-induced tumor growth either in the primary tumor or at metastatic sites on the omentum (Fig. 5C). We quantified the percentage of pHH3-positive proliferating KRT8-positive tumor cells, further confirming our observations of tumor growth rate with estradiol and progesterone treatment (Fig. 5D). Thus, we conclude that estradiol promotes primary and omental metastatic tumor growth in PKP53$^{H+/+}$ mice, and this effect is not blocked by progesterone in the context analyzed.
Figure 5.

Estradiol promotes tumor growth on the ovary and omentum in PKP53 mice. A, representative images of H&E and KRT8 staining of ovarian tissue sections from PKP53 mice. Right, regions in frames are shown at higher power. Scale bar, 750 and 150 μm. B, immunofluorescent staining of pHH3 and KRT8 on sections of ovaries and omentum from PKP53 mice with or without in vivo estradiol treatment. Yellow dashed lines, surface of the ovary. C, representative images of H&E and KRT8 and pHH3 staining of ovarian tissue sections from PKP53 mice implanted with progesterone (P4) pellets one day before estradiol (E2) pellets. Black arrowheads, transformed OSE cells; dashed lines, surface of the ovary. F, follicles. Scale bar, 100 and 50 μm. D, quantification of the percentage of KRT8+pHH3 cells among KRT8 cells with visible nuclei was performed on representative images taken from different mice of each genotype (n = 4). Data are represented as mean ± SEM. *, P < 0.05. Scale bar, 50 μm. E, estradiol promotes the growth of subcutaneously injected PKP53 OSE cells into syngenic mice (n = 5). *, P < 0.01. D, primary tumors on the ovary (top) and metastatic tumors on the omentum (bottom) showed extensive growth when the progesterone pellet was implanted one day before the estradiol pellet. Black arrowheads, ovarian bursa; dashed lines, surface of the ovary.
Expression of the Trp53R172H-mutant allele alters OSE tumor differentiation and promotes their proliferation and survival.

To obtain a comprehensive understanding of the molecular events occurring in the OSE tumor cells expressing Trp53R172H, we performed microarray analyses on RNA prepared using unpashed OSE cells isolated from PKP53+/+; PKP53H/+, and PKP53H/H mice (Fig. 6A). Heatmap analyses showed that gene expression patterns are quite similar between PKP53+/+ and PKP53H/+ tumor cells, despite the marked differences in the responses of tumor cells to estradiol in these mice. PKP53H/H cells had a markedly distinct gene expression profile compared with the other two genotypes.

To verify the gene expression profiles, real-time qPCR analyses were performed (Fig. 6B). Genes known to be associated with OSE stem cells (Lgr5, Aldh1a1, and Aldh1a7) exhibited significantly reduced levels of mRNA in the PKP53H/H tumor cells. Three epithelial cell differentiation–regulating transcription factors, Lhx9, Amhr2, and Foxa2, were also substantially dysregulated in these cells: Lhx9 was undetectable, Amhr2 was minimal, and Foxa2 was increased in the PKP53H/H tumors. The expression of several secretory factors was altered: Cxcl12 and Bdnf mRNAs increased in tumors of the PKP53H/H mice but not in the PKP53+/+; PKP53H/+ mice, whereas Igfbp6 mRNA was drastically decreased in these cells. Expression of apoptosis and cell survival regulators Ddit4 and Dapk2, among many other apoptosis regulators, was diminished in PKP53H/H cells, whereas cell cycle regulators, such as Ccnb1, were highly expressed in these cells. Finally, several tumorigenic regulators...
in OSE tumors had differentially regulated expression in the PKP53H/+/cells; Ddx39 and Eya4 were significantly increased. We further analyzed cell adhesion and proliferation in the PKP53H/+, PKP53H/-, and PKP53H/0 cells. As shown in Fig. 6C, cell adhesion was reduced in both PKP53H/+ and PKP53H/0 cells, and the PKP53H/0 cells were also significantly more proliferative than cells of the other genotypes.

Trp53R172H mutation alters TRP53 activity in OSE tumors

To determine TRP53 activity in the PKP53H/+, PKP53H/-, and PKP53H/0 cells, nutlin-3a, a small molecule stabilizer and activator of TRP53, was utilized (37). Nutlin-3a stabilized TRP53 protein in cells expressing WT Trp53 as expected (Fig. 7A; ref. 38). Expression of the Trp53R172H stabilized TRP53 protein with or without nutlin-3a treatment in PKP53H/+ and PKP53H/0-mutant cells. Enhanced stabilization of TRP53 protein was also demonstrated by TRP53 immunofluorescent staining (Fig. 7B). Despite the high levels of TRP53 protein, the activity of TRP53 signaling pathway was reduced and negligible, respectively, in PKP53H/+ and PKP53H/0, mutant cells, as measured by a luciferase assay containing an inducible p53 reporter, either with or without nutlin-3a treatment (Fig. 7C).

In addition, we analyzed basal and nutlin-3a-induced expression or repression of known TRP53 target genes in mutant cells of each genotype (Fig. 7D). Basal levels of Cdkn1a (P21) and Mdm2 were reduced in the PKP53H/+ and PKP53H/0-mutant cells compared with the PKP53H/+ cells. However, Cdkn1a, Mdm2, Btg2, and Bc3 (Puma) were highly induced by nutlin-3a in both the PKP53H/+ and PKP53H/0 cells, but not in the PKP53H/0-mutant cells, indicating that the presence of one mutant allele is not sufficient to suppress the activity of the WT allele. Likewise, expression of genes suppressed by TRP53, such as Birc5 and Brca1, was decreased by nutlin-3a treatment in the PKP53H/+ and PKP53H/0 cells, but not in the PKP53H/0-mutant cells. Notably, the expression of Cdkn2a (P16) was highly expressed in the PKP53H/0-mutant cells, as we have observed in Pten/Kras–mutant cells that are null for Trp53 (21). Interestingly, despite retained transactivation activity of the WT TRP53 in the PKP53H/0 cells, nutlin-3a treatment effectively caused death of PKP53H/+ cells in a dose-dependent manner, but this effect was abolished in PKP53H/+ cells (Fig. 7E), reflecting potential gain-of-function effect of the R172H allele in promoting tumor survival.

Discussion

Trp53R172H mutation impacts ovarian cancer cell initiation and progression

The R175H mutation in the human TP53 gene is one of the most frequently occurring mutations in human EOC and, based on recent analyses, is frequently expressed with one WT allele (10). These data raise a novel and critical question about the functional activity and impact of the mutant p53 allele in the presence or absence of the WT allele (11). Indeed, we demonstrate herein that tumors in the PKP53H/+-mutant mice exhibit some similarities but also some marked differences from tumors in the PKP53H+/- mice. Like the primary tumors in the PKP53H/- mice, those in the PKP53H/+-mutant mice develop extensive papillary-like structures on the ovarian surface that stain positively for KRT8 before 8 weeks of age. However, PKP53H/+-mutant tumors (i) develop faster and are more metastatic, (ii) present mucinous epithelial tumors between 8 and 12 weeks of age; (iii) express higher levels of ESR1 mRNA and protein, and (iv) are more responsive in vivo to estradiol than the PKP53H/- cells.

In line with the tumor-promoting effects of the Trp53R172H allele in our mouse model of EOC, homozygous PKP53H/0 OSE cells exhibit early lesion onset and undergo more advanced papillary transformation and prominent metastasis onto the omentum as early as 4 weeks of age (39). When we injected PKP53H/0 tumor OSE cells into the ovaries of syngenic host mice, they grew rapidly in the ovary and intraperitoneal cavity and invaded the pancreas. Thus, homozygous mutant alleles exert a potent effect on the initiation, early progression, and metastasis of ovarian tumor in the same genetic background. Gene profiles of the PKP53H/0 EOC cells further document that these cells are markedly distinct from those in the PKP53H/- or PKP53H/0 mice, as discussed below.

Trp53R172H mutation alters epithelial ovarian tumor differentiation

Particularly striking, and of high clinical relevance, was the emergence of KRT8-positive mucinous-like tumors in the PKP53H/0 mice that invade the ovary and present a variety of differentiation stages by 8 to 12 weeks of age. Thus, the Trp53R172H-mutant allele promotes, in a subset of cells, a phenotype that is distinct from the serious, papillary-like structures that develop earlier. To our knowledge, the PKP53H/0 mice may potentially provide the first mouse model of mucinous EOC subtype arising from OSE cells, a site far removed from the presumed cervical/gut origin of mucinous tumors in women (3). Among human patients, the mucinous subtype is considered relatively uncommon but often has worse outcomes and is more resistant to conventional chemotherapy (36, 40). Tumor development in the PKP53H/0 mice resembles features of human mucinous ovarian cancer in several aspects: (i) mutation in KRAS is a notable feature of the human ovarian mucinous subtype (in ~50% cases; refs. 10, 31, 41–43), (ii) concurrence of KRAS and TP53 mutations occurs in human mucinous ovarian cancer samples (36%; refs. 10, 31), (iii) a significant number of human mucinous tumors are heterozygous for mutant TP53 allele and a WT allele (Fig 3B and Supplementary Table S1; ref. 10), and (iv) mutations in the PI3K/PTEN pathway are also a prominent feature of human mucinous ovarian cancer (10). That OSE cells may have the potential to transdifferentiate into mucinous-like, secretory tumor cells highlights (i) the plasticity of these ovarian surface epithelial cells and (ii) the importance of the interactions among p53 status, cell-of-origin, and the ovarian microenvironment in determining ovarian tumor cell fate decisions.

TRP53 and steroid hormone receptor status impact tumor prognosis and responsiveness to steroid hormones

We show that tumors in the PKP53H/0 mice express elevated levels of ESR1 in vivo and in vitro and undergo marked proliferation in the primary tumor and at metastatic sites on the omentum in response to estradiol treatment in vivo. Although PGR (A and B) are present in these cells, progesterone treatment prior to estradiol does not block estradiol-induced tumor growth. These results differ from those described previously.
in the same mutant background (11), where estradiol potently stimulated proliferation and metastasis of Trp53-null PKP53/C0/C0 tumors, and these responses to estradiol were completely blocked by progesterone treatment. According to these observations, TP53 status and steroid hormone receptor status could be considered together for predicting tumor prognosis and responsiveness to steroid hormones in ovarian cancer patients.

Heterozygous state with coexpression of both WT and Trp53R172H mutation represents a distinct tumor phenotype

A number of reports indicate that the Trp53R172H mutation exerts dominant negative (DN) effects on the WT Trp53 allele (44, 45). However, our data indicate that this mutant has other effects on the WT allele in the PKP53/C0/C0 tumor cells that may be context specific. First, as mentioned above, tumor

**Figure 7.**

Mutant Trp53R172H alters TRP53 activity in OSE tumors. A, representative Western blot analyses of TRP53 and AKT in OSE cells of different genotypes treated with DMSO or nutlin-3a (10 μmol/L). B, representative immunofluorescent staining of TRP53 in cultured OSE cells. Scale bar, 25 μm. C, relative luciferase activity of a p53 reporter construct. Data are representative of experiments repeated four times, and in each experiment, luciferase activity was measured in triplicates. Bars without common superscript are significantly different. *, P < 0.05. D, expression of the Trp53R172H-mutant allele alters mRNA levels of both TRP53-induced (top) and -suppressed genes (bottom) in response to nutlin-3a treatment (n = 3). E, cell viability in response to nutlin-3a treatment (72 hours) as measured by the WST-1 assay. This experiment was repeated three times, and each experiment contains three replicates for each genotype. Data are represented as mean ± SEM.
morphology appeared to be quite similar between PKP53<sup>H/-</sup> mice and PKP53<sup>+/-</sup> mice, at least until later stages of tumor development when mucinous tumors arise in the PKP53<sup>H/-</sup> mice. Second, whereas microarray gene expression profiles are similar (but not identical) between OSE cells of these two genotypes, they are dramatically distinct from the gene expression profiles in the PKP53<sup>H/H</sup> cells. Third, in the presence of the R172H allele, p53 signaling seems to be active based on both luciferase TRP53 activity reporter assays and induction of P53 target genes upon nutlin-3a treatment. Collectively, our data and other studies provide evidence that it is possible that some ovarian cancer patients with heterozygous mutant TP53 expression will respond to therapeutic interventions, which activate WT p53 signaling (11, 46).

The vast number of genes that exhibit increased and decreased expression in the PKP53<sup>H/H</sup> tumor cells compared with PKP53<sup>H/-</sup> and PKP53<sup>+/-</sup> cells suggest that the homozygous mutants exert profound changes in the molecular events controlling these cells, some of which may mediate GOF mechanisms to ensure tumor survival in the absence of WT TRP53. The uniqueness of the gene profiles in the PKP53<sup>H/H</sup> tumor cells is further supported by the fact that they are also quite distinct from those in the p53-null cells of the Pten/Kras background (21). One example is Eya4, the overexpression of which has been reported in many different cancers (47–50). In comparison with cells expressing WT Trp53, the level of Eya4 mRNA is dramatically elevated in PKP53<sup>H/H</sup> cells, but reduced in PKP53-null OSE cells (21). Eya4 and other differentially regulated genes in OSE cells expressing homozygous R172H-mutant alleles versus null Trp53 alleles may explain the distinctly different behaviors of these two OSE tumors. In particular, whereas epithelial ovarian tumors in the PKP53<sup>H/H</sup> homozygous mice exhibit early onset and metastasis, epithelial ovarian tumors in the PKP53<sup>+/-</sup> homozygous mice exhibit slow growth and minimal transformation (21). Thus, the status of p53 in tumor cells impacts tumor cell behavior, growth morphology, and metastatic potential.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

**References**


Mutant p53 Promotes Epithelial Ovarian Cancer by Regulating Tumor Differentiation, Metastasis, and Responsiveness to Steroid Hormones


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