

A Role for BRCA1 in Uterine Leiomyosarcoma

Deyin Xing,^{1,2} George Scangas,^{1,2} Mai Nitta,^{1,2} Lei He,^{1,2} Xuan Xu,⁴ Yevgeniya J.M. Ioffe,⁴ Paul-Joseph Aspuria,⁴ Cyrus Y. Hedvat,⁵ Matthew L. Anderson,⁶ Esther Oliva,^{2,3} Beth Y. Karlan,⁴ Gayatry Mohapatra,^{1,2,3} and Sandra Orsulic^{1,2,3,4}

¹Molecular Pathology Unit and Center for Cancer Research, Massachusetts General Hospital, Charlestown, Massachusetts; ²Department of Pathology, Massachusetts General Hospital and ³Department of Pathology, Harvard Medical School, Boston, Massachusetts; ⁴Women's Cancer Research Institute, Cedars-Sinai Medical Center, Los Angeles, California; ⁵Department of Pathology, Memorial Sloan-Kettering Cancer Center, New York, New York; and ⁶Division of Gynecologic Oncology, Baylor College of Medicine, Houston, Texas

Abstract

Uterine leiomyosarcoma (ULMS) is a rare gynecologic malignancy with a low survival rate. Currently, there is no effective treatment for ULMS. Infrequent occurrences of human ULMS hamper the understanding of the initiation and progression of the disease, thereby limiting the ability to develop efficient therapies. To elucidate the roles of the *p53* and *BRCA1* tumor suppressor genes in gynecologic malignancies, we generated mice in which *p53* and/or *BRCA1* can be conditionally deleted using anti-Müllerian hormone type II receptor (*Amhr2*)-driven Cre recombinase. We showed that conditional deletion of *p53* in mice results in the development of uterine tumors that resemble human ULMS and that concurrent deletion of *p53* and *BRCA1* significantly accelerates the progression of these tumors. This finding led to our hypothesis that *BRCA1* may play a role in human ULMS development. Consistent with this hypothesis, we showed that the *BRCA1* protein is absent in 29% of human ULMS and that *BRCA1* promoter methylation is the likely mechanism of *BRCA1* downregulation. These data indicate that the loss of *BRCA1* function may be an important step in the progression of ULMS. Our findings provide a rationale for investigating therapies that target *BRCA1* deficiency in ULMS. [Cancer Res 2009;69(21):8231–5]

Introduction

Although uterine leiomyosarcoma (ULMS) is a rare tumor that accounts for <1% of all uterine malignancies, >80% of patients with ULMS that has spread beyond the uterus experience tumor recurrence after initial chemotherapy (1). The etiology associated with the carcinogenesis of ULMS is largely unknown. Frequently observed mutations and overexpression of *p53* in ULMS suggest that the loss of *p53* function may play a critical role in the development of this cancer (2–4). Mice without a functional *p53* tumor suppressor gene or with mutant *p53* gain-of-function develop a spectrum of tumors. However, leiomyosarcomas that reproduce corresponding human malignancies with the same cellular origin rarely occur.

Several transgenic mouse models have been reported to give rise to leiomyosarcoma. In one mouse model, Cre-dependent activation

and expression of an actin-cassette transgene encoding the T antigens of the SV40 early region resulted in the development of massive ULMS in all female mice at ~3 months of age (5). The second model was based on mouse mammary tumor virus (MMTV) promoter overexpression of *Cripto-1*. In addition to the development of mammary tumors, ULMS developed in ~20% of aged mice (6). Similarly, mammary tumors and ULMS arose in v-Ha-ras transgenic mice driven by the MMTV promoter (7). Disruption of *Pten* in the smooth muscle lineage with *Tag1n-Cre* caused the formation of widespread smooth muscle cell hyperplasia and abdominal leiomyosarcoma but not ULMS (8).

Materials and Methods

Mouse strains. *Amhr2*^{Cre/+} mice (9) were crossed with *Brcal*^{lox/lox} (10) or *p53*^{lox/lox} (11) mice. Triple transgenic *Amhr2*^{Cre/+}/*p53*^{lox/lox}/*Brcal*^{lox/lox} mice were generated by crossing *Amhr2*^{Cre/+}/*Brcal*^{lox/lox} and *Amhr2*^{Cre/+}/*p53*^{lox/lox} mice. The resulting transgenic mice were maintained on a mixed background. All mice were genotyped by PCR using tail or ear DNA. Kaplan-Meier survival curves were drawn using GraphPad PRISM software. Mean survival time was calculated using the Log-rank test.

Confirmation of gene recombination. Genomic DNA extracted from tumors or normal tissues of the female reproductive tract was used to detect Cre-mediated recombination of the *p53* and *Brcal* genes. Cre-mediated deletion of *p53* displayed a 612-bp PCR product amplified with primers *p53*-a (5'-CAC AAA AAC AGG TTA AAC CCA-3') and *p53*-c (5'-GAA GAC AGA AAA GGG GAG GG-3'). PCR amplification of the recombined *Brcal* gene resulted in a 621-bp product using the primers *Brcal*-d (5'-CTG GGT AGT TTG TAA GCA TCC-3') and *Brcal*-g (5'-CTG CGA GCA GTC TTC AGA AAG-3'), which flanked *Brcal* exon 11. The presence of wild-type *Brcal* was determined by PCR using primers within exon 11 (*Brcal*-e: 5'-ATC AGT AGT AGA AAT CCA AGC CCA CC-3'; *Brcal*-f: 5'-TGC CAC TCC CAG CAT TGT TAG-3').

Human specimens. Formalin-fixed paraffin-embedded archival human specimens were obtained from the following institutions: Massachusetts General Hospital, Boston, MA; Baylor College of Medicine, Houston, TX; Memorial Sloan-Kettering Cancer Center, New York, NY; Cedars-Sinai Medical Center, Los Angeles, CA; Olive View Medical Center, Los Angeles, CA; Inova Fairfax Hospital, Falls Church, VA; Università Cattolica, Rome, Italy; and Istituto di Anatomia e Istologia Patologica, Ancona, Italy.

H&E staining and immunohistochemistry. Tissues were fixed in 10% buffered formalin and embedded in paraffin. Tissue sections were deparaffinized in a graded xylene/ethanol series and used for H&E staining or immunohistochemistry (IHC) with an ABC antibody staining kit (Vector Laboratories) according to the manufacturer's instructions. After color development, the slides were counterstained with hematoxylin and mounted with mounting medium (Permount, Fisher Sciences). To determine the proliferative index of the tumors, mice were i.p. injected with 100 mg/kg bromodeoxyuridine (BrdUrd; Zymed Laboratories). Tissues and tumors were collected after 2 h and fixed in 10% formalin overnight. Paraffin-embedded sections were deparaffinized, followed by hydrogen chloride

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Requests for reprints: Sandra Orsulic, Women's Cancer Research Institute, Cedars-Sinai Medical Center, 8635 West 3rd Street, Suite 290W, Los Angeles, CA 90048. Phone: 310-423-9546; Fax: 310-423-9537; E-mail: orsulics@cshs.org.

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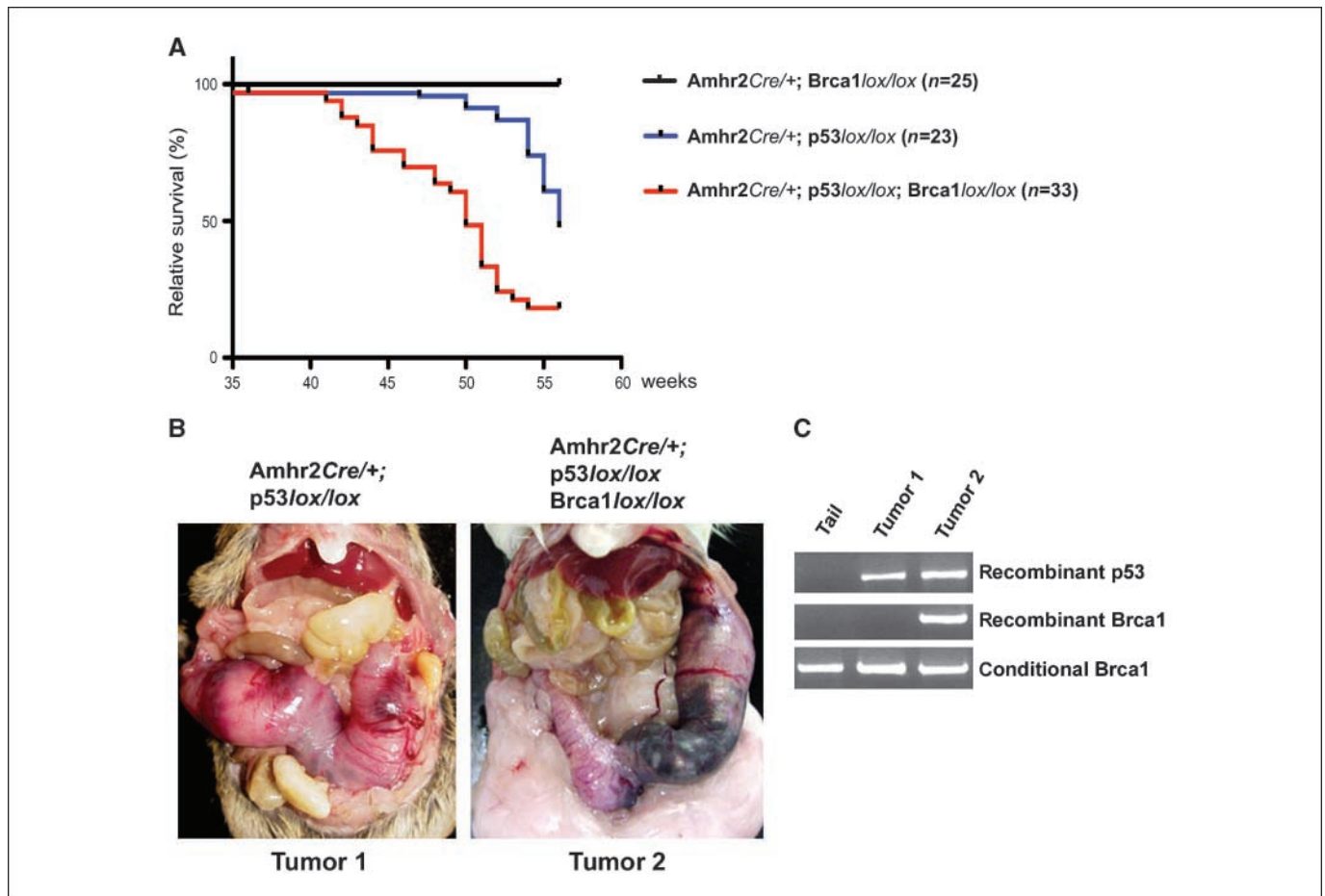


Figure 1. Characterization of uterine tumors in mice with conditional deletion of *p53* and/or *Brca1* using *Amhr2*-driven Cre recombinase. **A**, Kaplan-Meier survival curves for *Amhr2*^{Cre/+}/*Brca1*^{lox/lox}, *Amhr2*^{Cre/+}/*p53*^{lox/lox}, and *Amhr2*^{Cre/+}/*p53*^{lox/lox}/*Brca1*^{lox/lox} mice. **B**, uterine tumors in *Amhr2*^{Cre/+}/*p53*^{lox/lox} (*tumor 1*) and *Amhr2*^{Cre/+}/*p53*^{lox/lox}/*Brca1*^{lox/lox} mice (*tumor 2*). **C**, detection of Cre-mediated recombination of *p53* in tumors dissected from an *Amhr2*^{Cre/+}/*p53*^{lox/lox} mouse (*tumor 1*) and double recombination of *p53* and *Brca1* in a tumor dissected from an *Amhr2*^{Cre/+}/*p53*^{lox/lox}/*Brca1*^{lox/lox} mouse (*tumor 2*). Tail tissue from the *Amhr2*^{Cre/+}/*p53*^{lox/lox}/*Brca1*^{lox/lox} mouse was used as a control.

(2N HCl) digestion, trypsinization (0.1% Trypsin), and IHC with an ABC antibody staining kit. The following primary antibodies were used: α -smooth muscle actin (1:200 dilution, Sigma), β -catenin (H-102; 1:100 dilution, Santa Cruz), BRCA1 (Ab-1; 1:100 dilution, Calbiochem); BrdUrd (1:100 dilution, Vector Laboratories), p16 (M-156; 1:100 dilution, Santa Cruz), p53 (Ab-1; 1:100 dilution, Calbiochem), phospho-estrogen receptor α (Ser167; 1:100 dilution, Cell Signaling), and TROMA-1 (keratin 8; 1:25 dilution, Developmental Studies Hybridoma Bank at the University of Iowa). H&E and immuno-stained uterine tumor sections were reviewed by two independent observers (D.X. and E.O.).

BRCA1 promoter methylation analysis. The methylation status of ULMS specimens was determined using the EZ DNA Methylation Gold kit (Zymo Research) following manufacturer's instructions. Ovarian cancer tissues with known *BRCA1* methylation status (12) were used as a control. Bisulfite-modified DNA PCR amplification and primers have been previously described (12).

Results

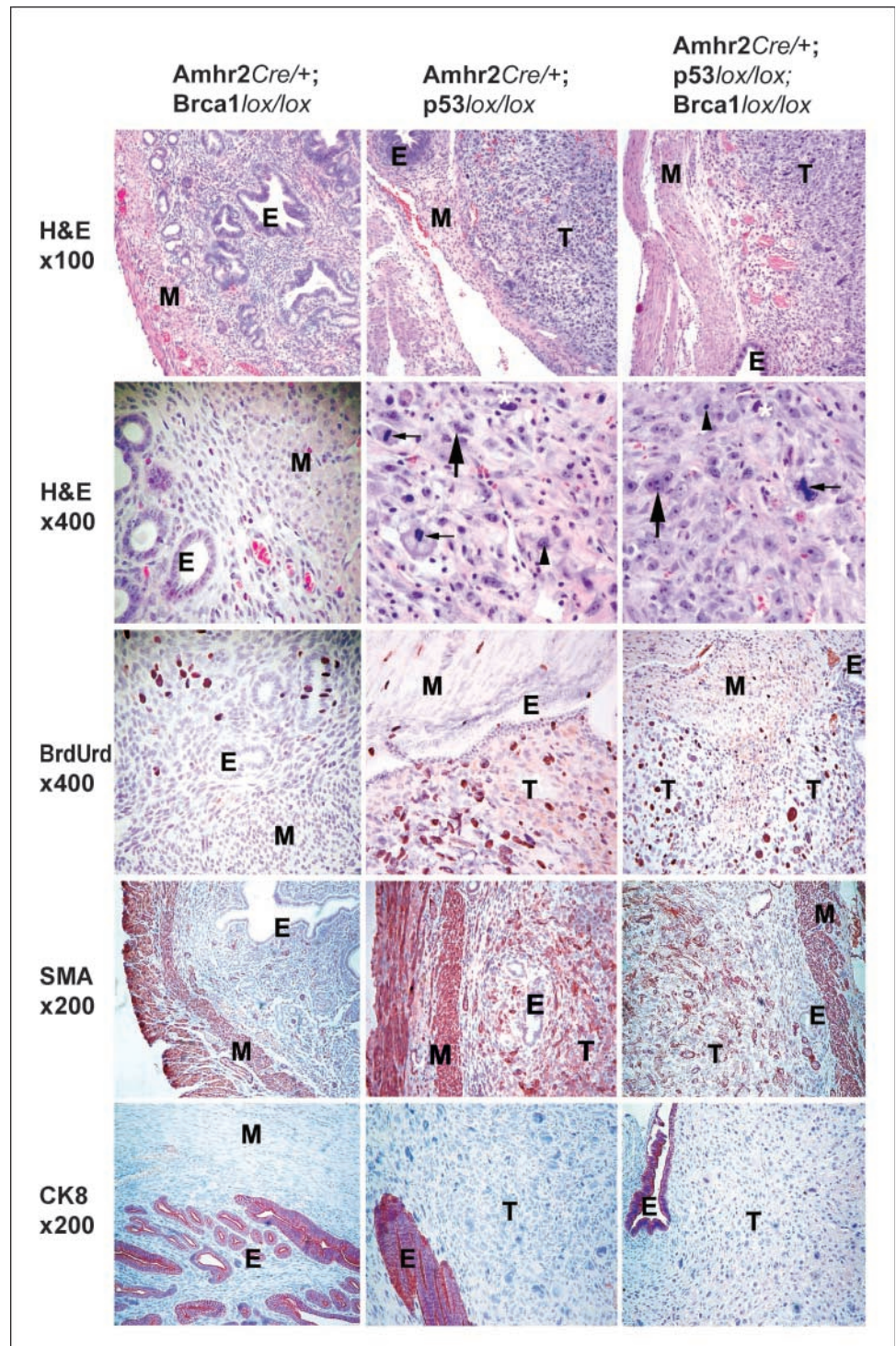
Conditional deletion of *p53* and *Brca1* in the female mouse reproductive tract. To define the roles of the *p53* and *Brca1* tumor suppressor genes in oncogenesis of the female mouse reproductive tract, we generated mice in which *p53* and *Brca1* can be conditionally deleted using Cre recombinase knocked into the anti-Müllerian hormone type II receptor (*Amhr2*) locus (*Amhr2*-

Cre; Supplementary Fig. S1A; ref. 9). Three individual strains of mice, *Amhr2*^{Cre/+}/*p53*^{lox/lox}, *Amhr2*^{Cre/+}/*Brca1*^{lox/lox}, and *Amhr2*^{Cre/+}/*p53*^{lox/lox}/*Brca1*^{lox/lox}, were generated (Supplementary Fig. S1B). *p53* and/or *Brca1* in these mice are expected to be inactivated by Cre recombinase in the *Amhr2*-expressing tissues, which include Müllerian duct mesenchymal cells, coelomic epithelium, and granulosa cells of the adult ovary (13). PCR of genomic DNA extracted from normal tissues of the female reproductive tract (ovary, oviduct, and uterus) was used to detect Cre-mediated recombination of the *p53* (deleted exons 2–10) and *Brca1* (deleted exon 11) genes. One 3-month-old female mouse from each genotype was selected for PCR analysis. As expected, Müllerian duct organs from *Amhr2*^{Cre/+}/*p53*^{lox/lox} mice harbored recombinant *p53* but not recombinant *Brca1*, whereas Müllerian duct organs from *Amhr2*^{Cre/+}/*Brca1*^{lox/lox} mice harbored recombinant *Brca1* but not recombinant *p53* (Supplementary Fig. S1B). Recombinant products for both *Brca1* and *p53* were detected in the ovaries, fallopian tubes, and uteri of *Amhr2*^{Cre/+}/*p53*^{lox/lox}/*Brca1*^{lox/lox} mice (Supplementary Fig. S1B). Primers within *Brca1* exon 11 (Supplementary Fig. S1A) were used to detect the presence of conditional *Brca1* in various non-*Amhr2*-expressing cell types in the ovary, oviduct, and uterus (Supplementary Fig. S1B).

Loss of *p53* and *Brcal* in the female mouse mesenchyme of the reproductive tract leads to the development of ULMS. Mice with deleted *p53*, *Brcal*, or both in the Müllerian duct tissues developed normally and histopathologic analyses did not reveal any specific anomalies in the Müllerian duct tissues or other organs of 3-month-old mice. However, uterine tumors developed in 12 of 23 (52%) of the *Amhr2^{Cre/+}/p53^{lox/lox}* female mice during the 13-month observation period. None of the 25 *Amhr2^{Cre/+}/*

Brcal^{lox/lox} female mice developed uterine masses during the same time period (Supplementary Table S1; Fig. 1A). However, the loss of *Brcal* synergistically accelerated the formation of tumors in mice lacking *p53*, with 27 of 33 (82%) of the *Amhr2^{Cre/+}/p53^{lox/lox}/Brcal^{lox/lox}* female mice developing uterine masses within 13 months (Supplementary Table S1; Fig. 1A). The median time of tumor-free survival was 56 weeks for *Amhr2^{Cre/+}/p53^{lox/lox}* mice and 50 weeks for *Amhr2^{Cre/+}/p53^{lox/lox}/Brcal^{lox/lox}* mice. Conditional

Figure 2. Immunohistochemical analysis of normal uteri derived from *Amhr2^{Cre/+}/Brcal1^{lox/lox}* mice and uterine tumors derived from *Amhr2^{Cre/+}/p53^{lox/lox}* and *Amhr2^{Cre/+}/p53^{lox/lox}/Brcal1^{lox/lox}* mice. Representative H&E staining ($\times 100$ and $\times 400$ magnification). Incorporation of BrdUrd indicates a high proliferation index. The IHC profile shows that the myometrium of the uterus and the uterine tumors are positive for smooth muscle actin (SMA) but negative for the epithelial marker Keratin 8 (CK8). T, tumor; E, endometrium; M, myometrium; small arrow, abundant mitoses; large arrow, marked cytologic atypia; arrowhead, prominent nucleoli; *, hyperchromatic nuclei.



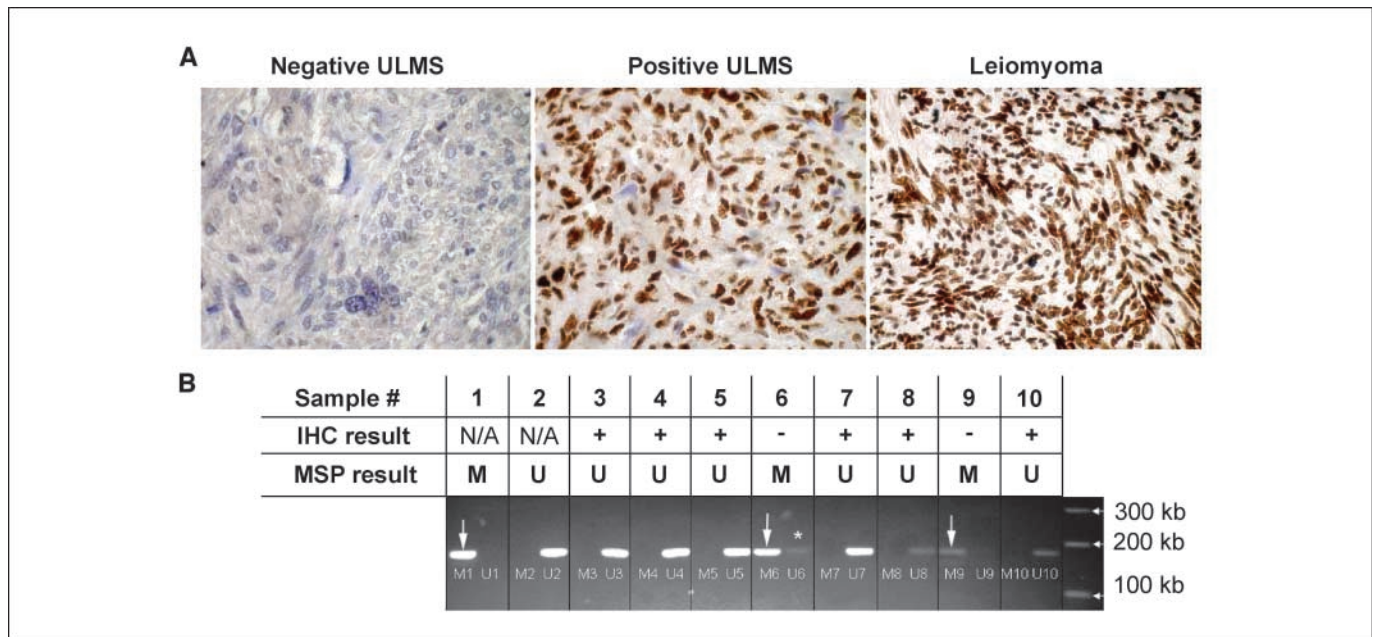


Figure 3. BRCA1 IHC and methylation status. *A*, representative immunohistochemical staining in BRCA1-negative ULMS, BRCA1-positive ULMS, and benign leiomyoma. *B*, methylation-specific PCR analysis of two BRCA1-negative (–) and six BRCA1-positive (+) samples. *Sample 1*, positive control for methylated BRCA1 promoter (ovary tumor in which methylation of the BRCA1 promoter has been previously shown); *sample 2*, negative control for methylated BRCA1 promoter (normal male DNA); *samples 3 to 10*, primary human ULMS samples in which the presence (+) or absence (–) of the BRCA1 protein has been determined by IHC. The IHC results are compared with the BRCA1 methylation status determined by methylation-specific PCR (MSP). *M*, methylated product; *U*, unmethylated product. Both methylated and unmethylated product size, 182 bp. *, an unmethylated product that is probably derived from stromal cells and connective tissues within the tumor.

deletion of *p53* and *Brcal* significantly accelerated tumor development compared with inactivation of *p53* alone (Log-rank test = 13.12; $P = 0.0003$). At gross examination, the uterine tumors in $Amhr2^{Cre/+}/p53^{lox/lox}$ and $Amhr2^{Cre/+}/p53^{lox/lox}/Brcal^{lox/lox}$ mice looked similar, although the tumors in the $Amhr2^{Cre/+}/p53^{lox/lox}/Brcal^{lox/lox}$ mice were typically associated with more hemorrhagic necrosis (Fig. 1*B*). PCR was used to confirm the presence of recombinant *p53* in tumors dissected from three $Amhr2^{Cre/+}/p53^{lox/lox}$ mice as well as the presence of recombinant *Brcal* and *p53* in tumors dissected from four $Amhr2^{Cre/+}/p53^{lox/lox}/Brcal^{lox/lox}$ mice. Representative PCR results for one tumor from each genotype are shown in Fig. 1*C*.

Histopathologic analysis showed that all of the tumors were ULMS, which were characterized by spindle-shaped cells with hyperchromatic nuclei, prominent nucleoli, abundant mitoses, and marked cytologic atypia (Fig. 2). Immunohistochemical analysis (Supplementary Table S2) revealed additional characteristics that are consistent with ULMS. BrdUrd staining indicated a high proliferation index (Fig. 2). The smooth muscle cell origin of the tumors was confirmed by the expression of smooth muscle actin and the absence of the epithelial marker Keratin 8 (Fig. 2). Several other characteristics of human ULMS, such as ER α positivity (14), nuclear localization of β -catenin (6, 8), and overexpression of cyclin-dependent kinase inhibitor p16 (4, 15, 16), were also present in mouse ULMS (Supplementary Table S2 and Fig. S2), indicating that mouse and human ULMS may arise through similar molecular pathways.

BRCA1 expression is downregulated in human ULMS. Our mouse model of ULMS indicates that BRCA1 may play a role in human ULMS carcinogenesis. This finding led to our hypothesis that BRCA1 expression may be altered in human ULMS. To address this question, we evaluated p53 and BRCA1 protein expression in a cohort of 85 ULMS and 76 benign uterine leiomyoma

tissue specimens organized in a tissue microarray. The slides were stained with antibodies against BRCA1 and p53 using the avidin-biotin immunoperoxidase method. Nuclear positivity was scored by two independent observers and quantified as either present or absent. Results were analyzed using a two-tailed Fisher's exact test. BRCA1 protein expression was absent in 29% (25 of 85) of ULMS samples and in 4% (3 of 76) of benign leiomyoma samples. Representative results of BRCA1 immunohistochemical detection are shown in Fig. 3*A*. This difference in BRCA1 protein expression between ULMS and benign leiomyoma samples was statistically significant with a P value of <0.0001 . Consistent with previous reports (17, 18), we found that p53 positivity was present in 50% (30 of 60) of ULMS and 0% (0 of 28) of benign leiomyomas (data not shown). There was no significant correlation between BRCA1 and p53 staining in ULMS, suggesting that the loss of BRCA1 in ULMS may collaborate with pathways other than the p53 pathway.

To identify a possible mechanism of BRCA1 protein downregulation in human ULMS, we selected two BRCA1-negative and six BRCA1-positive ULMS samples for which we had sufficient material to determine the *BRCA1* methylation status using bisulfite-modified DNA PCR amplification (Fig. 3*B*). One ovarian cancer sample in which methylation of the *BRCA1* promoter was previously confirmed (12) was used as a positive control, whereas normal male DNA was used as a negative control (Fig. 3*B*). *BRCA1* promoter methylation was present in both samples that were BRCA1 negative as determined by IHC and not present in the six samples that were BRCA1 positive as determined by IHC (Fig. 3*B*).

Discussion

The understanding of the molecular biology of ULMS is poor due to rare occurrences of human ULMS and the lack of molecularly

defined animal models. Therefore, there is a great need to generate genetically engineered mouse models that resemble the development of human ULMS. We investigated the role of p53 and Brca1 in the development and tumorigenesis of the female mouse reproductive tract based on a Cre/LoxP process in which the expression of Cre recombinase is under the control of the *Amhr2* locus. Mice with *p53* deletion in *Amhr2*-Cre-expressing tissues developed ULMS, indicating that p53 may play a causative role in the formation of ULMS. In contrast, mice lacking functional *Brca1* driven by *Amhr2*-Cre did not present any visible phenotype during the 13-month observation period. This result is consistent with the view that *Brca1* plays a general role in the maintenance of genomic integrity and that a long latency is required for the activation of oncogenes and the inactivation of additional tumor suppressor genes to form *Brca1*-associated tumors (19, 20). Therefore, we cannot rule out the possibility that *Brca1*-deficient mice could develop gynecologic tumors after 13 months. Unlike human ULMS, which are highly metastatic, metastasis of mouse ULMS to other organs was not identified at the time of tumor extraction, although it is unknown whether these tumors would metastasize after 13 months.

Germline *BRCA1* mutations have not been associated with a predisposition to human ULMS development, indicating that genomic alterations of *BRCA1* are unlikely to play a role in the development of this disease. It is possible, however, that genetic or epigenetic somatic inactivation of *BRCA1* contributes to the progression of ULMS. Our IHC results on patient samples indicate a significant

difference in *BRCA1* protein expression between ULMS and benign uterine leiomyoma. Consistent with the view that *BRCA1* silencing may play a role in the development or progression of ULMS, we showed that the *BRCA1* promoter is methylated in samples with negative *BRCA1* immunohistochemical staining. Together, our findings provide a rationale for the investigation of targeted therapies that take advantage of the absence of *BRCA1* expression in a subset of ULMS patients.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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