A Mouse Model for the Molecular Characterization of Brca1-Associated Ovarian Carcinoma

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Abstract

Little is known about the mechanisms that underlie Brca1-associated ovarian tumorigenesis, mainly due to the lack of an appropriate experimental model. We developed genetically defined primary mouse ovarian surface epithelial (OSE) cell lines in which the loss of functional Brca1 and p53 recapitulates the events that are thought to occur in early ovarian cancer development in patients with Brca1 mutations. This system allows for the introduction of additional oncogenes that are thought to cooperate with the loss of Brca1 and p53 to induce tumorigenesis. We showed that Myc is sufficient to induce transformation of ovarian cells that are deficient for both Brca1 and p53 but not sufficient for the transformation of cells that are deficient for either Brca1 or p53. The transformed Brca1-deficient OSE cells display an increased number of centrosomes, acquire complex chromosome aberrations, and lack Rad51 nuclear foci in the presence of DNA-damaging agents, such as mitomycin C and cisplatin. Immunocompetent mice injected with transformed OSE cells develop tumors that resemble human metastatic serous ovarian carcinoma, the most common type of ovarian cancer in women. Consistent with the reported platinum chemosensitivity in patients with Brca1-associated ovarian cancer, the Brca1-deficient OSE cells have increased sensitivity to the DNA-damaging agent cisplatin, whereas sensitivity to the microtubule poison paclitaxel is similar between Brca1 wild-type and Brca1-deficient cells. The Brca1 wild-type and Brca1-deficient mouse ovarian tumors and cell lines provide a new experimental system for the evaluation of therapies that target the Brca1 pathway. (Cancer Res 2006; 66(18): 8949-53)

Introduction

The majority of hereditary ovarian cancers are of a serous type and can be attributed to germ-line mutations in the Brca1 tumor suppressor gene. Hereditary and sporadic ovarian cancers are similar in many respects; however, patients with hereditary mutations in Brca1 develop ovarian cancers earlier than patients with sporadic cancers (1), suggesting that the loss of the Brca1 function lowers the threshold for oncogenic transformation. The loss of heterozygosity for Brca1 and the inactivation of the p53 function seem to be the early events in the induction of hereditary ovarian cancer (2). Consistent with this, familial ovarian cancers have a significantly higher frequency of p53 mutations than sporadic cancers (3), indicating that the loss of p53 function is required for a cell to tolerate the loss of the Brca1 function (4). However, little is known about the requirement for additional genetic alterations that collaborate with Brca1 and p53 in the transformation of ovarian surface epithelial (OSE) cells. Considering the distinct clinical, histopathologic, and molecular aspects of Brca1-associated ovarian cancers, there is a great need to generate ovarian cancer mouse models for the comparison of Brca1 wild-type and Brca1-deficient ovarian tumors.

Materials and Methods

Mouse strains. K5-TVA mice (5) were crossed with Brca1\textsuperscript{lox/lox} (6), p53\textsuperscript{lox/lox} (7), or p53\textsuperscript{lox/-} (8) mice. Triple transgenic K5-TVA; Brca1\textsuperscript{lox/lox}, p53\textsuperscript{lox/lox} mice were generated by crossing K5-TVA; Brca1\textsuperscript{lox/lox} and K5-TVA; p53\textsuperscript{lox/lox} mice.

Retroviral constructs and generation of genetically defined ovarian cancer cell lines. Replication-competent avian leukemia virus long terminal repeat with splice acceptor (RCAS) retroviral constructs and viral infection of mouse ovarian explants have been described (5, 9). For the generation of C1, C11, C2, C22, and C3 cell lines, ovarian explants from K5-TVA; p53\textsuperscript{lox/-} mice (5) were infected with different combinations of RCAS viruses carrying human Myc, mouse K-ras\textsuperscript{G12D}, and mouse myristoylated Akt1 oncogenes: C1 and C11 (Myc plus K-ras), C2 and C22 (Akt plus Myc), and C3 (Akt plus K-ras). The BR2, BR5, and BR6 cell lines were generated by RCAS-Cre and RCAS-Myc infection of ovarian explants from mice with K5-TVA; Brca1\textsuperscript{lox/lox}, p53\textsuperscript{lox/lox} genetic background. Tumors from nude mice injected with the Brca1 wild-type cell lines C1, C11, C2, C22, and C3 were used to generate T1, T11, T2, T22, and T3 tumor cell lines, whereas tumors from nude mice injected with the Brca1-deficient cell lines BR2, BR5, and BR6 were used to generate the TBR2, TBR5, and TBR6 tumor cell lines.

Confirmation of gene recombination and expression. Genomic DNA extracted from different cell lines was used to detect the Cre-mediated recombination of the p53 and Brca1 genes. The primers used for the PCR and reverse transcription-PCR (RT-PCR) analyses are described in Supplementary Materials and Methods.

Tumor production and immunodetection of proteins. Tumor production, Western blotting, immunohistochemistry, and immunofluorescence were done as described previously (9) The antibodies used for immunodetection of proteins are described in Supplementary Materials and Methods.

Cell treatment with mitomycin C, cisplatin, and paclitaxel. Equal numbers of cells were plated into six-well dishes in DMEM/F12 with 10% FCS. After 1 day of growth in culture, the medium was replaced with a medium that contained 1μg/ml mitomycin C (MMC; Roche, Indianapolis, IN) or gradually increasing concentrations of cisplatin (cis-diaminedichloroplatinum, Calbiochem, Darmstadt, Germany) or paclitaxel (Baccatin III N-Benzy1-N-(phenylsuximide ester, LC Laboratories, Woburn, MA). The cells were treated with MMC overnight or with cisplatin or paclitaxel for 8 days. Cell numbers were determined using a counting chamber (Hauser Scientific, Horsham, PA). To determine chromosome breakage, subconfluent cells were exposed to 1μmol/L cisplatin for 48 hours and metaphase chromosomes were prepared as described previously by Litman et al. (10).
Results and Discussion

Myc cooperates with the loss of Brca1 and p53 in transforming mouse OSE cells. We have developed a K5-TVA-RCAS system in which defined multiple genetic alterations can be introduced into mouse OSE cells (5, 9), a single layer of cells that is believed to be the precursor for ovarian carcinoma. We have previously shown that the minimal requirement for the transformation of OSE cells from p53-deficient mice is a combination of any two of the following oncogenes: Myc, K-ras, and Akt (5). To identify the minimal requirement for the transformation of mouse OSE cells that are deficient for both Brca1 and p53, we crossed K5-TVA (5), Brca1lox/lox (6), and p53lox/lox (7) transgenic mice. The triple transgenic system allows for conditional inactivation of Brca1 and p53 in OSE cells as well as the introduction of putative oncogenes that are thought to collaborate with Brca1 and p53 in

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<th>Transgenic background</th>
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<tr>
<td>K5-TVA: Brca1lox/lox, p53lox/lox</td>
<td>Cre AP</td>
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<td>Cre GFP</td>
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<td>K5-TVA: p53lox/lox</td>
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<tr>
<td>K5-TVA: Brca1lox/lox</td>
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Table 1. Identification of oncogenes that cooperate with the loss of p53 and/or Brca1 in inducing transformation of mouse OSE cells

Figure 1. Characterization of genetically defined mouse ovarian cancer cell lines. A, detection of deleted p53 (exons 2–10), conditional Brca1, deleted Brca1 (exon 11), and tva alleles by PCR using DNA from mouse tails (2, 5, 6) and the corresponding transformed ovarian cell lines with deleted p53 and Brca1 tumor suppressor genes (BR2, BR5, and BR6). B, RT-PCR detection of p53, Brca1, Myc, and β-actin in MEFs and engineered mouse ovarian cancer cell lines C1 (p53+/−; Myc; K-ras), C2 (p53−/−; Myc; Akt), C3 (p53+/−; K-ras; Akt), and BR2, BR5, and BR6 (p53+/−; Brca1−/−; Myc). C, Western blot of whole-cell extracts from Brca1 wild-type (C22) and Brca1-deficient (BR2) cell lines with a mouse Brca1 antibody. Asterisk, nonspecific band. D, Immunodetection of Rad51 nuclear foci formation after overnight exposure of Brca1 wild-type (C11) and Brca1-deficient (BR5) OSE cell lines to 1 μg/mL MMC or vehicle.
Consistent with the requirement for Brca1 in these processes, proteins, such as Rad51, to the DNA damage-induced foci (16). centrosome duplication (15) and localization of DNA repair.

p53lox/lox mice with a combination of RCAS-Myc, K-ras vectors carrying (Fig. 1 A). Because both Brca1 and p53 are located on chromosome 11, Cre-loxp-mediated recombination may result in recombination between Brca1 and loxP loci, potentially influencing carcinogenesis. PCR with four combinations of primers (Fig. 1) flanking p53 (Fig. 1A, a and c) and Brca1 (Fig. 1A, d and g) loxP sites did not detect such alterations (data not shown). OSE cell lines that are wild-type for Brca1 were generated by infection of the ovaries from K5-TVA; Brca1lox/lox; p53lox/lox transgenic mice were infected in vitro with RCAS-Cre along with RCAS vectors carrying K-ras, HER-2, Akt, or Myc genes. We determined that the Myc oncogene is capable of transforming cells that are deficient for both Brca1 and p53, whereas K-ras, HER-2, and Akt are not sufficient for the transformation of these cells (Table 1). Myc, however, is not sufficient for the transformation of ovarian cells with intact Brca1 or p53 (Table 1).

The mechanism by which Myc transforms Brca1- and p53-deficient mouse OSE cells is presently unknown. Tumor growth requires the activation of proliferative signals and amplification of the Myc gene might be sufficient to provide these signals. However, other oncogenes, such as activated Akt, K-ras, and HER-2, which are known to induce strong proliferative signals, were not sufficient for the transformation of the Brca1- and p53-deficient OSE cells in our assay. This suggests that Myc may play a different and possibly more direct role in the transformation of mouse OSE cells that are deficient for Brca1 and p53. Brca1 physically associates with Myc and represses its transcriptional and transformative activity (11). It has also been shown in transgenic mice that deregulated Myc expression leads to DNA damage and genomic instability, which induces p53 activation and apoptosis (12). However, in the absence of the p53 function, rather than leading to apoptosis, Myc-induced DNA damage may lead to uncontrolled genomic instability and cell proliferation. The amplification of Myc and the loss of the p53 function often accompany the Brca1 mutation in human and mouse breast tumors (13, 14), providing additional support for the role of Myc and p53 in Brca1-associated tumor progression. Use of high-throughput technology, such as microarray analysis and comparison of OSE cell lines with different genetic alterations, might shed light on the cooperative pathways among Myc, p53, and Brca1.

Serous ovarian carcinomas develop in mice injected with Brca1 wild-type and Brca1-deficient mouse OSE cells. Three independent OSE cell lines, designated as BR2, BR5, and BR6, were generated by infection of the ovaries from K5-TVA; Brca1lox/lox; p53lox/lox mice with a combination of RCAS-Cre and RCAS-Myc. PCR of mouse tail DNA and the corresponding transformed OSE cell line DNA was used to confirm the deletion of exons 2 to 10 in p53 and exon 11 in Brca1 in the transformed OSE cell lines (Fig. 1A). Because both Brca1 and p53 are located on chromosome 11, Cre-loxp-mediated recombination may result in recombination between Brca1 and loxP loci, potentially influencing carcinogenesis. PCR with four combinations of primers (Fig. 1) flanking p53 (Fig. 1A, a and c) and Brca1 (Fig. 1A, d and g) loxP sites did not detect such alterations (data not shown). OSE cell lines that are wild-type for Brca1 were generated by infection of the ovaries from K5-TVA; p53−/− mice with combinations of RCAS vectors carrying Myc, K-ras, and Akt oncogenes as described previously (5, 9). RT-PCR (Fig. 1B) was used to detect the presence of p53, Brca1, Myc, and β-actin in mouse embryonic fibroblasts (MEFs), mouse ovarian cancer cell lines deficient for p53 and wild-type for Brca1 (C1, C2, and C3), and mouse ovarian cancer cell lines deficient for both Brca1 and p53 (BR2, BR5, and BR6). A Western blot of whole-cell extracts from Brca1 wild-type and Brca1-deficient (exon 11 deleted) cell lines with a Brca1 antibody shows expression of the 210-kDa wild-type Brca1 and the 92-kDa Brca1Δ117 spliced product (Fig. 1C). Brca1 is known to play a role in the fidelity of centrosome duplication (15) and localization of DNA repair proteins, such as Rad51, to the DNA damage-induced foci (16). Consistent with the requirement for Brca1 in these processes, Brca1-deficient cell lines displayed an increased number of centrosomes (Supplementary Fig. S1) and lacked Rad51 nuclear foci in the presence of the DNA cross-linking agent MMC (Fig. 1D).

Lp. injection of Brca1 wild-type and Brca1-deficient cell lines into nude or FVB mice invariably resulted in metastatic tumor formation. Similar to human ovarian tumors, the mouse tumors were associated with the accumulation of hemorrhagic ascites and bloating (Fig. 2A). The tumor nodules grew on the surfaces of organs without significant invasion. Tumor spread sites included the mesothelial lining of the peritoneum, intestines, pancreas, and diaphragm, whereas the liver, spleen, and kidneys were typically spared from tumor spread. Both Brca1 wild-type and Brca1-deficient tumors were characterized by papillary structures typical of serous papillary carcinoma (Fig. 2B), which is the most common type of hereditary ovarian cancer. The OSE cell origin of the tumors was confirmed by the presence of the epithelial cell marker keratin 8 (Fig. 2C). The cell lines that were derived from the transformed primary ovarian epithelial cells and the mouse tumors maintained the typical epithelial cobblestone morphology of OSE cells (Supplementary Fig. S2).

Loss of Brca1 function increases sensitivity of mouse OSE cells to the DNA-damaging agent cisplatin. Administration of the microtubule-poise paclitaxel and the DNA-damaging agent cisplatin is a current standard of postoperative chemotherapy for patients with advanced ovarian cancer. Patients with hereditary ovarian cancers display a longer recurrence-free interval following
chemotherapy and have a longer overall survival rate (1). This phenomenon is likely due to induced chromosomal instability, cell cycle arrest, and subsequent apoptosis in cells that lack Brca1 and thus cannot repair the induced DNA damage. To test whether Brca1 status influences the sensitivity of OSE cells to drugs with different mechanisms of action, we compared the sensitivity of Brca1 wild-type and Brca1-deficient OSE cells to paclitaxel and cisplatin. OSE cell lines that are wild-type (C1, C2, C3, T1, T2, and T3) or deficient for Brca1 (BR2, BR5, BR6, TBR2, TBR5, and TBR6) were tested for their rate of survival in increasing concentrations of paclitaxel and cisplatin at doses that are effective in human ovarian cancer cell lines that harbor defined genetic alterations such as Brca2, p53, K-ras, TGFβ-RII, and CDNK2A (17). We showed that transformed Brca1 wild-type and Brca1-deficient mouse OSE cell lines have a similar sensitivity to the microtubule poison paclitaxel (Fig. 3A). However, the DNA-damaging agent cisplatin was significantly more effective in inducing cell death in the Brca1-deficient cell lines (Fig. 3A), presumably because these cells are unable to repair cisplatin-induced DNA damage. The exposure of ovarian cancer cell lines lacking functional Brca1 to 1 μmol/L cisplatin for 48 hours resulted in chromatid breaks and other complex chromatid aberrations, such as triradial and quadriradial chromosomes (Fig. 3B). We hypothesized that, in this case, Brca1 functioned as a chemotherapy sensitivity modulator independent of oncogenic composition. Consistent with this hypothesis, we showed that the introduction of Akt or H-ras into the Brca1-deficient TBR5 cell line results in a similar response to cisplatin or paclitaxel compared with that of TBR5 cells with an empty vector (Supplementary Fig. S3). Similarly, it has been shown that the K-ras status in human ovarian cancer cell lines does not affect chemosensitivity to paclitaxel and cisplatin (17, 18).

Although these studies were done in culture, our results concur with previous findings that Brca1-deficient human ovarian cancer cells have a greater sensitivity to cisplatin in vitro (16) and that...
patients with Brca1-associated ovarian carcinoma respond better to platinum-based therapies (1, 19). The increased sensitivity of the Brca1-deficient OSE cells to cisplatin and the reported platinum chemoinsensitivity displayed by patients with Brca1-associated ovarian cancer raise the question about whether platinum agents would be effective in the treatment of patients with Brca1-associated breast cancer. This biochemical difference between Brca1 wild-type and Brca1-deficient cells provides an opportunity to develop novel therapeutic agents that generate specific DNA lesions that require functional Brca1 for their repair. The compelling factor of this type of therapy is its selectivity; the nontumor cells that are heterozygous for Brca1 should have normal Brca1 function and, therefore, normal levels of DNA repair, whereas the Brca1-deficient tumor cells should be highly sensitive to DNA-damaging treatments (20). Unlike cell lines isolated from human tumors, the Brca1 wild-type and Brca1-deficient mouse OSE cell lines are capable of forming serous papillary carcinomas in immunocompetent mice, thus providing a unique experimental model to study the sensitivity of ovarian tumors to various therapies that may interfere with the immune system. We anticipate that this mouse model will facilitate the evaluation of various therapies that target the Brca1 pathway as well as assist in the identification of novel biochemical pathways that are associated with hereditary ovarian cancers.

Acknowledgments

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