Expression of OVCA1, a Candidate Tumor Suppressor, Is Reduced in Tumors and Inhibits Growth of Ovarian Cancer Cells

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ABSTRACT

Loss of all or part of one copy of chromosome 17p is very common in ovarian and breast tumors. OVCA1 is a candidate tumor suppressor gene mapping to a highly conserved region on chromosome 17p13.3 that shows frequent loss of heterozygosity in breast and ovarian carcinomas. Western blot analysis of extracts prepared from breast and ovarian carcinomas revealed reduced expression of OVCA1 compared with extracts from normal epithelial cells from these tissues. Subcellular localization studies indicate that OVCA1 is localized to punctate bodies scattered throughout the cell but is primarily clustered around the nucleus. Attempts to create cell lines that stably expressed OVCA1 from the cytomegalovirus promoter were generally unsuccessful in a variety of different cell lines. This reduction of colony formation was quantified in the ovarian cancer cell line A2780, where it was demonstrated that cells transfected with plasmids expressing OVCA1 had a 50–60% reduction in colony number as compared with appropriate controls, and only a few of these clones expressed OVCA1, albeit at low levels. The clones that expressed exogenous OVCA1 were found to have dramatically reduced rates of proliferation. Reduced growth rates correlated with an increased proportion of the cells in the G1 fraction of the cell cycle compared with the parental cell line and decreased levels of cyclin D1. The low levels of cyclin D1 appeared to be caused by an accelerated rate of cyclin D1 degradation. Overexpression of cyclin D1 was able to override OVCA1’s suppression of clonal outgrowth. These results suggest that slight alterations in the level of OVCA1, such as would occur after reduction of chromosome 17p13.3 to hemizygosity, may result in cell cycle deregulation and promote tumorigenesis.

INTRODUCTION

Ovarian cancer is the leading cause of death from gynecological malignancy and the fourth leading cause of cancer death among American women, yet little is known about the molecular evolution of ovarian tumors. Only a few candidate tumor suppressor genes in sporadic ovarian cancer have thus far been identified. Although two familial breast/ovarian cancer genes, BRCA1 and BRCA2, have been identified, mutations in sporadic ovarian cancers are rare in these genes. Other recently identified tumor suppressor genes that have been analyzed for mutations in ovarian tumors include TSG101, PTEN, DPC4, and BARD1. However, there has been little evidence reported suggesting that these genes are important in the pathogenesis of sporadic ovarian cancers (1–7). In addition, several interesting candidate tumor suppressor genes, DOC2, NOEY2, and LOT1, have recently been identified, and their roles in the development of ovarian cancer are currently being investigated (8–11). The TP53 tumor suppressor gene is, by far, the most frequently altered gene observed in ovarian cancer. In epithelial ovarian carcinomas, TP53 mutations are present in ~50% of advanced-stage cancers. However, the low frequency of TP53 mutations in cancers confined to the ovary and the near absence of mutations in benign and borderline ovarian neoplasms suggest that TP53 alterations may be a relatively late event in the progression of ovarian cancer (12).

LOH4 for markers on the short arm of chromosome 17 is one of the most common genetic abnormalities in ovarian cancer. Two regions on 17p13, including TP53 at 17p13.1 and a more telomeric region at 17p13.3 defined by markers D17S530 (equivalent to YN22.1) and D17S28 (equivalent to YNH37.3), have received the most attention (13). It has been reported that YNZ22.1 had a rate of LOH as high as 80%, and YNH37.3 showed >65% LOH in ovarian carcinomas. Loss at either D17S530 or D17S28 was observed in 43% of low malignant potential tumors, 80% of carcinomas without metastases, and 90% of advanced-stage carcinomas. Interestingly, in the low malignant potential tumors, allelic losses at YNZ22.1 and YNH37.3 were not accompanied by LOH at TP53, suggesting the loss of a more distal tumor suppressor gene in early tumorigenesis (14, 15).

Alterations involving the YNH37.3/YNZ22.1 region on chromosome 17p13.3 are not limited to ovarian cancer. A recent study by the European Breast Cancer Linkage Consortium of 1280 breast tumors found that the frequency of LOH observed on the p arm of chromosome 17 was much higher than that observed on the q arm (16). Up to two-thirds of breast tumors show LOH at the YNZ22.1 locus (17–23), and this finding has been associated with markers of tumor aggression (16, 23–25). Breast tumors with LOH at YNZ22.1 have been associated with a higher risk of recurrence than those showing retention of this region (23, 25). This same region shows frequent LOH in small cell lung cancers (26–28), colon cancers (29), primitive neuroectodermal tumors (30–32), carcinoma of the cervix uteri (33–36), medulloblastoma (37–40), astrocytoma (41, 42), follicular thyroid carcinoma (43), malignant melanoma (44), hepatocellular carcinoma (45), and leukemia and lymphoma (46). In many of these studies, changes on chromosome 17p13.3 occur in the absence of alterations involving TP53, suggesting that a tumor suppressor gene(s) residing in this region on chromosome 17p13.3 may be involved in the development of many types of cancers.

We have previously reported the identification of a common region of alleric loss on 17p13.3 in ovarian cancer defined by the markers D17S28 and D17S530 (47). These two loci span <20 kbp (47). By the use of positional cloning strategies, two candidate tumor suppressor genes, OVCA1 and OVCA2, have been identified that map to the region that is most commonly lost in ovarian and breast tumors, chromosome 17p13.3 (47, 48). OVCA1 demonstrates sequence similarity (20% identity) to one of the yeast enzymes in the diphthamide synthase.
MATERIALS AND METHODS

Reagents and Cell Lines. Cell culture reagents were from Life Technologies, Inc. (Gaithersburg, MD); unless otherwise indicated, most other reagents were obtained from Sigma Chemical Co. (St. Louis, MO). Cell lines were obtained from American Type Culture Collection (Manassas, VA) or were derived in our laboratory (HOSE, human ovarian surface epithelial cell lines grown in primary culture; and HOH cell lines, SV40-immortalized human ovarian epithelial cells). A2780 cells were maintained in DMEM supplemented with 10% FCS and 0.2% (v/v) porcine insulin. COS-7 cells were maintained in DMEM supplemented with 10% FCS. T47D cells were maintained in RPMI 1640 supplemented with 10% FCS and 0.2% (v/v) porcine insulin. SKBR3 cells were maintained in McCoy's 5a medium supplemented with 10% FCS. HOSE cells and HOH cell lines were maintained in a 1:1 mixture of medium 199 and MCDB-105 medium, supplemented with 5% FCS and 0.2% (v/v) porcine insulin. Unless otherwise stated, cells were transfected with Superfect (Qiagen, Chatsworth, CA), as described by the manufacturer. The A2780 clones that stably express OVCA1 were obtained after selection in G418 by standard methods and maintained in DMEM supplemented with 10% FCS and 0.5 mg/mL G418.

SSCP Analysis of OVCA1. PCR was carried out in a reaction volume of 10 µl containing 100 ng of genomic DNA template, 10 µM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl₂, 0.001% (v/v) gelatin, 1 µM forward and reverse primers, 60 µM dNTPs, 0.1 µg of [α-32P]dATP (DuPont-NEN, Boston, MA), 5% DMSO, and 0.5 unit of AmpliTaq DNA polymerase (Perkin Elmer Corp., Foster City, CA). Following an initial denaturation step at 94°C for 4 min, DNA was amplified through 20 cycles consisting of 5 s of denaturing at 94°C, 1 min of annealing at 65°C – 0.5°C/cycle, and 1 min of extension at 72°C. The samples were then subjected to an additional 25 cycles, consisting of 1 min of denaturation at 94°C, 1 min of annealing at 55°C, and 1 min of extension at 72°C and a final extension at 72°C for 5 min. PCR products were diluted 1:10 in 95% formamide, 10 mM NaOH, 0.25% bromophenol blue, and 0.25% xylene cyanol. Diluted products were denatured for 5 min at 95°C and flash-cooled on ice. Four µl were loaded onto a 0.5 × MDE gel (AT Biochem, Malvern, PA), prepared according to manufacturer’s specifications, and electrophoresed at 6 W for 12–16 h at room temperature in 0.6×TBE [1×TBE, 0.09 M Tris, 0.09 M boric acid, and 0.002 M EDTA (pH 8.0)]. Following electrophoresis, the gel was dried and exposed to autoradiography film at −80°C for 4–24 h. Variant and normal SSCP bands were cut out from the gels after alignment with the autoradiograph, and the DNA was eluted in 100 µl of distilled deionized H₂O at 37°C for 3 h. Two µl of the eluted DNA were used as template for secondary PCRs, as described above, except that radiolabeled dATP was omitted. Following amplification, the DNA was collected on Wizard resin (Promega, Madison, WI) and eluted in 50 µl of distilled deionized H₂O, and 50–100 fmol of purified PCR product were subjected to direct sequencing.

Plasmid and mRNA expression vectors pcDNA3 and pcDNA3-LacZ were obtained from Invitrogen. The HA antibody tag (YPYDVPDYA) was added to the COOH or NH₂ terminus of the OVCA1 cDNA by standard PCR technology, and the resulting tagged cDNAs were subcloned into pcDNA3 and are referred to as pcDNA3-HA-OVCA1 or pcDNA3-OVCA1HA, depending on the location of the HA tag. The plasmid pGFP-C1, which expresses green fluorescent protein, was obtained from Clontech. The cDNA of OVCA1 was fused to the COOH terminus of the green fluorescent protein at the BglII site to generate the plasmid pGFP-OVCA1. To prepare a GST fusion of OVCA1 in bacteria, we subcloned the OVCA1 cDNA, containing an NH₂-terminal HA tag, into pGEX-2T (Pharmacia).

Production of Anti-OVCA1 Antibodies. The 13-amino acid peptide, RDGDPRGRAPRGC, corresponding to amino acids 20–31 of OVCA1 (where the terminal cysteine was added for conjugation purposes) was synthesized [Research Genetics, Huntsville, AL]. Purity of the peptide was confirmed by high-performance liquid chromatography. The peptide was conjugated to mBSA followed by keyhole limpet hemocyanin (Pierce, Rockford, IL), and used to immunize a New Zealand White rabbit (Cocalico, Reamstown, PA). Two mg of antigenic peptide were covalently linked to Aminolink agarose (Pierce) and used to purify anti-OVCA1 antibody from crude serum by affinity chromatography. The final antibody is referred to as T13J2. The antibody FC21 was produced by immunizing a New Zealand White rabbit (Cocalico) with a bacterially expressed carboxyl terminal portion of OVCA1 (amino acids 330–443). The resulting antiserum was immunouflnity purified on Aminolink agarose covalently linked to bacterially expressed GST-OVCA1.

Purification of Bacterially Expressed OVCA1. BL21 bacteria were transformed with pGEX2T-OVCA1. Expression of the fusion protein was induced with 1 mM isopropyl-β-thio-galactopyranosidase (Stratagene, La Jolla, CA). The bacteria were lysed by sonication, and GST-OVCA1 was purified from the soluble fraction by binding to glutathione-Sepharose 4B (Pharmacia). Pure OVCA1 was released by digesting with thrombin (Pharmacia), or the GST-OVCA1 fusion was eluted with excess glutathione. PET-OVCA1 (nucleotides 1011–1350) was expressed in BL21 bacteria and purified as an insoluble inclusion body by repeated washing of the insoluble fraction with 1% Triton X-100. The insoluble pellet was solubilized in 8 M urea-2% SDS. The protein (OVCA1 amino acids 330–443) was further purified by SDS-PAGE. The gel slice containing the protein was homogenized and used to immunize rabbits.

Preparation of Protein Extracts from Human Tumor Specimens. Normal human tissues were obtained from Clontech. Tumors were snap-frozen after surgical removal and stored in liquid nitrogen until use. One g of tumor tissue was rinsed twice with cold PBS and minced finely into small pieces. Tissue pieces were suspended in 1 mL of PBSTD [0.137 M NaCl, 2.68 mM KCl, 10.6 mM Na₂HPO₄, 1.47 mM K₂H₂PO₄, 1% (v/v) Triton X-100, 0.5% (w/v) deoxycholate, 0.1% (v/v) SDS, 0.004% (w/v) NaF, 100 mg/mL phenylmethylsulfonyl fluoride, 1 mg/mL aprotinin, 1 mg/mL leupeptin, and 2 mM sodium orthovanadate (pH 7.4)] and ground with a Polytron tissue grinder at 300–400 rpm for two 30-s intervals at 4°C. Tissue homogenates were clarified by centrifugation at 100,000 × g for 1 hour at 4°C. Lipid layers were removed, and cytosolic extracts were aliquoted, snap-frozen in liquid nitrogen, and stored at −80°C. Quantitation of protein was achieved using a bicinchoninic acid/copper (II) sulfate assay (Sigma).

SDS-PAGE and Western Blot Analysis. Fifty µg of total protein extract from tissues or 20 µg of total protein from cell extracts, unless otherwise stated, were separated by standard SDS-PAGE and transferred to Immobilon-P (polyvinylidene difluoride; Millipore, Bedford, MA). The membranes were blocked with 3% BSA and probed with the anti-OVCA1 antibody T13J2, or blocked with 3% dried milk and probed with the indicated antibody. The signal was visualized using anti-rabbit antibodies coupled to HRP (Amer sham) and developed using ECL reagents, as recommended by the manufacturer (Amer sham).

Subcellular Fractionation. HOSE cells were homogenized in ice-cold hypotonic homogenization buffer [40 mM Tris (pH 7.4), 1 mM EDTA, 1 mM EGTA, 1 mM DTT, and 10% glycerol]. The nuclei were pelleted by centrifugation at 2500 rpm for 10 min. The supernatant was collected, and insoluble debris was pelleted at 180,000 × g for 30 min to give the cytosol fraction. The insoluble pellet was washed twice with homogenization buffer containing 0.1 M KCl. The nuclear pellet was then extracted with homogenization buffer plus 0.45 M KCl for 1 h on ice, with frequent vortexing. Insoluble debris was pelleted at 180,000 × g for 30 min to obtain the nuclear fraction.

Immunofluorescent Staining and Imaging. COS-1 cells were transfected with the indicated plasmids using Lipofectamine (Life Technologies, Inc.) or Superfect (Qiagen), as directed by the manufacturer. Forty-eight h after trans-
OVCA1 is reduced in tumors and inhibits growth

**RESULTS**

**Mutational Analysis of OVCA1 by SSCP.** SSCP analysis was conducted on 50 ovarian tumors independent of LOH status for markers on 17p13.3 and on 20 breast tumors demonstrating allelic loss of OVCA1 and retention of TP53. Multiple sequence variants were identified throughout the gene (Fig. 1; Table 1). These sequence variants were deemed to be polymorphisms because these same alterations were either found in the corresponding germ line or resulted in either conservative or silent amino acid substitutions. The frequency of these putative polymorphisms was determined by SSCP analysis of 100 chromosomes from control individuals (Table 1). In addition, we identified two nonconservative amino acid substitutions: alanine 34 changed to an aspartic acid residue and serine 389 changed to an arginine residue. Each alteration was detected in the germ line of a woman with early-onset breast cancer who reported a family history of the disease. In both cases, the missense mutation/rare polymorphism was retained in the corresponding breast tumor DNA and showed reduction to homozygosity (data not shown). Evaluation of >100 control chromosomes has failed to detect these sequence variants. The individual carrying the A34D missense variant was diagnosed with breast cancer at age 37 and reported a history of one

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**Table 1** Nucleotide sequence variants observed in OVCA1 in tumors

<table>
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<tr>
<th>Exon</th>
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<th>Base Change</th>
<th>Result</th>
<th>Frequency</th>
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<td>Leu→Leu</td>
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<td>11</td>
<td>13</td>
<td>C→G</td>
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*The variants shown are those that were detected in the coding and 3′ untranslated region segments. Sequence variants that were detected in the promoter and in introns 5, 6, 11, and 12 are not listed. Codon refers to the amino acid affected by the nucleotide change. Base indicates the nucleotide position of the codon affected. Change describes the nature of the nucleotide alteration. Result describes the effect the nucleotide alteration has on the amino acid. ND, not determined; NC, noncoding sequence.*
first-degree relative and two second-degree relatives with the disease (ages of onset unknown). The individual carrying the S389R missense variant was diagnosed with breast cancer at age 49. She reported that her mother was affected with breast cancer at age 55 and that two maternal aunts were diagnosed with the disease at 61 and 65 years of age. The functional significance of these mutations is not yet clear; preliminary experiments exploring their effect on the OVCA1 protein are presented below.

**Southern Blot Analysis of OVCA1.** To assess deletions or rearrangements within the OVCA1 gene, we performed Southern blotting of 60 normal/ovarian tumor DNA pairs using the full-length OVCA1 cDNA as the probe. The vast majority of the tumors had lost one copy of the OVCA1 gene. No rearrangements or large interstitial deletions were detected in the remaining copy. However, a 7-kbp EcoRI fragment was observed to be variable in length due to changes in the VNTR, i.e., YNH37.3, which is intragenic to OVCA1 (data not shown). We did not observe any correlation between the length of this fragment and an increased risk of developing ovarian cancer.

**Western Blot Analysis of OVCA1.** Conceptual translation of OVCA1 predicts a 443-amino acid protein with M_r ~50,000. An antibody that recognizes 11 amino acids at the NH_2 terminus of OVCA1 was prepared by immunizing rabbits with a peptide. The antiseraum was affinity-purified and was designated TJ132. Another antibody that recognizes the COOH terminus of OVCA1 (amino acids 330–443) was prepared by immunooaffinity purification following immunization of rabbits with a bacterially expressed polypeptide and was designated FC21. Both antibodies were able to recognize bacterially expressed OVCA1 by Western blotting (data not shown). In addition, these antibodies were able to recognize a protein of M_r ~50,000 in extracts prepared from COS-1 cells that had been transiently transfected with pcDNA3-HAOVCA1 and in whole-cell lysates from the ovarian tumor cell line A2780 (Fig. 2A). Recognition of this M_r 50,000 protein could be competed with a molar excess of the antigenic peptide, indicating that the antibodies recognize the authentic OVCA1 protein (data not shown). In addition to the M_r 50,000 protein, both antibodies detected proteins of M_r ~85,000, as observed in extracts prepared from a variety of sources, including normal human tissues, primary cultures of HOSE cells and a number of cell lines (Figs. 2 and 3; data not shown). The NH_2-terminal antibody TJ132 also recognized proteins of M_r ~70,000, but these species were variable in amount and presence and were not recognized by antibodies directed against the COOH terminus of the protein. The secondary antibody alone did not recognize any of the three proteins (M_r 50,000, 70,000, and 85,000; data not shown). The identity of the M_r 70,000 and M_r 85,000 proteins is unknown, as are their relationships with the M_r 50,000 OVCA1 protein; however, the available evidence suggests that the M_r 85,000 form is an alternatively spliced or posttranslationally modified form of OVCA1 and that the p70 form is either unrelated to OVCA1 or is a breakdown product of the p85 form. Alternatively, the p85/p70 forms could be unrelated, cross-reacting proteins. However, this is unlikely because completely different anti-OVCA1 antibodies recognize the p85 protein, and recognition of the M_r 85,000 protein by TJ132 can be competed with a molar excess of

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**Fig. 2. Characterization of OVCA1 expression.** A, extracts from the indicated tissues and cell lines were separated by 10% SDS-PAGE and transferred to Immobilon-P, as described in “Materials and Methods.” The blot was then probed with the anti-OVCA1 antibody TJ132. Lane IVT, in vitro translated pcDNA3-HAOVCA1; Lane COS, extract of COS-1 cells that had been transfected with pcDNA3-HAOVCA1; Lane A2780, extract of the ovarian tumor cell line A2780; Lane H, ovary, extract of whole human ovary. Arrowheads, three different polypeptides that TJ132 recognizes (p50, p70, and p85). B, 20 µg of each indicated cell line extract were separated in duplicate by 10% PAGE, transferred to Immobilon-P, and probed with the indicated antibodies, as described in “Materials and Methods.” One of the duplicate blots was probed with the anti-OVCA1 antibody TJ132, and the other was probed in parallel with the anti-OVCA1 antibody FC21. OVCA-5 is a cell line derived from an ovarian tumor, whereas T47D and SKBR3 are cell lines derived from breast tumors. C, 50 µg of extracts from various human tissues (Clontech) were separated by 12% SDS-PAGE and processed for Western blotting, as described in “Materials and Methods.” The blot was probed with the anti-OVCA1 antibody TJ132. Lane H, heart; Lane B, brain; Lane P, placenta; Lane L, lung; Lane Li, liver; Lane S, skeletal muscle; Lane K, kidney; Second Lane P, pancreas; Second Lane S, spleen; Lane T, thymus; Lane M, mammary gland; Lane Te, testis; Lane O, ovary.
the antigenic peptide. Because FC21 and TJ132 gave almost identical patterns by Western blotting (Fig. 2B), most of the data shown used only the antibody TJ132.

OVCA1 was found to be expressed in many different tissues (Fig. 2C). In some cases, the $M_r$ 70,000 and $M_r$ 85,000 proteins were very prominent, whereas the $M_r$ 50,000 protein was less so (notably the ovary and placenta) and, in other tissues, the p50 form was predominant (liver and thymus). Note that, although extracts from total breast tissue appeared to express little or no OVCA1 (Fig. 2C), breast epithelial cells did express the p50 OVCA1 protein (Fig. 3A, N.M.E.*). We explain this apparent discrepancy as being due to epithelial cells making up only a low percentage of the total breast. Analysis of breast and ovarian tumor extracts demonstrated variable expression levels of p50 and an almost complete absence of the p70/p85 species (Fig. 3). Expression levels of p50 were reduced as compared to normal epithelial cells in 21 of 59 ovarian (37%) and 18 of 46 breast (39%) carcinomas. p85 and p70 were not detected in the majority of tumors analyzed (100% of breast tumors and 85% of ovarian tumors) (Fig. 3). No correlation was evident between reduced expression and clinical prognostic factors.

**Subcellular Localization of OVCA1.** To aid in understanding the function of OVCA1, we determined its subcellular localization.

COS-1 cells were transfected with either an empty vector pcDNA3 or with pcDNA3-OVCA1HA, which expresses OVCA1 fused to a COOH-terminal HA tag. Immunostaining of transfected cells with an anti-HA antibody (Y-11; Santa Cruz Biotechnology) indicates that OVCA1 is located throughout the cell. A widespread diffuse staining was seen, in addition to strongly staining punctate bodies (Fig. 4A and B). These bodies were scattered throughout the cell and were heavily clustered around the nucleus. A similar pattern was obtained in immortalized HOSE cells transfected with pcDNA3-OVCA1HA and when the cells were immunostained with the specific anti-OVCA1 antibody, TJ132 (data not shown). To further confirm the localization, we fused OVCA1 to the COOH terminus of the green fluorescent protein. COS-1 cells expressing the green fluorescent protein-OVCA1 fusion again demonstrated a punctate, primarily perinuclear localization of the protein set against a weaker, diffuse staining throughout the cell (data not shown). Fractionation studies confirmed that the $M_r$ 50,000 OVCA1 protein is located throughout the cell (Fig. 4C). However, the $M_r$ 70,000 and $M_r$ 85,000 species appeared to be exclusively located within the nucleus (Fig. 4C).

**Suppression of Clonal Outgrowth.** Attempts to generate cell lines that stably expressed OVCA1 were unsuccessful. Very few clones...
were found to express OVCA1, and those that did expressed only low levels of the protein. This phenomenon was consistently observed in a number of different cell types (RAT-1, U2OS, MCF-7, HIO118, and T47D cells; data not shown). To quantitate this effect, we transfected equimolar amounts of a mammalian expression vector containing the NH2-terminal HA-tagged OVCA1 open reading frame (pcDNA3-HAOVCA1) and an empty expression vector (pcDNA3) into the ovarian cancer cell line A2780. A2780 cells were chosen for further analysis because they are well-characterized ovarian tumor cells that normally express fairly low levels of OVCA1 p50 and almost no p85/p70 OVCA1 (Fig. 2). As a positive control for growth suppression, an expression vector that expresses wild-type p53 protein was included in some of the colony number experiments. Evaluation of colony formation in the presence of geneticin (G418) consistently resulted in a 50–60% reduction in colony number in cells transfected with the OVCA1 expression construct compared with controls (Fig. 5A). This effect was reproducibly observed in more than four independent transfection experiments. Suppression of clonal outgrowth was independent of plasmid DNA purity (we tested three different preparations of plasmid DNA) whether equivalent molar amounts or microgram amounts of plasmid were transfected. Furthermore, experiments in which an expression vector containing the gene encoding for the β-galactosidase protein were cotransfected with OVCA1 indicate that the reduction in clonal outgrowth is not an artifact due to differences in transfection efficiency (data not shown).

The A34D and S389R alterations described above, detected in the germ line of women with breast cancer and with a strong family history of the disease, were rebuilt into the pcDNA3-OVCA1HA expression plasmid using standard PCR technology. Both altered proteins were expressed well in transient transfection assays (data not shown). However, both alterations were found to suppress colony formation 50–60%, as compared with controls, similar to wild-type OVCA1 (Fig. 5B).

**Growth Kinetics of Stable Transfectants.** To verify that the suppression effect was due to exogenous OVCA1 expression, individual colonies were clonally expanded after G418 selection. A total of seven colonies from pcDNA3 vector control transfected cells and 15 colonies from pcDNA3-HAOVCA1 transfected cells were Amplified following selection in G418 for 10 days. All colonies selected from pcDNA3 vector control plates expanded and formed stable cultures. In contrast, only 9 of 15 colonies selected from pcDNA3-HAOVCA1 transfected cells expanded to form a stable culture. Because an in-frame HA epitope was fused to the open reading frame of OVCA1 at the NH2 terminus, the level of exogenous protein produced in these clones could be monitored. Western blot analysis revealed that there was approximately equimolar expression of exogenous and endogenous OVCA1 in only four of nine stable pcDNA3-HAOVCA1 clones (OV-5, OV-6, OV-9, and OV-13; Fig. 6).

Of the HAOVCA1 transfectants with exogenous expression, no major differences in morphological features were observed when compared to parental A2780 cells (data not shown). Cells were plated at limited dilutions and monitored for growth kinetics. Two independent clones, OV-5 and OV-13, displayed 8- and 10-fold reductions in total cell number compared with expression vector controls and parental A2780 cells, respectively. A third clonal line, OV-9, demon-

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**Fig. 5.** Suppression of clonal outgrowth by OVCA1 in A2780 ovarian cancer cells. A2780 cells were transfected with the indicated plasmids and selected for resistance to G418, as described in “Materials and Methods.” Ten to 14 days posttransfection, the colonies were stained and counted. A, the percentage of G418 (NeoR) colonies that formed relative to the number formed after transfection with pcDNA3 (defined as 100% in each experiment) along the ordinate. Abcissae, data from eight independent transfection experiments. A wild-type TP53 positive control for colony suppression. B, the total number of colonies obtained after transfection with the indicated plasmids and selection with G418. pcDNA3 is the parent vector; OVCA1HA expresses the wild-type OVCA1 plus a COOH-terminal HA tag; and A34D and S389R refer to the point mutations, discussed in the text, that were introduced into the wild-type OVCA1HA construct using standard PCR technology. Columns, means of three independently repeated experiments; bars, SD.

**Fig. 6.** Maintenance of exogenous OVCA1 expression in stable transfectants of A2780 ovarian cancer cells. A2780 cells were transfected with an HA-OVCA1 expression vector and then selected for resistance to G418, as described in “Materials and Methods.” Clones were chosen at random and amplified. After amplification, extracts were prepared from the cells, as described in “Materials and Methods.” Ten μg of each extract were separated by duplicate 10% SDS-PAGE. The gels were transferred to Immobilon-P and probed with the indicated antibodies, as described in “Materials and Methods.” Top, total OVCA1 antigen was detected with the anti-OVCA1 antibody T2132. Bottom, exogenous OVCA1 antigen was detected with the anti-HA mAB 16B12 (BabCo, Richmond, CA). Lane COS, protein extract prepared from COS cells transiently transfected with an HA-OVCA1 expression vector; Lanes A2780, protein extract prepared from the parental cell line; Lanes CMV, cell lines derived from pcDNA3 transfected cells; Lanes OV, cell lines derived from HAOVCA1-transfected cells.
stratified a 4-fold reduction in total cell number over the same time interval compared with controls (Fig. 7A). On the basis of these growth curves, the cell doubling times between parental A2780 and OVCA1-expressing stable clones were found to be considerably different. A2780 cells doubled 2–2.5 times during a 24-h period, whereas OV-5, OV-9, and OV-13 doubled 1–1.5 times during the same time interval. Consistent with the reduced growth rate, the clones stably expressing OVCA1 had a dramatic reduction in cyclin D1 levels (Fig. 7B). The reduction of steady-state cyclin D1 levels appeared to be primarily due to an increased rate of degradation of cyclin D1 in cells expressing HAOVCA1 compared with the parental cell line (Fig. 7C).

FACS Analysis of Stable Transfectants. Two main mechanisms, apoptosis and cell cycle arrest, may account for the growth suppression observed in stable clones expressing exogenous OVCA1. To investigate the mechanism of growth suppression, we seeded parental A2780 cells and each of the stable transfectants at an equal number of cells. Seventy-two h postseeding, the cells were harvested, nuclei were stained with ethidium bromide, and cell cycle distribution was measured by FACS analysis. As illustrated in Fig. 8, a 10–20% increase in the number of cells in the G1 fraction was observed in clones OV-5, OV-9, and OV-13 compared with parental A2780 cells and stable vector control cells, CMV-5. No subdiploid cell peaks suggestive of apoptosis were observed. To further investigate the possibility of apoptosis playing a role in reduced cell number, we subjected clones OV-5 and OV-9 to TUNEL staining and compared them with the vector control cells. There were no TUNEL-positive cells on the vector control cell slides. A total of 1.2% of the OV-9 cells were TUNEL-positive, and 4% of the OV-5 cells were TUNEL-positive, suggesting that, although rates of apoptosis are slightly elevated in A2780 cells stably expressing OVCA1, apoptosis does not fully account for the drastic reduction in growth rates (data not shown).

Cyclin D1 Overexpression Can Partially Overcome OVCA1’s Suppression of Clonal Outgrowth. Reduction of cyclin D1 levels by OVCA1 may be the primary cause of OVCA1’s growth-suppressive function.
OVCA1 IS REDUCED IN TUMORS AND INHIBITS GROWTH

Fig. 9. Overexpression of cyclin D1 can override OVCA1’s suppression of clonal outgrowth. A2780 cells were transfected with the indicated plasmids, as described in “Materials and Methods.” Resistant colonies were selected in G418 for 14 days and then they were fixed and stained. Colonies with >50 cells were counted. The experiment was repeated three times. pcDNA-3, transfected with 1 μg of pcDNA3 and 1 μg of pskII; CMV-OVCA1, transfected with 1 μg of pcDNA3-OVCA1HA and 1 μg of pskII; CMV-cyclin D1, transfected with 1 μg of CMV-cyclin D1 (carries no selectable marker) and 1 μg of pcDNA3; CMV-OVCA1 + cyclin D1 was transfected with 1 μg of pcDNA3-OVCA1HA and 1 μg of CMV-cyclin D1.

effect. To test this theory, we cotransfected A2780 cells with pcDNA3-OVCA1HA and CMV-cyclin D1. The cells were transfected with pcDNA3 alone, pcDNA3-OVCA1HA alone, CMV-cyclin D1 alone, or both pcDNA3-OVCA1HA and CMV-cyclin D1. pcDNA3 and pskII were added as necessary to equalize the amount of selectable marker and plasmid DNA in each transfection. After selection for 14 days in G418, the colonies were fixed, stained, and counted. As noted previously, cells transfected with the OVCA1 expression construct formed ~50% fewer colonies than did cells transfected with pcDNA3 (Fig. 9). Cells transfected with the cyclin D1 expression vector formed almost as many colonies as did cells transfected with pcDNA3. Cells cotransfected with pcDNA3-OVCA1HA and CMV-cyclin D1 formed ~75% fewer colonies than did cells transfected with pcDNA3 and almost the same number of colonies formed by cells transfected with CMV-cyclin D1 alone, suggesting that overexpression of cyclin D1 can compensate for overexpression of OVCA1.

DISCUSSION

Molecular studies of human neoplasms suggest that a tumor suppressor locus exists on chromosome 17p13.3 near the VNTR markers YNH37.3 and YNZ22.2 (14–18, 20–25). To date, only two genes have been reported that map within the critical region of allelic loss on chromosome 17p13.3: OVCA1 and OVCA2 (47, 48). We have found that OVCA2 cannot suppress tumor cell proliferation.6 The amino acid sequence of OVCA1 contains little information with regard to its biological function. The only portion of the protein that is similar to one other human gene, DPH2L2, is more similar to the yeast dph2 than is OVCA1 (57).

Screening of a panel of primary breast (n = 20) and ovarian (n = 50) tumors for alterations of OVCA1 revealed two distinct missense changes and multiple polymorphisms in both the coding and noncoding regions. Both missense changes were detected in breast tumors, and each alteration was present in the germ line of a woman with a strong family history of this disease. In both cases, the missense mutation/rare polymorphism was retained in the corresponding breast tumor DNA and showed reduction to homozygosity. Evaluation of >100 control chromosomes failed to detect these sequence variants. The probands do not have unusual ancestries, indicating that the sequence alterations are unlikely to be related to a specific ethnic group. Unfortunately, the probands are deceased, and we do not have informed consent to contact other members of their respective families. Both of these probands have tested negative for germ-line mutations in BRCA1 and BRCA2.6 However, neither amino acid substitution alters OVCA1’s ability to suppress colony formation, suggesting that either these alterations are nonfunctional polymorphisms or that they affect some as yet undefined function of OVCA1 or perhaps alter the function of the p85 form of OVCA1. Our observation is of particular significance because, in a recent European Consortium study, an association between LOH at the OVCA1 locus and a positive family history of breast cancer was observed (16).

We also assessed ovarian tumors for large alterations involving the OVCA1 gene by Southern blotting; however, no rearrangements or large interstitial deletions were detected. One previous study has reported a homozygous deletion in an ovarian carcinoma that involved both D17S28 and D17S30 but not any other flanking markers (15).

Overall, no somatic mutations were detected within the coding region of OVCA1 at the DNA level in either primary breast or ovarian tumors.

Because OVCA1 does not appear to be commonly mutated in tumors and tumor cell lines, we sought to determine whether changes in its protein levels are more frequent in breast and ovarian cancer. Western blot analysis of extracts from breast and ovarian tumors suggested that expression of p50 OVCA1 is reduced in at least one-third of the tumor specimens evaluated. The larger putative forms of OVCA1 (p70/p85) are absent or highly reduced in almost 100% of the tumor specimens evaluated. The mechanism whereby the p70/p85 forms of OVCA1 are generated is as yet unclear. Several different antibodies raised against different regions of OVCA1 recognize the larger isoforms, confirming that they are closely related to the p50 OVCA1. Most likely, the p85 form is the product of an as yet undefined alternatively spliced exon or posttranslational modification. The p70 form is not recognized by antibodies directed against the COOH terminus of OVCA1, suggesting that it is either a degradation product of the p85 form or an unrelated, cross-reacting protein. If reduction of OVCA1 levels is important in tumorigenesis, then reintroduction of OVCA1 into tumor cell lines should revert, at least partially, the transformed phenotype. Because the p85/p70 isoforms are most consistently lost from tumors, reintroduction of these forms would be most informative. However, because they have not yet been completely defined, our experiments were confined to reintroduction of the p50 isoform. Attempts to stably express OVCA1 from the CMV promoter in a variety of cell lines were unsuccessful. This phenomenon crossed species lines, being apparent in cells derived from both rodents and primates; was independent of p53 status; and was evident in both immortalized and transformed cells, suggesting that overexpression of OVCA1 either blocks growth or is toxic to the cells.

Overexpression of OVCA1 in the ovarian cancer cell line A2780 provided some clues about the function of OVCA1. It was possible to

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6 A. K. Godwin, unpublished observations.
sppresses growth could fall into three categories: apoptosis, replicative senescence, or cell cycle arrest. On the basis of the amino acid sequence of OVCA1, it is unclear which, if any, of these pathways OVCA1 may affect. It is unlikely that OVCA1 promotes cell death to any great extent. TUNEL labeling and FACS analysis suggest that, although the OVCA1 stably expressing clones have a slightly elevated rate of apoptosis, it is not significant enough to account for the dramatic reduction in proliferation rates. It is also unlikely that exogenous OVCA1 restores replicative senescence because stable overexpression of OVCA1 did not affect rates of colony outgrowth (data not shown).

Cell cycle analysis of the OVCA1 stably expressing clones suggests that decelerated growth was associated with an increased percentage of the population in the G0-G1 phase of the cell cycle. We observed a reduction of cyclin D1 levels caused by destabilizing the protein, and this may be the direct cause of the slowed proliferation rates. In support of this hypothesis, cotransfection of cyclin D1 was able to override OVCA1’s suppression of clonal outgrowth. Cyclin D levels are primarly regulated at the transcriptional level in response to extraacellular mitogenic stimulation; however, in the absence of such stimulation, cyclin D is rapidly degraded by calpain proteases (Ref. 58; reviewed in Ref. 59). It is not yet clear as to how increased levels of OVCA1 leads to destabilization of cyclin D1. Deregulation of cyclin D1 has been implicated in the generation of many types of tumors. In some tumors, overexpression of cyclin D1 is achieved by amplification of the cyclin D1 gene (reviewed in Ref. 60). However, in other tumors, including ovarian tumors, overexpression of cyclin D1 is not associated with genetic alterations, suggesting that some other mechanism, perhaps an increase in stability, is the cause of the abnormality (61, 62).

Analyses of ovarian and other tumors clearly indicate that allelic loss of chromosome 17p13.13 is one of the more frequently observed molecular alterations; >70% of ovarian tumors, at least two-thirds of breast tumors, and many other types of tumors have lost part or all of one copy of chromosome 17 (see “Introduction”). It was previously thought that both alleles of a tumor suppressor gene must be inactivated, as addressed by the “two-hit” hypothesis for tumorigenesis of Knudson (63). However, it has recently been shown that genes such as the murine gene p27kip1 and the PTEN gene are haploinsufficient for tumor suppression (64, 65). Abnormal low levels of the p27 protein are frequently found in human carcinomas (66–70). They also spontaneously developed germ cell, gonadostromal, thyroid, and colon tumors, suggesting that PTEN haploinsufficiency plays a causal role in CD, LDD, and BZS (65). These studies, therefore, suggest that there is another class of tumor suppressor genes, in which genes that exhibit haploinsufficiency, leading to reduced levels of the protein, are important for tumorigenesis.

The data presented here and elsewhere, i.e., high rate of allelic loss observed for chromosome 17p13.13 in ovarian tumors, the reduced expression of OVCA1 in ovarian tumors, and the observation that an equimolar level of exogenous p50 OVCA1 suppresses the growth rate of tumor cells up to 10-fold, suggest that a slight reduction in the level of expression of OVCA1 is sufficient for loss of growth regulation. The high rate of loss of one copy of chromosome 17p in breast and ovarian tumors may contribute to carcinogenesis by reducing OVCA1 to hemizygosity, Future efforts aimed at clarifying the biochemical function of OVCA1 will aid in confirming the role that this gene has in tumorigenesis as well as its normal cellular function.

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REFERENCES


Expression of OVCA1, a Candidate Tumor Suppressor, Is Reduced in Tumors and Inhibits Growth of Ovarian Cancer Cells


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