

Reduction of BRCA1 Expression in Sporadic Ovarian Cancer¹

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Objective. The purpose of this study was to examine BRCA1 expression and its relationship to cell proliferation in sporadic ovarian epithelial tumors (OETs).

Methods. We investigated BRCA1 expression and cell proliferative activity in 72 unselected OETs. They comprised 16 benign cystadenomas, 18 borderline (low malignant potential) tumors, and 38 carcinomas (OECs). These patients had no known family history of breast and/or ovarian cancer. BRCA1 and the cell proliferation marker, MIB-1, expressions in fixed tissue were investigated in all 72 cases by immunohistochemistry (IHC). BRCA1 mRNA in fresh frozen tissue samples from 20 of these cases was measured by a semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR) method.

Results. The average percentage of BRCA1-positive cells was 5.6% in cystadenomas, 29.7% in borderline tumors, and 6.6% in OECs. The average decreased steadily with increasing grade of OECs: grade 1 (21.4%), grade 2 (1.1%), and grade 3 (0%). The average percentage of MIB-1-positive cells increased steadily from cystadenomas (7.5%) to borderline tumors (22.6%) to carcinomas (41.2%). BRCA1 expression was highly correlated with MIB-1 expression in cystadenomas and borderline tumors. Six of seven OECs negative for BRCA1 by IHC showed low levels of BRCA1 mRNA by RT-PCR.

Conclusions. BRCA1 expression paralleled cell proliferation in benign and borderline OETs, but not in OECs. Sporadic OECs showed significantly reduced levels, rather than complete loss, of BRCA1 expression. The reduction was closely related to tumor grade. Reduction of BRCA1 expression may be of etiologic significance in the occurrence and progression of sporadic ovarian cancer. © 2000 Academic Press

Key Words: BRCA1; cell proliferation; ovarian cancer.

INTRODUCTION

Women who carry BRCA1 mutations are at a significantly increased risk of developing ovarian epithelial cancer (OEC) [1, 2]. BRCA1 expression has been shown in a number of studies to be much reduced in sporadic breast cancers [3–8], but there is only a single report on BRCA1 expression in sporadic OECs [8]. Wilson *et al.* [8] found BRCA1 expression (distinct nuclear staining) by immunohistochemistry (IHC) in only 17 (49%) of 35 OECs; and loss of BRCA1 expression was significantly correlated with the pathological grade of the tumor.

High levels of BRCA1 mRNA expression are generally found in rapidly proliferating tissues [9], with maximal expression just before cells enter S phase [10]. Induced overexpression of BRCA1 in several breast and ovarian cancer cell lines resulted in growth inhibition [4]. Conversely, inhibition of BRCA1 expression by antisense oligonucleotides resulted in an increased rate of growth of the cell lines [4]. This evidence suggests that BRCA1 has a tumor suppressor function.

We report here our finding of reduced levels of BRCA1 expression in sporadic OECs, and that this reduction in expression represents in the majority of cases a reduction in BRCA1 gene function and not a loss of gene function. We also report our findings on cell proliferation in sporadic benign (cystadenomas) and borderline (low malignant potential, LMP) ovarian epithelial tumors (OETs), as well as in OECs, and the relationship between BRCA1 expression and cell proliferation in these OETs. There have been no previous reports on the expression of BRCA1 protein in relation to cell proliferation in sporadic OETs.

MATERIALS AND METHODS

Tumor Specimens

Formalin-fixed, paraffin-embedded tumor specimens were studied from 72 patients with OETs comprising 16 benign cystadenomas (10 serous and 6 mucinous), 18 borderline tumors (10 serous and 8 mucinous), and 38 carcinomas (22 serous, 6 mucinous, 5 endometrioid, and 5 other). All tumor

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samples were collected from 1995 through 1998 at the Department of Pathology, Women's and Children's Hospital, University of Southern California School of Medicine, from patients treated at the hospital. Tissues were obtained from total abdominal hysterectomy and bilateral salpingo-oophorectomy. Medical charts were reviewed and no family history of breast or ovarian cancers was recorded for any of these patients. Slides from all tumor samples were reviewed independently by two pathologists (FL, WZ). Histologic types and tumor grades (1 to 3) were assigned using International Federation of Gynecology and Obstetrics criteria.

Freshly frozen tumor samples were available from 20 of these 72 patients. These 20 samples comprised 5 cystadenomas, 5 borderline tumors, and 10 carcinomas.

Approval for use of these tissues was obtained from the Institutional Review Board at the University of Southern California School of Medicine.

BRCA1 and MIB-1 IHC Analyses

Mouse monoclonal antibody against human BRCA1 protein (Ab-1, IgG2a) generated from amino acid sequence 1–304 of BRCA1 was obtained from Oncogene Research Products (Cambridge, MA). The specificity of this BRCA1 antibody has been characterized by Wilson *et al.* [8]. MIB-1 (Ki-67 paraffin), a mouse monoclonal antibody (IgG1) recognizing a nuclear antigen expressed in all phases of the cell cycle except G₀ [11], was obtained from Immunotech, Inc. (Westbrook, ME). Five-micrometer parallel sections of the ovarian sections showing OETs were cut and placed on Super Plus slides (Fisher Scientific, Pittsburgh, PA) for IHC. A section of each specimen was stained with hematoxylin and eosin and examined microscopically to confirm the diagnoses. IHC analysis was performed on formalin-fixed and paraffin-embedded sections using the streptavidin–biotin–peroxidase methodology. BRCA1 and MIB-1 antigens were unmasked with the heat-mediated antigen retrieval method [8, 12]. The tissue sections were incubated overnight at 4°C with the monoclonal antibodies at a dilution of 1:200 (10 µg/ml) in PBS with 1% bovine serum albumin. The LSAB+ reagents (DAKO, Carpinteria, CA) were used for BRCA1 as recommended. Secondary antibodies for MIB-1 were provided by BioGenex (San Ramon, CA). Visualization was carried out with diaminobenzidine tetrahydrochloride as chromogen (BioGenex).

MCF-7 breast cancer cells and developing follicles in human ovarian sections served as positive controls for BRCA1 expression by IHC [13]. Ab-1 BRCA1 antibody was also validated in two ovarian cancers containing germline BRCA1 del185AG mutations. This specific mutation results in the deletion of most of the protein, including the Ab-1 epitope. As expected, no BRCA1 immunoreactivity was detected in these two cases. Cytokeratin IHC was done in all BRCA1-negative cases to rule out false negatives. Proliferative endometrial tissue sections served as positive controls for MIB-1 staining.

Negative controls were carried out by replacing primary antibodies with class-matched mouse IgGs on parallel ovarian sections.

Assessment of Staining for BRCA1 and MIB-1

Quantitative assessment of IHC results for both BRCA1 [8] and MIB-1 [14, 15] was based on distinct nuclear staining (Fig. 1). Moderate or strong nuclear staining was considered positive. Weak nonspecific staining was defined as negative, as was the occasional cytoplasmic staining for BRCA1 observed in some cases. Percentages of positively stained cells were calculated after counting a total of 500 epithelial tumor cells from each OET case. No cytoplasmic staining for MIB-1 was noted.

BRCA1 mRNA Measurement by Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

Total RNA was extracted from 0.5 mg of each of the 20 frozen specimens after mincing with Trizol (Gibco BRL, Rockville, MD) according to the manufacturer's instructions. RNA integrity was checked by agarose gel electrophoresis and ethidium bromide staining as well as by monitoring absorbance at A₂₆₀/A₂₈₀ before each RT-PCR. RNA concentration was determined by spectrophotometric analysis at 260 nm before each RT-PCR; RNA (0.5 µg) from each sample was used for a single RT-PCR. RT-PCR was performed as previously described [12]. The synthetic oligonucleotides used as BRCA1 PCR primers were 5'-GAT TTG ACG GAA ACA TCT TAC-3' (sense) and 5'-CCA GCA TCA GTA GTA TGA-3' (antisense). This pair of primers, which was derived from the BRCA1 sequence in GenBank (No. U14680), amplifies a DNA product from exons 14–15 with a fragment size of 236 bp. The annealing temperature was 57°C and 35 cycles were performed. β -Actin served as internal control. A semi-quantitative method was used to examine the BRCA1 mRNA expression level compared to β -actin mRNA expression level in different OET samples [16]. Relatively accurate quantitation can be achieved by serial dilution of multiple replicates of tumor cDNA samples; each cDNA sample for BRCA1 measurement was serially diluted five times (at least eight times for β -actin) prior to PCR. Twenty microliters of the PCR product was electrophoresed in a polyacrylamide gel (8%) in Tris–borate EDTA buffer. The 236-bp band was visualized under ultraviolet light after ethidium bromide staining. Each sample was scored as the number of bands seen. Each experiment was repeated three times with little variation. The numbers of positively visualized bands given in the tables were the medians of the triplicate experiments. The 540-bp product of β -actin from each sample was analyzed simultaneously in the same way. β -Actin was visualized up to the seventh dilution with all tumor samples. Three human ovaries containing developing follicles served as positive controls for BRCA1 expression by RT-PCR [13]. RNA from one of these ovaries was included with each RT-PCR experiment. The 236-bp product of BRCA1

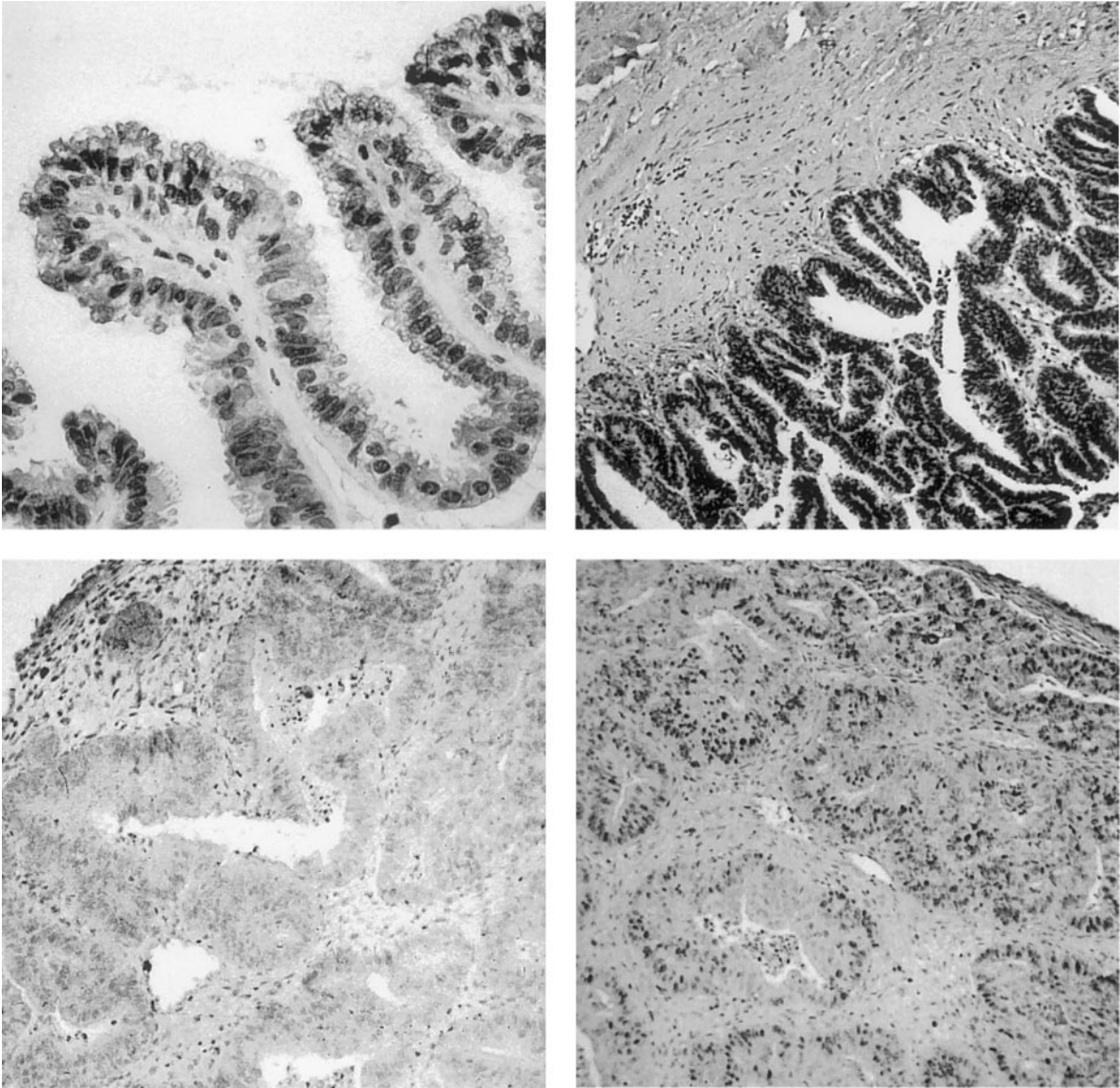


FIG. 1. Representative expression of BRCA1 and MIB-1 by immunohistochemistry in OETs. Nuclear staining of BRCA1 is illustrated in a cystadenoma (top left), a borderline tumor (top right), and a carcinoma (bottom left). No BRCA1 immunoreactivity was observed in this carcinoma case. This is in contrast to the diffuse MIB-1 nuclear immunoreactivity detected in the same ovarian cancer case (bottom right). No cytoplasmic, but occasional stromal staining is identified in these cases. Original magnifications: cystadenoma (400 \times), borderline tumor (200 \times), carcinoma (100 \times).

from the follicle-containing ovaries was still visualized after the fifth dilution. Negative controls included treatment with RNase A before the reverse transcription and absence of specific primers for BRCA1 under identical conditions for PCR. These were included with each RT-PCR experiment.

Statistical Analysis

Statistical analysis was carried out using standard methods with the SAS computer package system [17]. Differences between groups were analyzed by analysis of variance methods (equivalent to Student's *t* test when only two groups are in-

cluded). Standard Pearson correlation coefficients (*r* values) were calculated. All *P* values quoted are two-sided.

RESULTS

BRCA1 and MIB-1 IHC Expression

The BRCA1 immunoreactivity results (percentage of cell nuclear stained) are shown in Fig. 2. BRCA1 expression was found in 12 (75%) of the 16 benign cystadenomas and in 18 (100%) of the 18 borderline OETs, but in only 13 (34%) of the 38 OECs. There was a sharp decline in immunoreactivity with

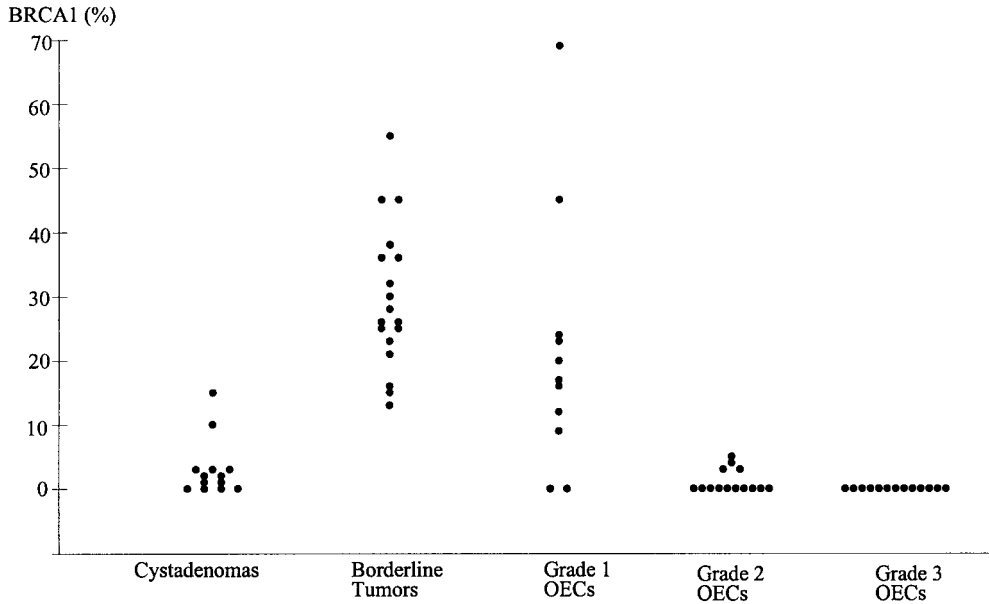


FIG. 2. BRCA1 detection by IHC in OETs. The percentage of OET cells positive for BRCA1 immunostaining was expressed after counting 500 tumor cells in each case. Compared to cystadenomas, borderline tumors showed increased BRCA1 expression. OECs showed significant reduction of BRCA1 expression in grade 2 cases and no expression in grade 3 cases; grade 1 tumors showed reduced expression when expressed relative to MIB-1 expression (see Fig. 3).

increasing grade of OEC: 9 (82%) of the 11 grade 1 (well differentiated) tumors were positive, compared to 4 (29%) of the 14 grade 2 (moderately differentiated) tumors, and none of the 13 grade 3 (poorly differentiated) tumors. Representative pictures of BRCA1 immunostaining are shown in Fig. 1. All BRCA1-negative OECs stained positive for cytokeratin.

The percentage of BRCA1-positive cells averaged 5.6% in cystadenomas, 29.7% in borderline tumors, and 6.6% in OECs (Table 1). Grade 1 OECs had an average of 21.4% positive cells, grade 2 OECs 1.1% positive cells, and grade 3 OECs were uniformly negative by IHC. The differences in percentage of BRCA1 staining between the OET types was highly statistically significant ($P < 0.001$).

MIB-1 expression was evaluated in parallel sections of the OET samples. MIB-1 immunoreactivity was detected in all 72

cases. The percentage of MIB-1-positive cells increased steadily from cystadenomas (average 7.5%) to borderline tumors (22.6%) to OECs (41.2%) (Table 1). This trend was highly statistically significant ($P < 0.001$). A representative picture of MIB-1 immunoreactivity is shown in Fig. 1.

There was a close association between BRCA1 and MIB-1 expression in cystadenomas and borderline tumors ($r = 0.72$, $P < 0.001$; Fig. 3). The BRCA1/MIB-1 ratio did not differ significantly between cystadenomas and borderline tumors when only the cystadenomas showing BRCA1 expression were considered. The average ratio for these cystadenomas plus

TABLE 1
BRCA1 and MIB-1 Expression in OETs

OET type	N	BRCA1 (average \pm SE) ^a (%)	MIB-1 (average \pm SE) (%)	BRCA1/MIB-1 ratio (average \pm SE) (%)
Cystadenoma	16	5.6 \pm 1.7	7.5 \pm 2.2	0.75 \pm 0.19
LMP tumor	18	29.7 \pm 2.6	22.6 \pm 3.0	1.60 \pm 0.19
Carcinoma	38	6.6 \pm 2.3	41.2 \pm 3.4	0.19 \pm 0.07
Grade 1	11	21.4 \pm 6.1	40.0 \pm 6.1	0.59 \pm 0.19
Grade 2	14	1.1 \pm 0.5	34.0 \pm 4.4	0.04 \pm 0.02
Grade 3	13	0.0	50.1 \pm 6.8	0.00

^a SE, standard error.

Correlation of BRCA1 and MIB-1 Expression in Benign and Borderline Ovarian Epithelial Tumors

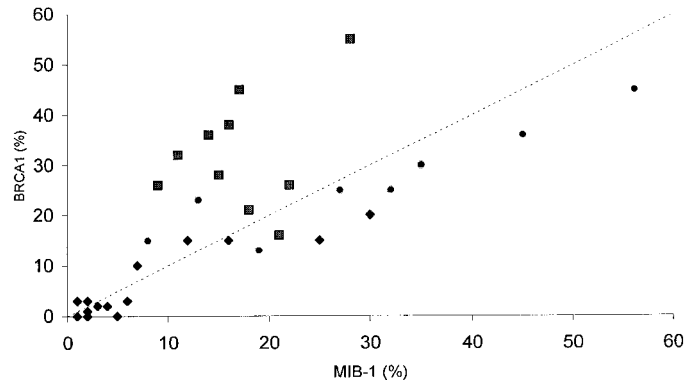


FIG. 3. Relation of BRCA1 and MIB-1 expression in cystadenomas, borderline tumors, and grade 1 OECs: diamonds, cystadenomas; circles, borderline tumors; and squares, grade 1 OECs.

borderline tumors combined was 1.36 ± 0.15 (average \pm standard error). This ratio was much reduced in OECs showing BRCA1 expression: grade 1 OECs, 0.72 ± 0.19 ; and grade 2 OECs, 0.16 ± 0.03 ($P < 0.01$ for comparison with BRCA1+ cystadenomas plus borderline tumors). The reduced BRCA1/MIB-1 ratio in grade 1 OECs can be easily seen in Fig. 3.

BRCA1 expression in normal ovarian tissue was mainly localized in granulosa and theca interna cell nuclei of developing follicles. The pattern of staining in ovarian follicles was uniform (data not shown). No BRCA1 immunoreactivity was observed in the stroma of normal ovaries. A small percentage (1–3%) of BRCA1-positive cells was seen in ovarian epithelial inclusions (data not shown).

For OETs, BRCA1 immunoreactivity was mainly observed in the nuclei of the epithelial cells (Fig. 1). Two (12%) of the 16 cystadenomas, 2 (11%) of the 18 borderline tumors, and 4 (11%) of the 38 OECs showed moderate cytoplasmic staining. Occasional stromal staining for BRCA1 was also noted in 3 (4.2%) of 72 OETs.

TABLE 2
BRCA1 Levels in Ovarian Epithelial Tumors

Band: ^a	RT-PCR Products in serial dilution					IHC positive ^b (%)
	1	2	3	4	5	
Cystadenomas						
6 ^c	+	+	+	–	–	15
7	+	+	+	+	–	20
8	+	+	+	–	–	3
14	+	+	–	–	–	3
16	+	+	+	–	–	10
Borderline tumors						
1	+	+	+	+	–	38
2	+	+	+	+	–	36
3	+	+	+	+	–	55
5	+	+	+	+	–	45
12	+	+	+	+	–	30
Carcinomas						
1	–	–	–	–	–	0
2	+	–	–	–	–	0
3	+	–	–	–	–	0
4	+	–	–	–	–	0
5	+	–	–	–	–	0
6	+	+	–	–	–	12
9	+	+	–	–	–	9
10	+	+	+	–	–	17
31	+	–	–	–	–	0
32	+	–	–	–	–	0

^a Bands 1 to 5 represent the serial dilution numbers of cDNA samples prior to PCR amplification. +, band visualized; –, no band visualized.

^b Percentages of tumor cells stained in the corresponding samples studied by IHC.

^c Case number.

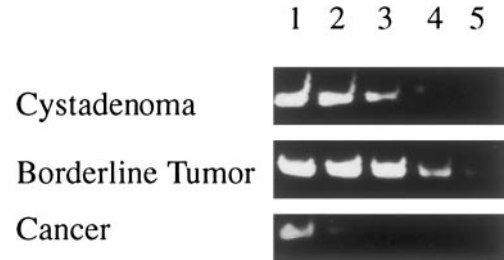


FIG. 4. Representative expression of BRCA1 mRNA levels in OETs with semi-quantitative RT-PCR. Results from a representative cystadenoma (case 16), a borderline tumor (case 12), and a carcinoma (case 3) are shown. Numerical numbers, 1 to 5, represent serial dilutions of the cDNA samples after reverse transcription. BRCA1 product was not visualized after the third dilution (three bands) for the cystadenoma, after the fourth dilution (four bands) for the borderline tumor, and after the first dilution for the carcinoma (one band). The 540-bp product of β -actin from the above three representative samples was visualized up to the seventh dilution (data not shown).

BRCA1 mRNA Detection

All but 1 of the 20 OETs showed at least one band for BRCA1 mRNA by RT-PCR (Table 2). Borderline tumors had slightly higher levels than benign or malignant OETs. Of the 5 cystadenomas, 3 showed three bands, 1 showed two bands, and 1 showed four bands. All 5 borderline tumors showed PCR product in the fourth dilution (four bands). All 5 benign cystadenomas and 5 borderline tumors examined by RT-PCR were positive for BRCA1 by IHC. One of the carcinomas showed no bands; only 3 showed more than one band, and these were the only ones positive for BRCA1 by IHC. Representative RT-PCR results are shown in Fig. 4. β -Actin was still visualized at the seventh dilution with all tumor samples but died thereafter with no differences being noted between the 20 OET samples (data not shown).

DISCUSSION

BRCA1 expression was detected by IHC in 9 (82%) of 11 grade 1 OECs, but only in 4 (29%) of 14 grade 2 OECs, and in none of the 13 grade 3 OECs. Although a high proportion of the grade 1 OECs expressed BRCA1 at a relatively high level (Fig. 1), the level of expression in these BRCA1+ OECs was much reduced relative to MIB-1 expression when compared to the results in borderline tumors, suggesting that BRCA1 function was also reduced in these low grade BRCA1+ OECs. Six of 7 OECs negative for BRCA1 by IHC were positive for BRCA1 expression by RT-PCR. (All 7 showed positive expression for cytokeratin by IHC, which eliminates the possibility of false IHC negatives for BRCA1). This suggests that normal BRCA1 may be being expressed at very low levels in OECs negative for BRCA1 by IHC, rather than there being a complete loss of function. These results confirm and extend the finding of Wilson *et al.* [8] of reduced BRCA1 expression in ovarian carcinomas.

The mechanisms of the reduced levels of BRCA1 expression in sporadic OECs are unknown. They could be due to a combination of several genetic and epigenetic changes. Although mutations of BRCA1 are rare in sporadic OECs [18–20], loss of heterogeneity of chromosome 17q markers, including the BRCA1 region, is relatively common [21–23]. Alternatively, lower BRCA1 activity may be related to epigenetic changes such as methylation of its promoter region [24, 25]. Methylation of the BRCA1 promoter has been shown to be associated with reduced gene activity in sporadic breast cancers [6, 26, 27].

BRCA1 expression was highly correlated with MIB-1 expression in cystadenomas and borderline tumors (Fig. 3). This is in agreement with evidence suggesting that BRCA1 acts in concert with DNA repair enzymes to maintain the integrity of the genome during periods of rapid growth [28–31]. Borderline tumors are recognized as a distinct subset of OETs that display clinical and histologic features intermediate between benign and malignant OETs. In contrast to malignant OETs, borderline tumors tend to occur in younger patients and have an excellent 95% overall survival [32]. The proliferative activity of borderline tumors was significantly higher than that of benign cystadenomas. There was no evidence that BRCA1 expression was reduced in borderline tumors. These findings suggest that BRCA1 function is still intact in the majority of cystadenomas and borderline tumors and may contribute to the indolent biological behavior of borderline tumors.

BRCA1 immunoreactivity in normal ovary was concentrated in the nuclei of granulosa and theca interna cells of the developing follicles, the vast majority of which were BRCA1 positive; this is consistent with observations made on mouse ovary [13]. BRCA1 immunoreactivity was also mainly observed in the nuclei of OET cells. However, we did find occasional cytoplasmic staining in OECs. This latter finding is in contrast to the report of Wilson *et al.* [8] who, using the same antibody (Ab-1), found BRCA1 immunoreactivity exclusively in the nucleus. We did not detect BRCA1 immunoreactivity in stroma from normal ovaries. However, we observed occasional BRCA1 immunoreactivity in the stroma of several cases of OETs. The nondetectable or rare immunoreactivity of BRCA1 in ovarian stroma is possibly due to the mitotically inactive nature of these stromal tissues. This finding is again in contrast to that of Wilson *et al.* [8] who reported that BRCA1 was expressed uniformly in all types of ovarian cells. We have no explanations for these discrepant findings. Further studies by other investigators are needed to clarify these issues.

Cell proliferation rate and tumor grade are important prognostic factors in ovarian cancer [33, 34]. Compared with the benign and borderline OETs, OECs showed significantly increased proliferative activity. However, there was no difference in cell proliferation between grade 1 and grade 2 OECs in this study, although grade 3 OECs did have the highest proliferation rate. Generally, increased cell proliferation is observed in tumors with increasing grade. This slight inconsistency in

our data may simply be due to relatively small sample sizes. We found that reduced BRCA1 expression correlates with the known poor biological behavior of the high-grade sporadic ovarian cancers. This is consistent with the recently reported finding that hereditary BRCA1-associated ovarian cancer cases tend to be of high grade [35]. It is, however, not clear whether this is compatible with the previous report of a more favorable clinical outcome in hereditary BRCA1-associated ovarian cancers [36].

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