BRCA1 is Expressed in Uterine Serous Carcinoma (USC) and Controls Insulin-Like Growth Factor I Receptor (IGF-IR) Gene Expression in USC Cell Lines

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Objective: The insulin-like growth factor I receptor (IGF-IR) and BRCA1 affect cell growth and apoptosis. Little information is available about BRCA1 activity on the IGF signaling pathway. This study evaluated the effect of BRCA1 on IGF-IR expression.

Methods: BRCA1 and IGF-IR immunohistochemistry on archival tissues (35 uterine serous carcinomas [USCs] and 17 metastases) were performed. USPC1 and USPC2 cell lines were transiently cotransfected with an IGF-IR promoter construct driving a luciferase reporter gene and a BRCA1 expression plasmid. Endogenous IGF-IR levels were evaluated by Western immunoblotting.

Results: We found high BRCA1 and IGF-IR protein expression in primary and metastatic USC tumors. All samples were immunostained for BRCA1—71% strongly stained; and 33/35 (94%) were stained positive for IGF-IR—2 (6%) strongly stained. No difference in BRCA1 and IGF-IR staining intensity was noted between BRCA1/2 mutation carriers and noncarriers. Metastatic tumors stained more intensely for BRCA1 than did the primary tumor site ($P = 0.041$) and with borderline significance for IGF-IR ($P = 0.069$). BRCA1 and IGF-IR staining did not correlate to survival. BRCA1 expression led to 35% and 54% reduction in IGF-IR promoter activity in the USPC1 and USCP2 cell lines, respectively. Western immunoblotting showed a decline in phosphorylated IGF-IR and phosphorylated AKT in both transiently and stably transfected cells.

Conclusions: BRCA1 and IGF-IR are highly expressed in USC tumors. BRCA1 suppresses IGF-IR gene expression and activity. These findings suggest a possible biological link between the BRCA1 and the IGF-I signaling pathways in USC. The clinical implications of this association need to be explored.

Key Words: Insulin-like growth factor I (IGF-I), IGF-I receptor, BRCA1, Uterine serous carcinoma

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The insulin-like growth factors (IGFs) IGF-I and IGF-II are a family of mitogenic polypeptides with important roles in cell growth and differentiation. The biological actions of the IGFs are mediated by the IGF-I receptor (IGF-IR), a tyrosine kinase-containing cell surface receptor that is structurally and functionally related to the insulin receptor.[1,2] The IGF system has a pivotal role in cellular growth, differentiation, proliferation, and prevention of apoptosis, as well as in cancer transformation, metastasis, and angiogenesis.[3,4] Consistent with its prosurvival role, IGF-IR expression and signaling are enhanced in most tumors and transformed cells, including breast, prostate, colorectal, thyroid, central nervous system, lung, ovarian, and endometrial cancers.[5]

Endometrial cancer is the most widespread gynecologic cancer in Western countries. Uterine serous carcinoma (USC) represents 10% of all endometrial carcinomas, is usually diagnosed at an advanced stage, and accounts for 50% of all endometrial cancer relapses, with a 5-year survival rate of 55%. An important body of evidence has identified a correlation between some of the major components of the IGF system and endometrial cancer risk. Thus, Ayabe et al[6] reported higher endocrine IGF-I and lower IGF-binding protein 1 (IGFBP-1) levels in postmenopausal patients with endometrial cancer. McCampbell et al[7] reported a large increase in IGF-IR levels in biopsies from hyperplastic endometrium and endometrial carcinoma compared with proliferative endometrium. Finally, and consistent with its potent antiapoptotic activity, Hirano et al[8] reported high IGF-IR expression in all gynecological cancers, with messenger RNA expression detected in 91% of endometrial cancers. Taken together, these studies suggest a crucial role for IGF-IR in endometrial cancer and emphasize the importance of altered IGF-IR gene expression in the development of a malignant phenotype.

BRCA1 is a tumor suppressor gene whose mutation has been associated with the appearance of breast and/or ovarian cancer at young ages. The BRCA1 gene product participates in multiple biological pathways including DNA damage repair, transcriptional control, cell growth, and apoptosis. Of interest, BRCA1 has been shown to inhibit IGF-IR transcription in breast, osteosarcoma, and ovarian cancer cell lines, suggesting that a potential mechanism of action of BRCA1 involves suppression of IGF-IR gene expression.[9,10] In contrast, mutant BRCA1 proteins lacking transcriptional activity are impaired in their ability to suppress the IGF-IR promoter, with resulting increases in IGF-IR messenger RNA and IGF binding in mammary tumors.[9,10] Finally, and consistent with the postulate that mutant BRCA1 may lead to dysregulated IGF-IR expression, a recent immunohistochemical analysis revealed significantly elevated IGF-IR levels in primary breast tumors derived from BRCA1 mutation carriers compared with sporadic tumors.[11]

In a recent study, we investigated the rate of 3 predominant BRCA1/2 mutations in Jewish patients with USC and the relevance of carrier status to clinicopathological features and survival.[12] Overall, 8 (25.8%) of 31 patients were mutation carriers: 4 BRCA2 (6174delT) carriers and 2 patients each carried the BRCA1 (185delAG) and the BRCA1 (5382InsC) mutations. These data suggested that USC could be considered an integral component of the hereditary breast-ovarian cancer syndrome. To the best of our knowledge, no information is available in the literature regarding the expression of IGF-IR and BRCA1 in USC, the most aggressive type of endometrial cancer. Furthermore, no studies have addressed the potential interactions between the BRCA1 and IGF signaling pathways in USC. In view of the putative roles of BRCA1 and IGF-IR in USC biology and to expand our previous studies on the interaction between these important proteins, in the present study, we evaluated the expression of BRCA1 in a collection of USC specimens and explored the regulation of IGF-IR gene expression and action by BRCA1.

MATERIALS AND METHODS

Patients and Tissue Samples

Samples from 35 consecutive patients who underwent surgery after USC diagnosis at the Gynecologic Oncology Division, Meir Medical Center, Kfar Saba, Israel, were analyzed. Pathologic reevaluation was performed in all cases, and the diagnosis of USC was based on accepted criteria described by Hendrickson et al.[13] Cases were excluded if the papillary serous component accounted for less than 25% of the surgical specimen,[14] if the predominant histological component was clear cell,[13] and if a primary site of origin could not be definitively determined. Of note, 30 patients were examined for the 3 predominant Jewish germ line BRCA1/2 mutations as previously described.[12] Five patients refused to be analyzed for the presence of BRCA1/2 mutations.

Insulin-like growth factor I receptor and BRCA1 protein expression were evaluated using formalin-fixed, paraffin-embedded tissues from patients with USC. The specimens were collected from the pathology department at Meir Medical Center. For each tumor, slides containing adjacent nonneoplastic endometrium (for internal controls) were chosen. Archival paraffin blocks were retrieved and 4-μm slices were placed on SuperFrostR Plus glass slides (Thermo Fisher Scientific, Waltham, MA). One slide from each specimen was stained by hematoxylin and eosin. The institutional review board of the hospital approved the study, and written informed consent was obtained from the participants. Pertinent clinical information was extracted from inpatient and outpatient charts.

Immunohistochemical Stains

The following primary antibodies were used: BRCA1 (H-100; SC-7867, Santa Cruz Biotechnology, Santa Cruz, CA; dilution 1:10) and IGF-IRβ (C-20; SC-713, Santa Cruz Biotechnology; dilution 1:100). Immunohistochemical stains were carried out on a Benchmark XT automatic immunostainer (Ventana Medical Systems, Tucson, AZ).

Evaluation of Immunohistochemical Staining

The *intensity* of staining was graded as 0, no staining; 1, weak staining; 2, moderate staining; or 3, strong staining. Adjacent endometrial tissue was considered as strong staining grade 3 and was used as an internal control. The *extensiveness* of staining (with no reference to intensity) was graded either 1, less than 50%; or 2, 50% or greater. The *staining* was graded according to both intensity and extensiveness using the following scheme:

I	No staining	= Negative
II	Weak to moderate staining in <50% of tumor cell	= Weak/mild
III	Weak to moderate staining in ≥50% of tumor cells	= Moderate/medium
IV	Moderate to strong staining in ≥50% of tumor cells	= Strong

Of note, all cases of strong staining had at least 50% of tumor cells stained. All staining was graded separately by 2 experienced pathologists. Differences in more than 1 grade were present in only 5% of cases. Differences derived from fading of tumor and inconsistent staining throughout large areas of tumors. Final scores were agreed upon by both examiners.

Cell Cultures

The uterine serous papillary carcinoma cell lines USPC1 and USPC2 were kindly provided by Dr. Alessandro Santin (Yale University School of Medicine, New Haven, CT). Cell lines were maintained in RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum, 2-mmol/L glutamine, 50-μg/mL gentamicin sulfate, and 5.6-mg/L amphotericin B (Sigma-Aldrich Co, St. Louis, MO). Cells were incubated at 37°C in a humidified atmosphere containing 5% carbon dioxide.

Luciferase Reporter Plasmids, Transfections, and Promoter Activity Measurements

An expression vector encoding wild-type BRCA1 was constructed by cloning the BRCA1 complementary DNA (cDNA) into artificially engineered *Hin*dIII and *Not*I sites in the pcDNA3 vector (Invitrogen Corp, Carlsbad, CA).[15] The BRCA1 vector was kindly provided by Dr. Lawrence Brody (NIH, Bethesda, MD). For transient cotransfection experiments, an IGF-IR promoter luciferase reporter construct that included 476 base pairs (bp) of 5′-flanking region and 640 bp of 5′-untranslated region of the rat IGF-IR gene [p(−476/+640)LUC] was used. The promoter activity of this genomic fragment has been previously described.[16] USPC1 and USPC2 cells were transfected with 1 μg of the [p(−476/+640)LUC] construct, along with 1 μg of the BRCA1 expression vector and 0.3 μg of a β-galactosidase expression plasmid (pCMV-β, Clontech, Palo Alto, CA), using the Jet-PEI (Polyplus Transfection, Illkirch, France) transfection reagent. Control cells were transfected with an empty pcDNA3 vector. Promoter activities were expressed as luciferase values normalized for β-galactosidase activity.

Western Immunoblots

Cells were serum starved overnight, and IGF-I (50-g/mL) was added for 10 minutes. Then, cells were lysed in a buffer containing protease inhibitors (Cell Signaling Technology, Beverly, MA). Protein content was determined using the Bradford reagent (Bio-Rad, Hercules, CA) and bovine serum albumin as a standard. Samples were electrophoresed through 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis followed by blotting of the proteins onto nitrocellulose membranes. After blocking with 5% skim milk and/or 3% bovine serum albumin, the blots were incubated overnight with the antibodies listed below, washed, and incubated with the appropriate horseradish peroxidase-conjugated secondary antibody. Antibodies against phospho-IGF-IR (3024), IGF-IR β-subunit (3027), and phospho-AKT (9271) were obtained from Cell Signaling Technology. An antibody against BRCA1 (C20) was purchased from Santa Cruz Biotechnology. An anti-tubulin antibody (Clone: B-5-1-2) was purchased from Sigma-Aldrich Co. The secondary antibodies were horseradish peroxidase-conjugated goat antirabbit IgG (1:50,000) and donkey antimouse IgG (1:25,000; Jackson ImmunoResearch Laboratories, West Grove, PA). Proteins were detected using the SuperSignal West PicoChemiluminescent Substrate (Pierce, Rockford, IL). The expression of tubulin was used as a loading control for total proteins.

Statistical Analysis

The statistical significance of the differences observed between groups was assessed using the $\chi 2$, *t* test, or Mann-Whitney rank test, each when appropriate. Correlation between continuous parameters was assessed using Pearson or Spearman correlations, each when appropriate. Kaplan-Meier was used for survival analyses.

Primary and metastatic tissue specimens were matched samples from the same patient. For the matched samples from the same patient, paired *t* test, Wilcoxon nonparametric test, and McNemar test were used, each when appropriate. $P < 0.05$ was considered statistically significant. The data were analyzed using SPSS-17.

RESULTS

Immunohistochemical Analysis of BRCA1 and IGF-IR Expression in USC

The potential involvement of tumor suppressor BRCA1 in the etiology of USC has been the topic of controversial research. To investigate the expression of BRCA1 in USC, and its presumed correlation with the IGF system, we performed an immunohistochemical staining of these proteins in USC paraffin blocks. The study group comprised 35 patients with

TABLE 1. Distribution of immunohistochemical staining for BRCA1 and IGF-IR

	BRCA1 n (%)				IGF-IR n (%)			
Staining	**None**	**Mild**	**Moderate**	**Strong**	**None**	**Mild**	**Moderate**	**Strong**
Uterus n = 35	0	1 (2.8)	9 (25.7)	25 (71.4)	2 (5.7)	18 (51.4)	13 (37.1)	2 (5.2)
Metastases n = 17	0	0	3 (17.6)	14 (82.3)	1 (5.9)	3 (17.6)	12 (70)	1 (5.9)

IGF-IR and BRCA1 levels were assessed by immunohistochemical staining. Scoring was determined as described in the "Materials and Methods" section.

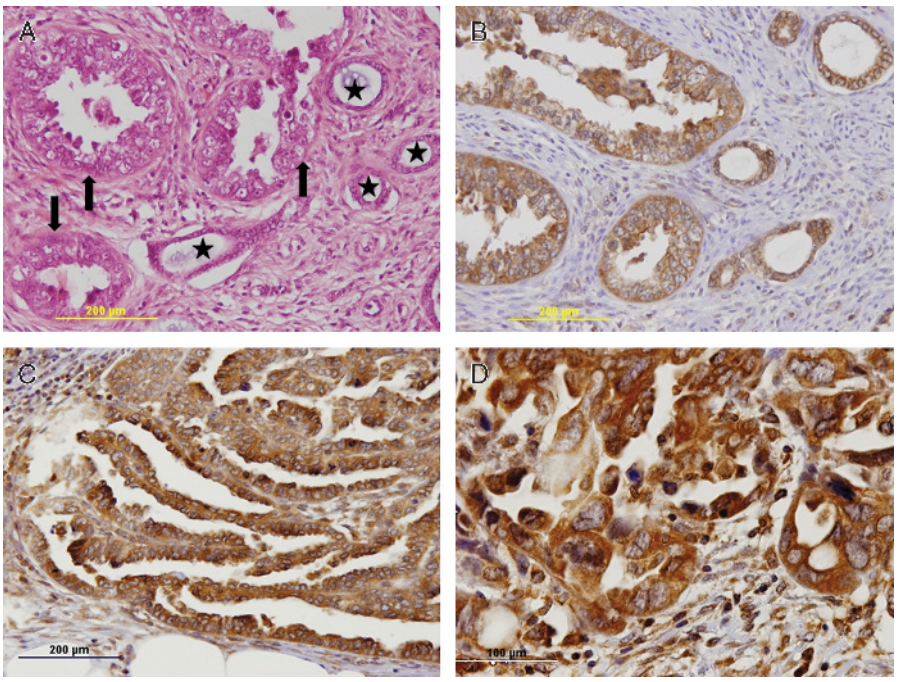

FIGURE 1. Distribution of immunohistochemical staining for BRCA1 in primary and metastatic USC. Metastatic tumors stained more intensely than primary tumors for BRCA1 ($P = 0.041$). A, Primary tumor, neoplastic glands (arrows) adjacent to nonneoplastic endometrial glands (asterisks); hematoxylin and eosin, original magnification ×200. B, Primary tumor; moderate cytoplasmic staining in greater than 50% of cells in neoplastic and nonneoplastic glands. C, Metastasis; moderate and strong cytoplasmic staining in nearly all neoplastic cells. D, Metastasis, a focus of increased atypia; strong staining in all neoplastic cells.

histologically confirmed USC. These patients' condition was diagnosed, and they underwent surgery at Meir Medical Center between April 1 and December 31, 2007. All 35 patients had archival uterine tissue, and 17 patients had additional archival metastasis tissue.

Results of immunohistochemical staining revealed a very high expression of BRCA1 and IGF-IR in primary and metastatic USC tumors (Table 1). BRCA1 immunostaining was cytoplasmic. BRCA1 was expressed in all primary and metastatic tumors with strong staining in 71% (25/35) of primary tumors and 82.4% (14/17) of metastatic tumors., Insulin-like growth factor I receptor immunostaining was also cytoplasmic. Ninety-four percent (33/35) of the primary tumors stained positively for IGF-IR and 6% (2/35) exhibited a strong staining. Of the metastatic tumors, 94% (16/17) stained positively for IGF-IR, while 6% (1/17) displayed strong IGF-IR staining.

Metastatic tumors stained more intensely for BRCA1 (82.3% strong staining) compared to the primary tumor site (71.4% strong staining; $P = 0.041$; Fig. 1). Staining was more intense for IGF-IR, expressed with borderline statistical significance, as more blocks with moderate staining (71% vs 37%; $P = 0.069$; Fig. 2). No significant correlation was observed between BRCA1 and IGF-IR protein expression. Moreover, there was no significant difference in BRCA1 and IGF-IR staining intensity in BRCA1/2 mutation carriers compared with noncarriers. Finally, there was no significant correlation between BRCA1 and IGF-IR staining and survival.

Regulation of IGF-IR Promoter Activity by BRCA1 in USC Cells

Because BRCA1 was previously shown to negatively control IGF-IR levels in prostate and breast cancer cells,[9,10,17] we examined the potential control of IGF-IR expression by BRCA1 in 2 USC-derived cell lines. To this end, cotransfection experiments were performed in the USPC1 and USPC2 cell lines using a BRCA1 expression vector along with construct p(−476/+640)LUC, which contains most of the proximal region of the IGF-IR promoter fused to a luciferase reporter gene. Cells were harvested 48 hours after transfection, and luciferase and β-galactosidase activities were measured. As shown in Figure 3, BRCA1 overexpression led to a 65% reduction in IGF-IR promoter activity in the USPC1 cell line and a 46% decrease in the USPC2 cell line compared with pcDNA3-transfected cells.

Regulation of Endogenous IGF-IR Levels by BRCA1 in USC Cells

Next, we studied whether BRCA1 was able to suppress the expression of the endogenous IGF-IR gene. For this purpose, USPC2 cells were transfected with a BRCA1 expression vector, and IGF-IR levels were measured by Western blotting. The results obtained revealed that IGF-IR levels were largely reduced in BRCA1, overexpressing USPC2 cells in comparison to pcDNA3-transfected (control) cells (Fig. 4A). In addition,

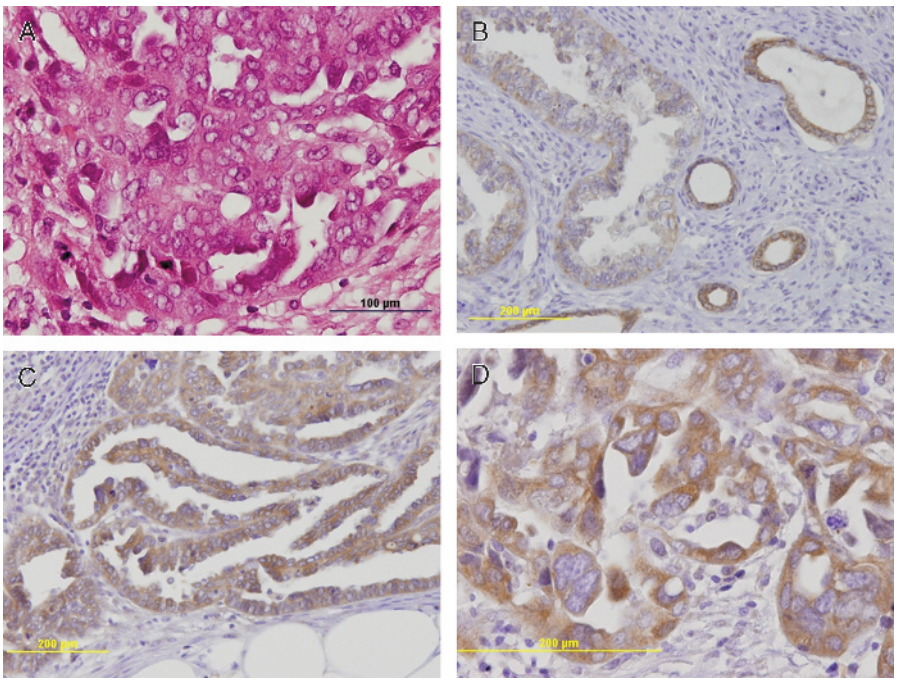

FIGURE 2. Distribution of immunohistochemical staining for IGF-IR. Metastatic tumors stained more intensely, with borderline significance for IGF-IR, expressed as more moderately stained blocks (71% vs 37%; $P = 0.069$). A, A focus of increased atypia in metastatic tumor; enlarged, pleomorphic and bizarre nuclei, atypical mitoses. Hematoxylin and eosin, original magnification ×400. B, Primary tumor; weak cytoplasmic staining in less than 50% of cells (grade 2) in neoplastic glands, moderate staining in greater than 50% of cells in adjacent nonneoplastic glands. C, Metastasis; moderate cytoplasmic staining in nearly all neoplastic cells. D, Metastasis, a focus of increased atypia; moderate and strong staining in all neoplastic cells.

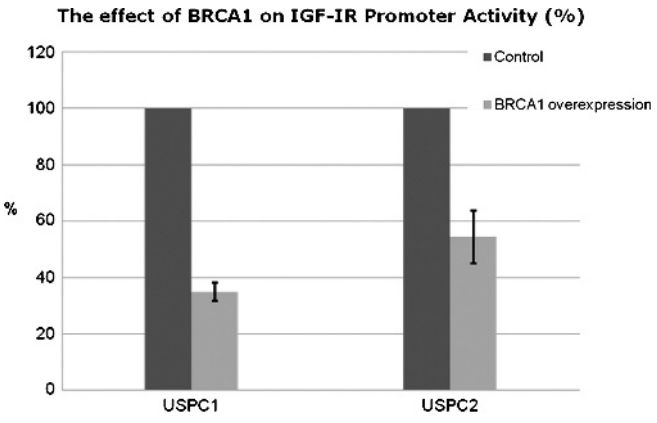

FIGURE 3. Repression of IGF-IR promoter activity by BRCA1 in USC cell lines. A BRCA1 expression vector (or empty pcDNA3) was transiently transfected into USPC1 and USPC2 cell lines, along with the p(−476/+640) LUC reporter plasmid, containing most of the proximal region of the IGF-IR promoter upstream of a luciferase reporter, and a β-galactosidase control plasmid. Luciferase and β-galactosidase activities were examined after 48 hours. BRCA1 overexpression led to a 65% reduction in IGF-IR promoter activity in the USPC1 cell line and a 46% decrease in the USPC2 cell line compared to pcDNA3-transfected cells.

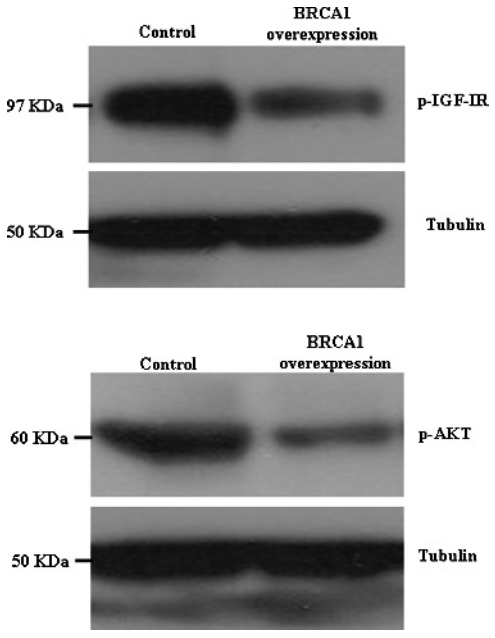

FIGURE 4. Repression of endogenous phospho-IGF-IR and phospho-AKT levels by BRCA1. Endogenousphos pho-IGF-IR (top) and phospho-AKT (bottom) levels were largely reduced in BRCA1-overexpressing USPC2 cells in comparison with USPC2 control cells.

BRCA1 overexpression led to a major reduction in phospho-AKT, an important downstream mediator of IGF-IR action (Fig. 4B), suggesting that BRCA1 has a negative effect on IGF-IR signaling in USC cells.

DISCUSSION

A solid body of evidence supports the notion that IGF-IR action has a crucial role in endometrial cancer and emphasizes the importance of altered IGF-IR gene expression in the development of the disease.[6–8] Most studies, however, were performed on type I endometrial cancers; and, to the best of our knowledge, no information is available regarding the involvement of the IGF system in USC. The clinical course and the histological picture of USC are similar to those of ovarian serous papillary cancer (OSPC). Interestingly, Gotlieb et al[18] recently confirmed the presence of an in vitro mitogenic autocrine loop involving the IGF system in OSPC, suggesting that the IGF system might also play a role in USC.

BRCA1 mutation correlates with the appearance of breast and ovarian cancer at very young ages. Given the clinical and histological similarity between OSPC and USC, a controversy arose concerning the role of BRCA1 mutations in the pathogenesis of USC. Lavie et al[19] found 20% to 27% BRCA mutation carriers among a group of patients with USC, whereas Goshen et al[20] reported no carriers among a group of 56 patients with USC. We recently demonstrated a high frequency (25%) of BRCA1/2 germ line mutations in patients with USC.[12] No significant differences in clinical survival parameters were noticed between mutation carriers and noncarriers. If confirmed by future larger studies, our data are consistent with the concept that USC constitutes an expression of the hereditary breast-ovarian cancer syndrome.

Very few information is available regarding a potential functional correlation between BRCA1 and the IGF system. A previous study showed that at least part of the biological actions of IGF-I in mammary gland cells can be mediated through BRCA1 and that dysregulated BRCA1 expression resulting from aberrant IGF signaling may have important consequences relevant to breast cancer pathogenesis.[21] Furthermore, we recently provided evidence that BRCA1 is expressed in prostate cancer and that its mechanism of action in prostate cancer cells involves modulation of IGF-IR gene transcription.[22] This is the first report demonstrating a high expression of BRCA1 in USC tumors, with positive staining of 100%. This finding suggests that the BRCA1 and IGF signaling pathways may play an important role in the biology of USC.

Results of cotransfection experiments revealed that BRCA1 overexpression led to a 65% decline in IGF-IR promoter activity in the USPC1 cell line and to a 46% decline in USPC2 cells. These results are consistent with previous data suggesting an inhibitory effect of BRCA1 on IGF-IR gene transcription. Measurements of the endogenous IGF-IR protein in the USPC2 cell line corroborated the inhibitory role of BRCA1 on IGF-IR expression and emphasized the physiological relevance of our data. Furthermore, the fact that BRCA1 overexpression led to a reduction in phospho-AKT, an important downstream mediator of IGF-IR, further emphasizes the notion that BRCA1 is involved in controlling the IGF system in USC. We previously reported similar results with prostate cancer.[22] Specifically, we observed an inverse correlation between BRCA1 and IGF-IR levels in the P69 and M12 prostate cancer-derived cell lines. Coexpression experiments revealed that BRCA1 was able to suppress IGF-IR promoter activity and endogenous IGF-IR levels.

However, we found no correlation between BRCA1 and IGF-IR staining intensities in paraffin blocks. Moreover, no correlation was observed between BRCA1 mutational status and staining intensity of either BRCA1 or IGF-IR. We speculate that this lack of correlation stems from the fact that transcriptional regulation of the IGF-IR gene is a complex mechanism involving a large series of transcription factors in addition to BRCA1. Therefore, it is often difficult to detect an inverse correlation in vivo. Of interest, Maor et al[11] reported a significant elevation in IGF-IR levels in breast tumors from BRCA1 mutation carriers compared with noncarriers. Hudelist et al[23] reported that IGF-I protein expression was significantly up-regulated in tumors of BRCA mutation carriers when compared with matched sporadic tumors. A recent study demonstrated a significant association between breast cancer risk and IGF-IR and IRS1 variants in BRCA1 carriers and with IGFBP2 variants in BRCA2 carriers.[24] Of interest, metastatic tumors stained more intensely for BRCA1 compared to their primary tumor site, and more intensely, with borderline significance, for IGF-IR. This may reconfirm the involvement of the IGF system and BRCA1 in metastatic processes in general and more specifically, the involvement of the IGF system and BRCA1 in the metastatic process in USC.

Finally, targeted therapies in cancer represent a specific unique way of treating cancer with minimal adverse effects. In recent years, the IGF-IR has emerged as one of the most promising molecular targets in cancer treatment.[25] Our data regarding IGF-IR expression and its correlation with BRCA1 in USC may pave the way for further translational developments with this promising mode of treatment.

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