Tumor Suppressor BRCA1 Is Expressed in Prostate Cancer and Controls Insulin-like Growth Factor I Receptor (*IGF-IR*) Gene Transcription in an Androgen Receptor–Dependent Manner

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Abstract

Purpose: The insulin-like growth factor (IGF) system plays an important role in prostate cancer. The *BRCA1* gene encodes a transcription factor with tumor suppressor activity. The involvement of BRCA1 in prostate cancer, however, has not yet been elucidated. The purpose of the present study was to examine the functional correlations between BRCA1 and the IGF system in prostate cancer.

Experimental Design: An immunohistochemical analysis of BRCA1 was done on tissue microarrays comprising 203 primary prostate cancer specimens. In addition, BRCA1 levels were measured in prostate cancer xenografts and in cell lines representing early stages (P69 cells) and advanced stages (M12 cells) of the disease. The ability of BRCA1 to regulate IGF-I receptor (IGF-IR) expression was studied by coexpression experiments using a BRCA1 expression vector along with an IGF-IR promoter-luciferase reporter.

Results: We found significantly elevated BRCA1 levels in prostate cancer in comparison with histologically normal prostate tissue ($P < 0.001$). In addition, an inverse correlation between BRCA1 and IGF-IR levels was observed in the androgen receptor (AR)–negative prostate cancer–derived P69 and M12 cell lines. Coexpression experiments in M12 cells revealed that BRCA1 was able to suppress IGF-IR promoter activity and endogenous IGF-IR levels. On the other hand, BRCA1 enhanced IGF-IR levels in LNCaP C4-2 cells expressing an endogenous AR.

Conclusions: We provide evidence that BRCA1 differentially regulates IGF-IR expression in AR-positive and AR-negative prostate cancer cells. The mechanism of action of BRCA1 involves modulation of *IGF-IR* gene transcription. In addition, immunohistochemical data are consistent with a potential survival role of BRCA1 in prostate cancer.

The insulin-like growth factors, IGF-I and IGF-II, are a family of mitogenic polypeptides with important roles in normal growth and differentiation as well as in tumor development and progression (1–3). In the specific context of prostate cancer, a significant amount of data has been accumulated over the last 20 years suggesting that the IGF axis plays an important role in the transformation of the prostate epithelium (4–7). The contribution of IGF action to prostate cancer development is further supported by epidemiologic studies showing a significant increase in serum IGF-I levels in patients who later developed prostate cancer (8). Acquisition of the malignant phenotype is initially IGF-I receptor (IGF-IR) dependent; however, the progression of prostate cancer from an organ-confined, androgen-sensitive disease to a metastatic one is associated with dysregulation of androgen receptor (AR)–regulated target genes and with a significant decrease in IGF-IR mRNA and protein levels (9, 10). Likewise, IGF-IR expression is extinguished in a majority of human prostate cancer bone marrow metastases (11). The molecular mechanisms that are responsible for regulation of the *IGF-IR* gene in prostate cancer, however, remain largely unidentified.

The familial breast and ovarian cancer susceptibility gene-1 (*BRCA1*) gene encodes a 220-kDa phosphorylated transcription factor with tumor suppressor activity (12). BRCA1 mutation was correlated with the appearance of breast and ovarian cancer at very young ages, although BRCA1 has been also implicated in the etiology of sporadic types of cancer (13–15). BRCA1 is normally targeted to the nucleus via two nuclear localization signals (16). The BRCA1 polypeptide participates in multiple biological pathways, including gene transcription, DNA damage repair, cell growth, and apoptosis (17). Both direct and indirect types of evidence support a tumor suppressor role of BRCA1. Direct evidence was provided by studies showing that transfer of BRCA1 protein arrested growth

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Translational Relevance

The breast and ovarian cancer susceptibility gene-1 (BRCA1) was originally identified as a protein whose mutated form was associated with familial breast and/or ovarian cancer. However, it is clear that the nonmutated (wild-type) form of BRCA1 has distinct cellular functions, including activity as an androgen receptor (AR) coactivator as well as inhibition of insulin-like growth factor-I receptor (*IGF-IR*) gene expression. In this study, we were interested in determining the role BRCA1 may have in regulation of the *IGF-IR* gene in prostate cancer. We have shown that BRCA1 protein expression is increased in prostate cancer, but rather than suppressing IGF-IR expression, as we show in AR-negative prostate epithelial cell lines, we show that BRCA1 is positively correlated with IGF-IR. Further we show that the mechanism responsible for this correlation involves enhancing AR transactivation. These findings are of relevance because they show a new mechanism for IGF and AR stimulation of prostate cancer and further support the relevance of targeting AR and IGF-IR in prostate cancer with BRCA1 expression as a marker for defining the target activity.

of breast and ovarian cancer cells, whereas inactivation of the endogenous *BRCA1* gene induced cellular transformation (18). On the other hand, indirect evidence was provided by studies showing somatic allelic loss of 17q21 in breast and ovarian tumors (19).

The involvement of BRCA1 in prostate cancer etiology has been the focus of controversial debate. Previous studies have suggested that BRCA1 functions as an AR coregulator and plays a positive role in androgen-induced cell death (20, 21). Consistent with a potential tumor suppressor role, prostate cancer cells DU-145 transfected with a wild-type BRCA1 exhibited decreased proliferation rate, increased sensitivity to chemotherapy drugs, increased susceptibility to drug-induced apoptosis, and alterations in expression of key regulatory proteins (22). Furthermore, BRCA1 splice variant BRCA1a was recently shown to display antitumoral activity in triple-negative prostate cancer cells (23). Linkage studies have provided conflicting data about a potential correlation between *BRCA1/BRCA2* status and a familial history of prostate cancer. Thus, Struewing et al. (24) reported that by the age of 70 years, the estimated risk of prostate cancer in Ashkenazi Jewish men carrying mutations in the BRCA1 or BRCA2 genes was 16%. The hypothesis that deleterious mutations in BRCA2 are associated with an increased risk of prostate cancer was further substantiated by studies showing that this type of mutations is more likely to be found in unselected individuals with prostate cancer than in age-matched controls (25). In a recent study, the Icelandic BRCA2 999del5 founder mutation was strongly associated with rapidly progressing lethal prostate cancer (26). Specifically, patients carrying this mutation had a lower mean age of diagnosis, more advanced tumor stages, and shorter median survival times. On the other hand, a study by Vazina el al. (27) concluded that the rate of predominant Jewish BRCA1 and BRCA2 mutations in prostate cancer patients was not significantly different from that in the general population. Likewise, no strong evidence for a role of BRCA1 or BRCA2 mutations in the development of prostate cancer was provided by other reports (28, 29).

In view of the putative role of BRCA1 in prostate cancer, and to expand our previous studies on the interactions between BRCA1 and the IGF system, we evaluated in the present study (*a*) the potential correlation between BRCA1 expression and tumor status in a collection of prostate cancer specimens, and (*b*) the capacity of BRCA1 to control IGF-IR expression in prostate cancer cells with different AR status. Results obtained indicate that BRCA1 is expressed at relatively high levels in prostate cancer compared with a very low BRCA1 immunostaining in normal prostate epithelium. There is a significant negative relationship between IGF-IR and BRCA1 expression levels in AR-negative prostate cancer cell lines, whereas in cancer with an active AR this relationship is positive. In addition, we provide evidence that the *IGF-IR* gene is differentially regulated by BRCA1 in prostate cancer cells with different AR status.

Materials and Methods

Tissue acquisition. The tissue samples used in this study were tissue microarrays made from human radical prostatectomy specimens acquired and used in conformity with an Institutional Review Board–approved protocol at the University of Washington. Median patient age was 58 y (range, 48-74 y). The prostates ranged in weight from 21 to 123 g (median, 42 g). At presentation, 58% of patients were clinical stage *c*T1 and 42% *c*T2. The range of serum PSA was 2.2 to 24 ng/mL (median, 5.4 ng/mL).

Tissue microarrays. Two tissue microarrays were used for these studies. All samples in all arrays were provided in duplicate as 0.6-mm-diameter tissue cores. These arrays contained 203 prostate carcinomas exhibiting a range of Gleason grades (72% Gleason pattern 3, 27% Gleason pattern 4, 1% Gleason pattern 5) and 80 samples of nonmalignant prostate tissue of different biological states (normal, atrophy, and benign prostatic hyperplasia).

Immunohistochemistry. Antibodies recognizing BRCA1 (Santa Cruz Biotechnology, Inc.) and IGF-IR α-subunit (Santa Cruz Biotechnology) were used to stain the tissue microarrays. BRCA1 blocking peptide was purchased from Abcam. Specificity of staining was confirmed by omission of the primary antibody, by immunostaining the sections with a primary antibody against an irrelevant antigen, and by preincubating the anti-BRCA1 in a 5-fold molar excess concentration of BRCA1 peptide before incubating the sections with primary anti-BRCA1 antibody. In addition, specificity was determined by Western blot of a human prostate xenograft, LuCaP 35, which expresses BRCA1 protein. The Western blot was stained with BRCA1 antibody or a 10-fold excess of the BRCA1 blocking peptide plus BRCA1 antibody. Antigen was localized using a three-step avidin-biotin-peroxidase method. In brief, deparaffinized sections were rehydrated in PBS and subjected to antigen retrieval using a microwave (15 min in citrate buffer solution). Sections were then incubated sequentially in solutions of 5% albumin in PBS, 10% hydrogen peroxide in water, primary antibody, secondary antibody [biotinylated antirabbit IgG (BA-1000, Vector Labs)], and avidin-biotin-peroxidase solution (Vector Labs) with interval washes in PBS. Reaction product was detected by incubating the sections in an aqueous solution of 0.05% diaminobenzidine and 0.3% hydrogen peroxide. The sections were counterstained with hematoxylin. Nuclear BRCA1 localization was assessed by staining the tissue microarray without hematoxylin counterstain to more clearly show only BRCA1 staining in the nucleus.

Quantitation and statistical analysis. The immunohistochemical stains were evaluated in a blinded fashion by two independent

pathology reviewers using the following scale: 0, no expression; 1, faint/focal/equivocal staining; 2, <50% of the cells express the antigen; 3, >50% of cells express the antigen. The following cell types were evaluated: secretory and basal epithelial cells; high-grade prostate intraepithelial neoplasia; and Gleason pattern 3, Gleason pattern 4, and Gleason pattern 5 tumor cells. Statistical analysis was done using two-way ANOVA and Bonferonni correction for multiple comparisons. Statistics were done using the Statview statistical program.

Cell cultures. Derivation of the P69 and M12 cell lines has been previously described (30, 31). Briefly, the P69 cell line was obtained by immortalization of prostate epithelial cells with SV40 T antigen, and the M12 cell line was derived by injection of P69 cells into athymic nude mice and serial reimplantation of tumor nodules into nude mice. P69 and M12 cells were cultured in RPMI 1640 supplemented with 10 ng/mL epidermal growth factor, 0.1 nmol/L dexamethasone, 5 μg/mL insulin, 5 μg/mL transferrin, and 5 ng/mL selenium. P69 cells are responsive to IGF-I and are rarely tumorigenic, whereas M12 cells are highly tumorigenic and metastatic and exhibit a reduced IGF-I responsiveness (32). P69 and M12 cells express extremely low levels of AR. The LNCaP C4-2 cell line was maintained in T-Medium (Invitrogen) containing 5% fetal bovine serum.

Western immunoblots. Cells were harvested with ice-cold PBS containing 5 mmol/L EDTA and lysed in a buffer containing 150 mmol/L NaCl, 20 mmol/L HEPES (pH 7.5), 1% Triton X-100, 2 mmol/L EDTA, 2 mmol/L EGTA, 1 mmol/L phenylmethylsulfonyl fluoride, 2 μg/mL aprotinin, 1 mmol/L leupeptin, 1 mmol/L pyrophosphate, 1 mmol/L vanadate, and 1 mmol/L DTT. Protein content was determined using the Bradford reagent. Samples were electrophoresed through 10% SDS-PAGE, followed by blotting of the proteins onto nitrocellulose membranes. After blocking with 5% skim milk in T-TBS [20 mmol/L Tris-HCl (pH 7.5), 135 mmol/L NaCl, and 0.1% Tween 20], blots were incubated with a rabbit polyclonal anti-human IGF-IR β-subunit antibody (Santa Cruz Biotechnology), washed with T-TBS, and incubated with an horseradish peroxidase–conjugated secondary antibody. In addition, blots were incubated with antibodies against BRCA1 (C20, Santa Cruz Biotechnology), tubulin (T-5168, Sigma-Aldrich Co.), Akt and phospho-Akt (Ser473) (#9272 and #9271, respectively, Cell Signaling), extracellular signal–regulated kinase (Erk)-1 and phospho-Erk1/2 (Thr202/Tyr204) (SC-94, Santa Cruz Biotechnology, and #9101, Cell Signaling, respectively), and actin (A-5060, Sigma-Aldrich). Proteins were detected using the SuperSignal West Pico Chemiluminescent Substrate (Pierce).

Plasmids and DNA transfections. An expression vector encoding wild-type BRCA1 was constructed by cloning the BRCA1 cDNA into artificially engineered *Hin*dIII and *Not*I sites in the pcDNA3 vector (Invitrogen; ref. 33). The BRCA1 vector was kindly provided by Dr. Lawrence Brody (NIH, Bethesda, MD). For transient cotransfection experiments, an IGF-IR promoter luciferase reporter construct was used that includes 476 bp of 5′-flanking region and 640 bp of 5′-untranslated region of the *IGF-IR* gene [p(-476/+640)LUC]. The promoter activity of this genomic fragment has been previously described (34). P69 and M12 cells were transfected with 1 μg of the p(-476/+640)LUC reporter construct, along with 1 μg of the BRCA1 expression vector and 0.3 μg of a β-galactosidase expression plasmid (pCMV-β, Clontech), using the Jet-PEI (Polyplus) transfection reagent. Promoter activities were expressed as luciferase values normalized for β-galactosidase activity.

For stable transfections, parental P69 and M12 cells were plated in six-well plates and transfected with a wild-type BRCA1 expression vector (or pcDNA3 as a control) using the Jet-PEI reagent. After 24 h, selection by 500 μg/mL G418 (geneticin, A.G. Scientific, Inc.) was started. After 2 wk of G418 selection, independent colonies were picked up and BRCA1 expression was assessed by quantitative reverse transcription-PCR, as described below. Stable-transfected clones used in this study expressed at least 50% more BRCA1 mRNA than control cells.

Quantitative real-time PCR. Quantitative Real-time PCR was done using TaqMan Universal PCR MasterMix and Assay-on-Demand Gene Expression primers and probes (Applied Biosystems). An ABI Prism 7000 Sequence Detection System was used. The glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA levels were analyzed as an internal control and used to normalize the BRCA1 mRNA values. Amplification was carried out after an incubation of 2 min at 50°C and 10 min at 95°C, followed by 20 cycles at 95°C for 15 s, 1 min at 55°C, and 30 s at 72°C. The number of PCR cycles to reach the fluorescence threshold was the cycle threshold (C_t). Each cDNA sample was tested in triplicate and mean C_t values are reported. Furthermore, for each reaction, a "no template" sample was included as a negative control. Fold differences were calculated using the $2^{\Delta\Delta C}$t method.

Proliferation assays. Cells were plated in six-well plates (2×10^5 per well) in complete medium. After 24 h, the medium was changed to fresh, serum-containing medium. Cells were counted daily by trypsin treatment, followed by trypan blue staining and manual counting with a hemocytometer. At least four fields were counted at each time point and ligand dose. Proliferation experiments were replicated using 2,3-bis[2-methoxy-4-nitro-5-sulfophenyl]-2*H*-tetrazolium-5-carboxanilide inner salt staining (Biological Industries) with similar results.

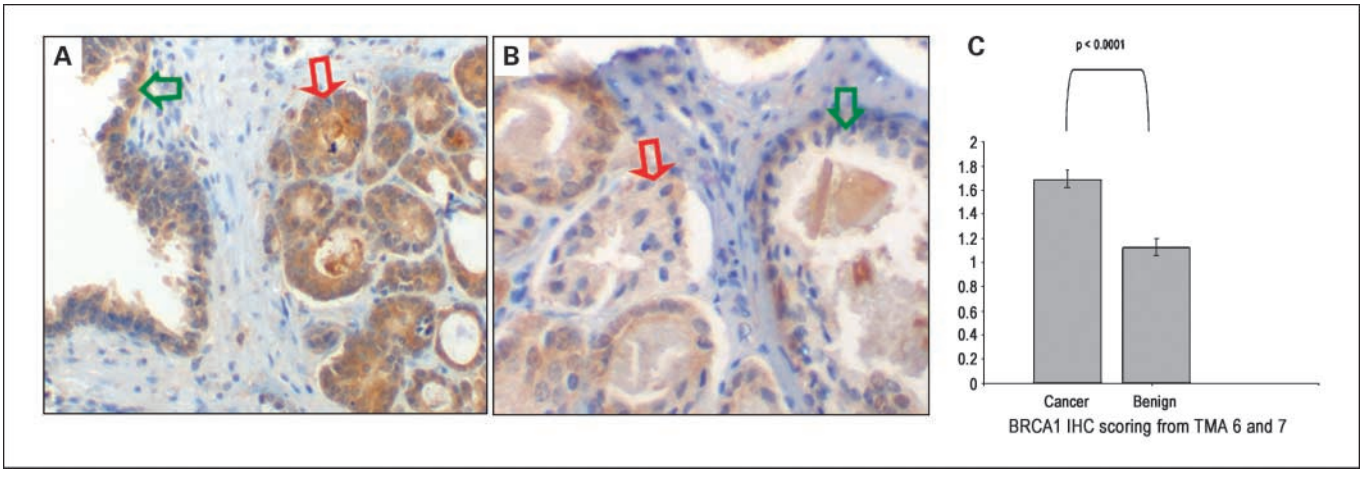

Fig. 1. Expression of BRCA1 in prostate cancer. Two tissue microarrays including 203 specimens were immunostained with BRCA1 antibody C20 as described in Materials and methods. *A,* Gleason score 6 cancer glands (*red arrows*) expressing intense immunoreactivity and a benign gland (*green arrow*) expressing faint immunoreactivity. *B,* Gleason score 6 cancer glands expressing faint immunoreactivity (*red arrow*) and adjacent normal gland (*green arrow*) with intraluminal crystalloid lacking immunoreactivity. *C,* statistical analysis of BRCA1 staining in prostate cancer versus normal adjacent prostate epithelium (average of the respective scores with SDs).

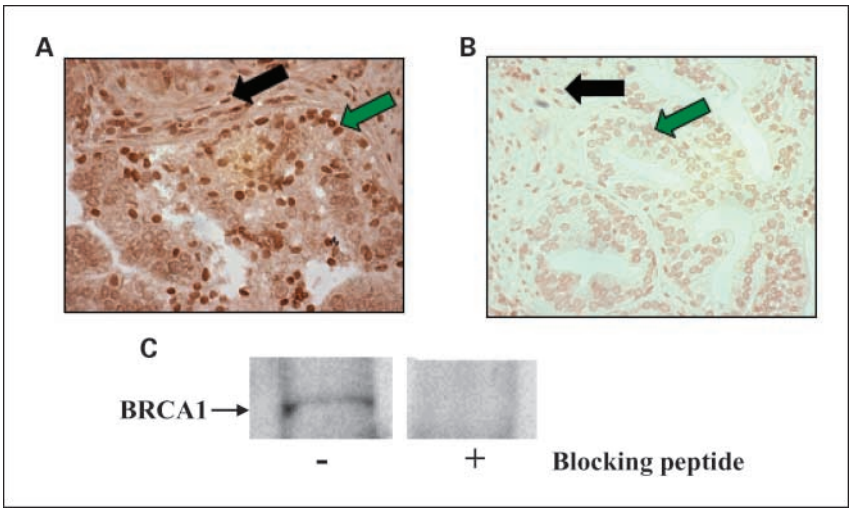

Fig. 2. Nuclear BRCA1 staining in prostate cancer. Nuclear staining by anti-BRCA1 of both benign epithelial (*green arrow*) and stromal (*black arrow*) cells in a section of prostate (*A*) was abolished when the primary anti-BRCA1 antibody was preincubated with a BRCA1 blocking peptide (*B*). *C,* tissue lysate from LuCaP 35 human prostate cancer xenograft was electrophoresed through SDS-PAGE and immunoblotted with anti-BRCA1 antibody in the absence or presence of a BRCA1 blocking peptide. Note loss of BRCA1 band with a 10-fold molar excess of the blocking peptide.

Results

Immunohistochemical analysis of BRCA1 expression in prostate cancer. The potential involvement of tumor suppressor BRCA1 in the etiology of prostate cancer has been the topic of controversial research. To investigate the expression of BRCA1 in primary prostate tumors, immunoreactive BRCA1 was measured in two tissue microarrays, which contained 203 prostate cancer specimens (Fig. 1). Only specimens including both tumor and normal prostate epithelium were included in our analysis. In general, no to very faint BRCA1 immunoreactivity was observed in benign glands, whereas variably intense staining was seen in prostate cancer. Statistical analysis of the data indicates a highly significant difference ($P < 0.001$) between BRCA1 expression levels in prostate cancer compared with normal adjacent tissue (Fig. 1C). IGF-IR immunostaining revealed no correlation between BRCA1 and IGF-IR staining in the benign luminal cells of the 203 specimens on the tissue microarrays that stained positively for both proteins ($r = -0.11$, $P > 0.05$). In contrast, in the malignant tissue from the same tissue microarrays, there was a significant positive correlation between IGF-IR and BRCA1 ($r = 0.21$, $P < 0.02$). IGF-IR levels were significantly higher in the malignant epithelium compared with normal luminal cells ($P < 0.01$). With respect to AR expression in the tissue samples by benign prostate luminal cells, 6% lacked AR immunoreactivity, 18% expressed AR at a level of 1, 31% at 2, and 45% at 3. All cancers expressed AR: 24% at 1, 34% at 2, and 42% at 3. BRCA1 was mainly localized to the nucleus, as shown by staining of the tissue microarray without hematoxylin counterstain (Fig. 2A). Furthermore, the specificity of the BRCA1 staining was addressed by preincubating the BRCA1 antibody in a 5-fold molar excess concentration of BRCA1 peptide before immunostaining. As shown in Fig. 2B, the intensity of the BRCA1 staining was significantly reduced in the presence of the peptide. Likewise, the intensity of the ~220-kDa BRCA1 band in a Western blot of a prostate cancer xenograft was largely reduced in the presence of a 10-fold molar excess of the BRCA1 blocking peptide (Fig. 2C).

BRCA1 expression in prostate cancer xenografts. To further examine BRCA1 levels in prostate cancer, protein expression was measured by Western blot in a collection of 27 human prostate cancer xenografts, kindly provided by Dr. Robert Vessella (University of Washington, Seattle, WA). Results obtained showed that BRCA1 was expressed in most of the xenografts; however, the levels varied over a wide range between xenografts. Likewise, large variations were seen in IGF-IR levels between xenografts. Equivalent amounts of protein were loaded in each lane and equal loading was confirmed by Erk loading (Fig. 3).

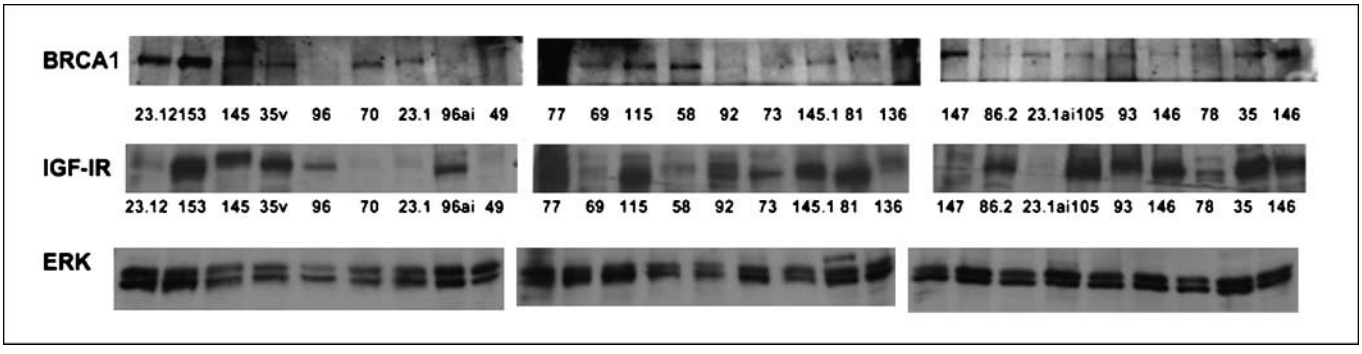

Fig. 3. Western immunoblots with BRCA1, IGF-IR, and Erk antibodies of cell extracts of 27 individual human prostate cancer xenografts grown in severe combined immunodeficient mice. Xenografts with a "v" or "ai" after the number are androgen-independent lines grown in castrated mice. All of the other lines were from intact mice. Tissue was kindly supplied by Dr. Robert Vessella.

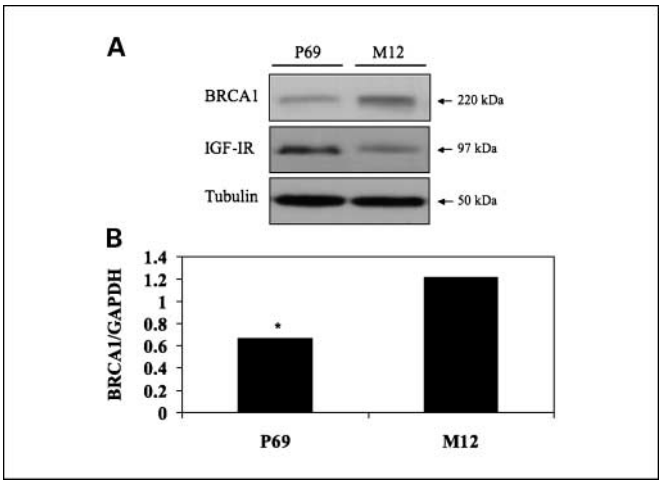

Fig. 4. Expression of endogenous IGF-IR and BRCA1 in P69 and M12 prostate cancer cells. *A,* Western blot analysis of IGF-IR and BRCA1 expression in prostate cancer cells. Untransfected M12 and P69 cells were lysed in the presence of protease inhibitors, as indicated in Materials and Methods. Equal amounts of protein (100 μg) were separated by 8% SDS-PAGE, transferred to nitrocellulose filters, and blotted with anti-BRCA1 (*top*), anti – IGF-IR (*middle*), and anti-tubulin (*bottom*) antibodies. The positions of the ~220-kDa BRCA1, ~97-kDa IGF-IR β-subunit, and ~50-kDa tubulin proteins are indicated. The figure shows a typical Western blot repeated at least thrice with similar results. *B,* quantitative real-time PCR of BRCA1 mRNA levels in prostate cancer cells. Total RNA was prepared from P69 and M12 cells, and BRCA1 mRNA and GAPDH mRNA values were measured using the TaqMan real-time PCR system. Analysis of the data was done as described in Materials and Methods. *, $P < 0.01$, versus M12 cells.

BRCA1 expression in prostate cancer cell lines. In view of the fact that progression to advanced stage disease is usually associated with a reduction in IGF-IR levels (9), and given that BRCA1 was previously shown to control IGF-IR levels in breast cancer cells in a negative fashion (35–37), we examined the pattern of expression of BRCA1 in two AR-negative prostate cancer–derived cell lines with different IGF-IR levels. As previously shown, the poorly tumorigenic P69 cell line expressed high IGF-IR levels, whereas the tumorigenic and metastatic M12 derivative exhibited significantly reduced IGF-IR values (32). Western blot analysis of BRCA1 revealed a diametrically opposite pattern of expression. Thus, BRCA1 levels were ~4-fold lower in P69 than in M12 cells (Fig. 4A). To assess whether the increased BRCA1 levels in M12 cells were associated with corresponding changes in mRNA levels, BRCA1 mRNA levels were measured in both prostate cell lines using quantitative real time-PCR. Results obtained showed that BRCA1 mRNA levels in M12 cells were ~1.5-fold higher than in P69 cells (Fig. 4B).

Regulation of IGF-IR promoter activity by BRCA1 in prostate cancer cells. To examine whether the reciprocal pattern of *BRCA1* and *IGF-IR* gene expression in prostate cancer cells could be due to transcriptional repression of the IGF-IR promoter by endogenous BRCA1, cotransfection experiments were done in M12 cells 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 gene. Forty-eight hours after transfection, cells were harvested and luciferase and β-galactosidase activities were measured. As shown in Fig. 5A, BRCA1 induced a significant reduction in IGF-IR promoter activity in comparison with pcDNA3-transfected cells (~50% suppression).

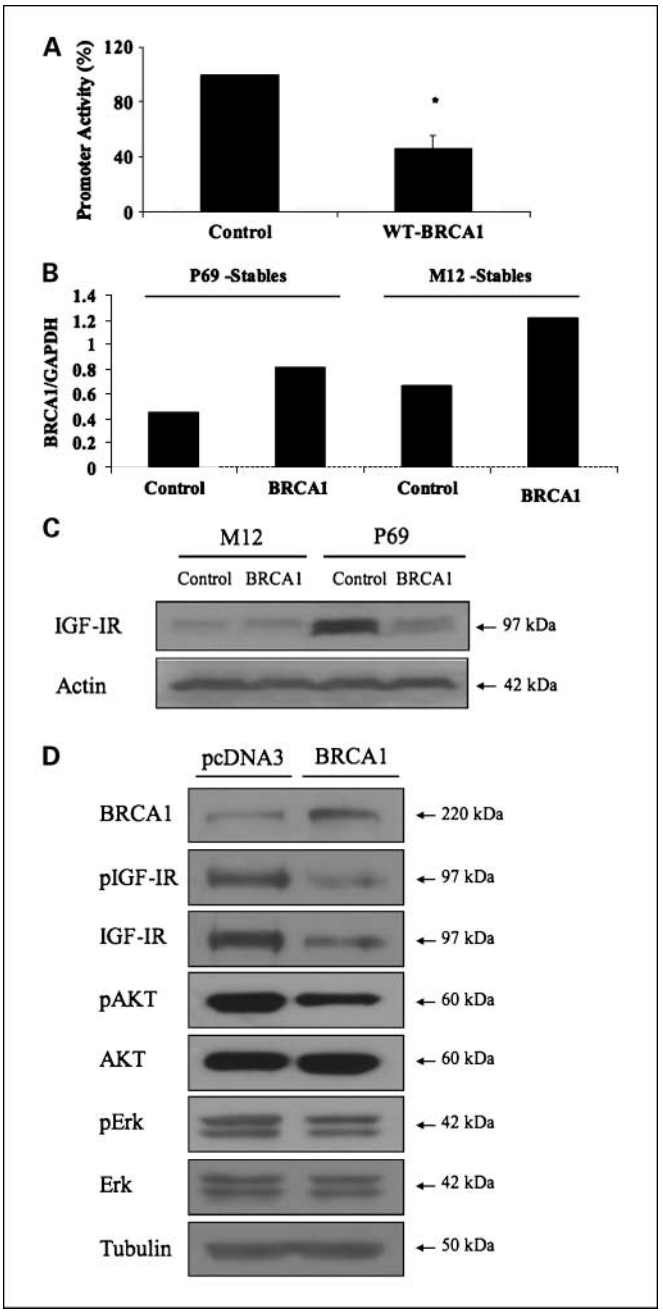

Fig. 5. Regulation of *IGF-IR* gene expression by BRCA1 in prostate cancer cells. *A,* regulation of IGF-IR promoter activity by BRCA1. M12 cells were transiently transfected with 1 μg of the p(-476/+640)LUC IGF-IR promoter-luciferase reporter construct, along with 1 μg of the BRCA1 expression vector (or empty pcDNA3) and 0.3 μg of the pCMVβ plasmid, using the Jet-PEI reagent. Forty hours after transfection, cells were harvested and the levels of luciferase and β-galactosidase were measured. Promoter activities are expressed as luciferase values normalized for β-galactosidase levels. Columns, mean of three independent experiments done in duplicate dishes; bars, SE. *, $P < 0.01$, versus pcDNA3-transfected cells. *B,* quantitative real time-PCR of BRCA1 mRNA in P69-derived and M12-derived stable BRCA1-overexpressing clones. BRCA1 mRNA levels were normalized to GAPDH mRNA levels and expressed in arbitrary units. Analyses were done as indicated in the legend to Fig. 4. Control, pcDNA3-transfected clones; BRCA1, full-length BRCA1-transfected clones. *C,* regulation of endogenous IGF-IR levels by BRCA1. Stable BRCA1-overexpressing (or pcDNA-3 transfected) P69 and M12 cells were lysed and endogenous IGF-IR levels were measured by Western blots. Blots were reprobed with anti-tubulin as a loading control. *D,* regulation of total and phosphorylated IGF-IR levels and downstream mediators by BRCA1. M12 cells were transfected with a BRCA1 expression vector (or empty pcDN3 vector), and after 48 h, cells were lysed and Western blots were done with antibodies against BRCA1, tubulin, and total and phospho-IGF-IR, Akt, and Erk.

Next we studied whether BRCA1 could suppress the expression of the endogenous *IGF-IR* gene. For this purpose, P69 and M12 cells were stably transfected with a BRCA1 expression vector followed by selection with G418. Total RNA was prepared from individual clones and BRCA1 mRNA levels were assessed by quantitative real-time PCR. Clones used in this study expressed at least 50% higher BRCA1 mRNA levels than control, pcDNA3-transfected P69 and M12 cells (Fig. 5B). Western blot analysis revealed that endogenous IGF-IR levels were largely reduced in BRCA1-overexpressing P69 cells in comparison with P69 control cells (Fig. 5C). On the other hand, no effect was seen on the already reduced endogenous IGF-IR levels in BRCA1-overexpressing M12 cells. An inhibitory effect of BRCA1 in M12 cells, however, was observed in cells that were transiently transfected with a BRCA1 vector. As shown in Fig. 5D, BRCA1 expression led to a reduction in total and phospho-IGF-IR, as well as in phospho-Akt, but not in total and phospho-Erk.

Effect of AR expression on BRCA1 action. Because most prostate cancer cells contain an AR and because BRCA1 is a recognized enhancing coregulator of the AR, we determined the response to BRCA1 in the AR-positive LNCaP C4-2 line, which expresses an endogenous AR, although mutated in the androgen-binding domain. The results of these studies are shown in Fig. 6. These studies show by both an AR reporter assay and measurement of the AR responsive gene TSC22 and IGF-IR mRNA by quantitative PCR that in the presence of a functional AR, enhancement of AR signaling by BRCA1 results in increased IGF-IR and TSC22 gene expression (Fig. 6A). In addition, BRCA1 expression enhanced AR transcriptional activity, as shown by cotransfection experiments using an AR-responsive luciferase reporter plasmid (AAR3; Fig. 6B).

Effect of BRCA1 expression on cell proliferation. To assess the potential effect of BRCA1 expression on cell proliferation, BRCA1-overexpressing P69 and M12 cells were plated in six-well plates at a density of 1×10^5 per well and counted after 24, 48, and 72 hours using a hemocytometer. Results obtained indicate that BRCA1-overexpressing P69 and M12 cells consistently displayed an enhanced proliferation rate in comparison with pcDNA3-transfected cells (~ 1.5-fold increase at 72 hours; $P < 0.05$, in three independent experiments; Fig. 6C).

Discussion

Tumor suppressor BRCA1 has been shown to be involved in the regulation of a number of biological processes in various cellular and animal models (17). A potential role for BRCA1 in prostate cancer was suggested by both epidemiologic and experimental studies (21, 38), although its mechanisms of action and potential targets have not yet been identified. The IGFs have been recognized as important regulators of prostate epithelial cell growth and differentiation as well as prostate

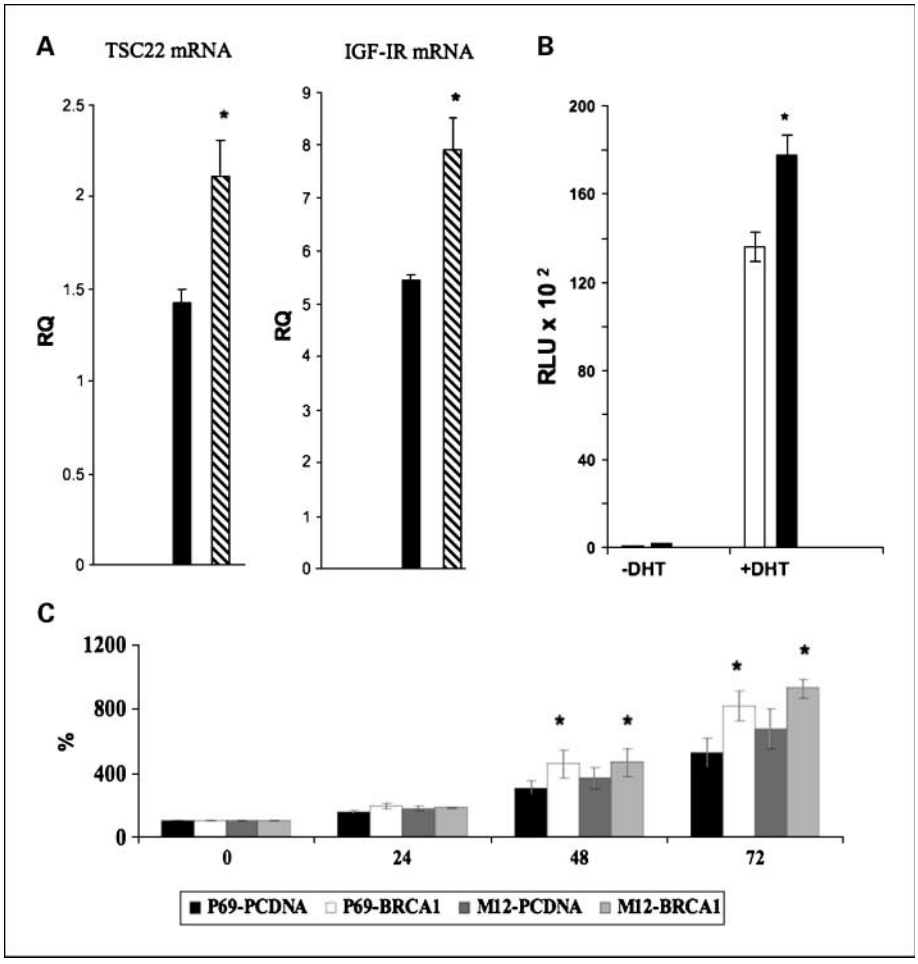

Fig. 6. Effect of AR status on BRCA1 action. *A,* LNCaP C4-2 cells were transfected with a BRCA1 expression vector (*hatched columns*) or an empty vector (*solid columns*) as described in Materials and Methods. Twenty-four hours after transfection, dihydrotestosterone 10^{-9} mol/L was added to the medium and total RNA was collected after an additional 3 h. Quantitative real-time PCR was run for the androgen-regulated gene TSC22 and IGF-IR mRNAs. *, $P < 0.01$, versus control. *B,* the AAR3 luciferase reporter construct was cotransfected onto LNCaP C4-2 cells along with a BRCA1 expression vector (*solid columns*) or an empty vector (*open columns*). Twenty-four hours after transfection, dihydrotestosterone 10^{-9} mol/L (or diluent) was added to the cells for an additional 3 h, after which luciferase activity was measured. Note the significant increase in reporter activity in the BRCA1-containing cells compared with control. RLU, relative luciferase units. *C,* effect of BRCA1 on cellular proliferation. BRCA1-expressing and control P69 and M12 cells were plated in six-well plates at a density of 2×10^5 per well in complete medium. Cells were trypsinized every 24 h, stained with trypan blue, and counted with a hemocytometer. The number of cells at time 0 was assigned a value of 100%. The *y*-axis denotes cell numbers (percentage of cells at time 0). Columns, mean ($n = 3$ independent experiments); bars, SD. Proliferation rates of BRCA1-expressing P69 and M12 cells at 72 h were significantly higher compared with control, pcDNA3-transfected cells ($P < 0.05$).

cancer development (39). The IGF-IR, which mediates the mitogenic and antiapoptotic actions of IGF-I and IGF-II, has been identified as a pivotal player in prostate cancer initiation and progression (10). The pattern of expression of the *IGF-IR* gene through the various stages of the disease, however, remains a controversial subject. Thus, whereas studies have shown that progression of prostate cancer xenografts to androgen independence is associated with a large increase in IGF-IR mRNA levels (compared with the original androgen-dependent tumors; ref. 40), other reports provided substantial evidence that IGF-IR levels were decreased in human prostate carcinoma compared with benign prostate epithelium (9). Consistent with this later study, and as shown in the present article, IGF-IR levels are much higher in the nonmetastatic prostate epithelial cell line P69 compared with its metastatic derivative, the M12 cell line (32). Furthermore, whereas IGF-IR mRNA levels were shown to be largely suppressed in bone marrow metastases (11), other studies reported a persistent expression of the *IGF-IR* gene in prostate metastases (41). Our data, showing a negative correlation between IGF-IR and BRCA1 levels in benign luminal cells and a positive correlation in the malignant tissues, suggest that in the transition from benign to malignant prostate epithelium, there is a potential enhancement of the IGF system with up-regulation of IGF-IR by BRCA1. Whether this is a direct interaction cannot be determined by this type of correlation analysis.

The present study identifies BRCA1 as a novel player in prostate cancer and establishes a functional link between BRCA1 and the IGF-IR with potentially relevant physiologic and pathologic implications in the prostate. Immunohistochemistry revealed that BRCA1 levels were ~60% higher in transformed epithelium in comparison with normal tissue ($P < 0.001$). This paradoxical pattern of expression is consistent with the results of assays showing that BRCA1-overexpressing cells exhibit an enhanced proliferation rate. In addition, data are also consistent with the results of ontogenetic analyses in rodents showing that BRCA1 is highly expressed in rapidly proliferating cells (42). BRCA1 expression is induced by positive growth signals at the cell cycle point where cells become committed to replicate their DNA and undergo cell division (17). Maximal BRCA1 expression was detected during the pre-replicative (G_1) phase of the cell cycle (43), and it was proved that BRCA1 is involved in the control of the G_1-S and G_2-M transition checkpoints (44). Furthermore, we have recently shown that IGF-II, whose levels are largely enhanced in prostate carcinoma, is a potent stimulator of BRCA1 expression (9, 45). On the other hand, BRCA1 overexpression in DU-145 prostate cancer cells was previously shown to cause a very small decrease in proliferation rate, as measured by [^{3}H]thymidine uptake (46). However, BRCA1 expression was associated with constitutive activation of STAT-3, a transcription factor with crucial roles in cell transformation and tumor formation. Moreover, the fact that reduction of STAT-3 levels with antisense oligomers inhibited cell proliferation suggests that BRCA1 expression may elicit a cell survival signal with importance in prostate cancer progression. Further support to the notion that BRCA1 may be involved in early (androgen-dependent) stages of the disease is provided by studies showing that BRCA1 directly interacts with AR and stimulates the activity of androgen response elements in prostate cancer cells (20). Of interest, a recent study has shown that the *BRCA1* gene is overexpressed in conjunction with a network of genes related to BRCA1 function in aggressive prostate, breast, and lung cancers in transgenic models associated with integrated SV40 T/t antigen expression (47). The apparent paradox between the increased BRCA1 levels in prostate cancer and a putative tumor suppressing activity may potentially stem from the multiple and often opposite cellular pathways elicited by BRCA1 (21).

Whereas the biological significance of IGF-IR reduction in prostate cancer is still unclear, the data presented here show that the *IGF-IR* gene is a downstream target for BRCA1 action in this organ. In prostate cancer cells not expressing an AR, BRCA1 expression resulted in an ~50% reduction in the activity of a cotransfected IGF-IR promoter construct, probably by a direct effect at the IGF-IR promoter. The physiologic relevance of these results is highlighted by the fact that the endogenous *IGF-IR* gene, as well as IGF-IR and Akt phosphorylation, was reduced in BRCA1-expressing M12 prostate cancer cells. However, in prostate epithelial cells that express an active AR, the effect of BRCA1 on *IGF-IR* gene expression is mediated through its enhancement of AR transcription and subsequent AR-mediated IGF-IR expression. These results are consistent with studies showing an interplay between BRCA1 and AR in transcriptional regulation (48). In terms of the mechanism of action of BRCA1, we have previously identified a proximal IGF-IR promoter region that mediated the effect of BRCA1 (35, 36). Specifically, this region included a cluster of four GC boxes, which are bona fide binding sites for zinc finger protein Sp1. Whereas we were unable to show direct binding of the *in vitro* translated BRCA1 protein to this promoter region, we identified a BRCA1 domain involved in Sp1 binding. Physical interaction between BRCA1 and Sp1 prevented binding of the zinc finger to *cis*-elements in the IGF-IR promoter, with ensuing reduction in promoter activity. A related mechanism of action was recently reported for the von Hippel-Lindau tumor suppressor in the specific context of IGF-IR regulation in clear cell renal carcinoma (49). Thus, similar to BRCA1, von Hippel-Lindau was shown to reduce IGF-IR promoter activity and mRNA levels via a mechanism that involves functional and physical interactions between von Hippel-Lindau and Sp1.

In summary, we have shown that BRCA1 regulates *IGF-IR* gene expression in prostate cancer cells via a mechanism that involves repression of *IGF-IR* gene transcription. In addition, immunohistochemical data are consistent with a potential survival role of BRCA1 in prostate cancer. Regulation of IGF-IR expression by BRCA1 may constitute a novel control mechanism that allows the IGF system to engage in both differentiative and proliferative types of actions.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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