Intratumoral IGF-I protein expression is selectively upregulated in breast cancer patients with BRCA1/2 mutations

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Abstract

BRCA1/2 mutations predispose to early onset breast and ovarian cancers. The phenotypic expression of mutant alleles, however, is thought to be modified by factors that are also involved in the pathogenesis of sporadic breast cancer. One such protein is IGF-I, one of the strongest mitogens to breast cancer cells in vitro. We have utilized immunohistochemistry to compare the intratumoral IGF-I and IGF-I receptor (IGF-IR) protein expression in 57 BRCA1/2 mutation carriers and 102 matched breast cancer patients without a family history in a nested case–control study. BRCA1 silencing by siRNA was used to investigate the effect of BRCA mutations on IGF-I protein expression. IGF-I protein expression was detected in tumoral epithelium and surrounding stroma, and was significantly upregulated in tumors of BRCA mutation carriers when compared with matched sporadic tumors (epithelial: 87.7% vs 61.8%, P<0.001; stromal: 73.7% vs 34.3%, P<0.001). By contrast, IGF-IR protein expression was confined to malignant epithelium and was unchanged in mutation carriers (52.6% vs 39.2%, P=0.310). While in mutation carriers IGF-IR protein expression was significantly correlated with both epithelial (P=0.003) and stromal IGF-I (P=0.02), this association was less pronounced in sporadic breast cancer (P=0.02 respectively). siRNA-mediated downregulation of BRCA1 in primary human mammary gland cells triggered upregulation of endogenous intracellular IGF-I in vitro. The increased intratumoral IGF-I protein expression in BRCA mutation carriers suggests an involvement of the IGF-I/IGF-IR axis in the biological behavior of breast cancers in this population and could define a potential therapeutic target.

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Introduction

The insulin-like growth factor (IGF) system comprises a well characterized family of two pro-mitogenic growth factors (IGF-I and IGF-II), their respective receptors (IGF-IR and IGF-IIR), and six IGF-binding proteins (IGFBP-1 to 6), which modulate the biological action of the two ligands. While the physiological functions of IGF-II are largely confined to fetal development, IGF-I-associated receptor activation has been shown to play an essential role in postnatal cell cycle regulation and apoptosis (Werner et al. 2000, Shalita-Chesner et al. 2001). The biological effects of both growth factors are thought to be exerted through IGF-IR, which, through activation of the ras-raf-MAPK and PI3-K/Akt signal transduction cascades, lead to proliferation, angiogenesis, and increased cell motility (Werner & Le Roith 1997, Yakar et al. 1999, Werner et al. 2000, Desbois-Mouthon et al. 2001, Singer et al. 2003, Helle 2004).

The biological data are supported by epidemiological evidence which suggests that upregulated IGF-IR
activation is associated with an increased risk of developing malignancies such as breast and colorectal cancers. A meta-analysis of six major studies analyzing the relationship between breast cancer risk and serum IGF levels has recently confirmed the observations of increased IGF-I serum concentrations in premenopausal women who develop breast cancer (Renehan et al. 2004). Furthermore, elevated IGF levels in premenopausal women are also associated with larger breast volume and increased breast density (Byrne et al. 2000, Jernstrom et al. 2005), which in turn has been linked to an increased risk of breast cancer after hormonal exposure (Boyd et al. 2001).

The risk of developing malignant breast tumors is particularly high in women with germ line mutations within BRCA1 and 2 genes, which account for a large proportion of families with inherited breast and ovarian cancers (Futreal et al. 1994, Struwing et al. 1996). Within this population of BRCA1 or 2 mutation carriers, elevated IGF-I serum levels have been detected in women with a family history of breast cancer compared with those without such a history (Lukanova et al. 2001). The synthesis of IGFs in adults predominantly occurs in the liver but may also be accomplished by other tissues, since liver-specific disruption of the IGF-I gene has been demonstrated not to affect growth development and maturation in mice (Liu et al. 1993, Yakar et al. 1999). Several studies have investigated the systemic and tumoral expression of IGF family members in sporadic breast cancer patients and found elevated IGF levels in patients with breast cancer (Peyrat et al. 1993, Rocha et al. 1997), and a positive correlation with breast tumor size (Singer et al. 2004). However, data on the tumoral expression of members of the IGF axis in patients with inherited breast tumors are sparse, and have not been demonstrated to influence systemic IGF-I levels. In order to better define the role of the IGF axis in these tumor entities, we have examined tumoral expression levels of IGF-I and its target receptor IGF-IR in sporadic and BRCA1/2-mutated breast cancers.

### Materials and methods

#### Patient population and breast tumor specimens

Breast cancer specimens from 58 BRCA1/2 mutation carriers (49 BRCA1 and 9 BRCA2) and 102 breast cancer patients without a family history of breast or ovarian cancer were included in this nested case–control study. Breast tumor samples from patients with BRCA1 and 2 mutations were matched 1:2 with samples from malignant control biopsies according to age, menopausal status, receptor status oestrogen receptor (ER and/or PgR + versus ER −/PgR −), and histological tumor type (ductal versus lobular versus others), since the expression of IGF family members has been shown to be strongly influenced by sex steroids (Table 1; Eppler et al. 2002, Jorgensen et al. 2004, 2005, Christiansen et al. 2005). All patients had undergone local treatment for early breast cancer. None of the women in our study had received primary systemic treatment. The retrospective analysis of stored tumor tissue has been authorized by the local IRB, and informed consent was obtained from all patients prior to the analysis. Only tissue specimens with a percentage of tumor cells ≥70% were included in the present analysis. Tumor grade, ER, PgR, p53, and Her-2/neu status were available from pathological records.

Human embryonic kidney (HEK293) and CRL-7347 human primary mammary gland cells, derived from apparently normal tissue (obtained from the American Type Culture Collection), were grown in Dulbecco’s Modified Eagle’s Medium supplemented with 10% calf serum.

#### Immunohistochemistry

Sections of paraffin-embedded, formalin-fixed tissue blocks were deparaffinized with xylene for 5 min each, followed by two washes with 100% ethanol for 10 min each. Then the slides were incubated in 95% ethanol for 10 min and washed twice with dH2O for 5 min. Antigen retrieval was performed by placing slides in 10 mmol/l citrate buffer (pH 6.0) and microwave treatment for 15 min. Tissue sections were cooled to room temperature (RT) and washed with PBS and distilled water. Then sections were blocked with Ultra V Block (Lab Vision, Westinghouse Drive, Fremont, CA, USA) for 4 min. After a consecutive PBS wash, slides were incubated with the anti-IGF-IR antibody (mouse monoclonal #MS-641-P0, 1:100, NeoMarkers, Fremont, CA, USA) and anti-IGF-I antibody (rabbit monoclonal antibody #RB-9240-P, 1:50, NeoMarkers) for 2 h at RT. Negative controls were performed on all tissue sections by replacing primary antibodies with diluted isotype immunoglobulin (ImmunoCruz Staining System, Santa Cruz Biotechnology, Santa Cruz, CA, USA). Human placenta and pancreas sections served as positive controls. Then the slides were incubated with goat anti-polyvalent and streptavidin–HRP (both Lab Vision, Charlottesville, VA, USA) for 60 min, followed by incubation with 3-amino-9-ethylcarbazole. Finally, slides were washed with PBS, counterstained with hematoxylin for 5 s, and coverslipped.

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**Table 1:**

<table>
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<tr>
<th>Study</th>
<th>Patients</th>
<th>Mutations</th>
<th>Tumor Type</th>
<th>ER Status</th>
<th>PgR Status</th>
<th>p53 Status</th>
<th>HER-2 Status</th>
<th>Induction Factor</th>
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<td>PgR+</td>
<td>p53+</td>
<td>HER-2+</td>
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<tr>
<td>Jorgensen et al. 2004</td>
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<td>Lobular</td>
<td>ER-</td>
<td>PgR-</td>
<td>p53+</td>
<td>HER-2+</td>
<td>3.0</td>
</tr>
<tr>
<td>Christiansen et al. 2005</td>
<td>102</td>
<td>BRCA1/2</td>
<td>Others</td>
<td>ER+</td>
<td>PgR+</td>
<td>p53+</td>
<td>HER-2-</td>
<td>4.5</td>
</tr>
</tbody>
</table>
Transfections and siRNA treatment specific for BRCA1

HEK293 cells were transiently transfected using Lipofectamine 2000 (Invitrogen) with the empty pcDNA3 vector control or pcDNA3 harboring HA-tagged full-length wild-type human BRCA1 cDNA. RNA silencing was achieved using ON-TARGETplus SMARTpool human BRCA1 (NCBI accession no. NM007298) and ON-TARGETplus siCONTROL reagents obtained from Dharmacon at a final concentration of 100 nM for 24 h. siRNA was delivered to the cells using Lipofectamine 2000 reagent (Invitrogen) following the transfection protocol provided by the manufacturers.

Immunoblotting

For preparing total lysates, cells were washed with PBS, collected by scraping, and lysed in a buffer containing 20 mM HEPES (pH 7.9), 0.4 M NaCl, 2.5% glycerol, 1 mM EDTA, 1 mM phenylmethylsulphonyl fluoride, 0.5 mM NaF, and 0.5 mM Na3VO4 supplemented with 2 μg/ml aprotinin, 2 μg/ml leupeptin, 0.3 μg/ml benzamidinchloride, and 10 μg/ml trypsin inhibitor by repeated freezing and thawing. Supernatants were collected by centrifugation and stored at −80 °C. Protein concentrations were determined using the Bio-Rad protein assay with BSA as the standard. For the detection of endogenous BRCA1 protein levels and human recombinant IGF-I, proteins were run on 10 and 15% standard SDS–polyacrylamide gels respectively, and transferred to nitrocellulose. Blots were stained with Ponceau S to visualize the amount of loaded protein. For the detection of endogenous IGF-I levels, precast Tris–Tricine gels with an effective separation range of 26.6–1.4 kDa were used. For immunodetection, antibodies specific for the following proteins were used: BRCA1 (Cell Signaling Technology, Danvers, MA, USA) at a dilution of 1:1000 or IGF-I (clone Sm1.2, Upstate Technologies, Charlottesville, VA, USA) at a dilution of 1:200. Signals were detected with appropriate HRP-conjugated secondary antibodies and the enhanced chemiluminescence method.

Immunostaining quantification

IGF-I and IGF-IIR protein expression levels were independently assessed by two investigators (G H and K C) who were blinded to the BRCA mutation status.
Membranous IGF-IR staining was evaluated using the HercepTest (DAKO Corp., Carpinteria, CA, USA) scoring guidelines, according to the manufacturer’s recommendations. Cytoplasmatic protein staining was evaluated with the semiquantitative immunoreactive score (IRS, staining intensity) (negative = 0, weak = 1, moderate = 2, and strong = 3) multiplied by percentage of positive cells in quartiles) and ranged from 0 to 12; the points 0–2 were considered negative, 3–5 weak, 6–8 moderate, and 9–12 strong staining (Remmele & Schicketanz 1993). Tumors were considered IGF-IR positive only in the following two cases: (a) when the receptor was detected in the cell membrane exclusively and (b) when the intensity of membrane staining clearly exceeded cytoplasmatic staining. Cases with weak (1+), moderate (2+), or strong (3+) staining intensities were considered to have an elevated expression of IGF-I and/or IGF-IR and were considered ‘positive’. BRCA1/2 status was determined as elevated expression of IGF-I and/or IGF-IR and were considered IGF-IR positive in 18 (31.6%) cases. Overall, BRCA1/2-mutated specimens expressed significantly higher IGF-I levels than sporadic breast cancer cases ($P = 0.001$, $\chi^2$ test). IGF-I was also detected in stromal cells surrounding malignant epithelia in both sporadic (35 out of 102 cases, 34.3%) tumors and breast cancer specimen from women with BRCA1/2 mutations (42 out of 57 cases, 73.7%). Stromal protein expression was weak in 10 (9.8%), moderate in 21 (20.6%) and strong in 4 (3.9%) of sporadic breast cancer cases, but was significantly more pronounced in BRCA1/2 mutation carriers with 15 (26.3%), 22 (38.6%) and 5 (8.7%) positive cases respectively ($P < 0.001$, $\chi^2$ test). Photomicrographs demonstrating different intensities and patterns of IGF-I expression are depicted in Fig. 2A–D.

**Results**

**siRNA-mediated downregulation of BRCA1 in primary human mammary gland cells triggers upregulation of endogenous intracellular IGF-I**

In order to investigate the role of BRCA1 in the regulation of IGF-I, we silenced the endogenous BRCA1 gene expression in CRL-7347 cells using siRNA specific for BRCA1. The resulting downregulation of endogenous BRCA1 protein is shown in Fig. 1C. We then used an IGF-IR-specific antibody to detect IGF-I protein expression in siRNA-treated cells and observed an upregulation of endogenous intracellular IGF-I protein (Fig. 1D). The specificity of the anti-BRCA1 antibody was confirmed by transiently transfecting HEK293 cells with a full-length wild-type human BRCA1. Figure 1A shows a band which is upregulated in the transfected cells and which corresponds to BRCA1. The specificity of the anti-IGF-I antibody was confirmed by loading 1 μg of recombinant human IGF-I onto a 15% standard SDS–polyacrylamide gel and utilizing the anti-IGF-I antibody as described in the Materials and Methods section (Fig. 1B). The recombinant IGF-I used here is a tagged fusion protein purified from bacterial lysates. Its molecular mass (about 35 kDa) makes up approximately four times that of endogenous IGF-I (about 7.6 kDa).

**Intratumoral IGF-I protein expression in sporadic and BRCA1/2-mutated breast cancers**

Intratumoral IGF-I protein expression was detected in the epithelium in 63 out of 102 (61.8%) sporadic breast cancer cases (Table 2). The expression levels were weak in 14 (13.7%), moderate in 32 (31.3%), and strong in 17 (16.7%) cases. By contrast, 50 out of 57 (87.7%) malignant breast epithelia from BRCA1/2 mutation carriers expressed IGF-I, and staining was weak in 15 (26.3%), moderate in 17 (29.8%) and strong in 18 (31.6%) cases. Overall, BRCA1/2-mutated specimens expressed significantly higher IGF-I levels than sporadic breast cancer cases ($P = 0.001$, $\chi^2$ test). IGF-I was also detected in stromal cells surrounding malignant epithelia in both sporadic (35 out of 102 cases, 34.3%) tumors and breast cancer specimen from women with BRCA1/2 mutations (42 out of 57 cases, 73.7%). Stromal protein expression was weak in 10 (9.8%), moderate in 21 (20.6%) and strong in 4 (3.9%) of sporadic breast cancer cases, but was significantly more pronounced in BRCA1/2 mutation carriers with 15 (26.3%), 22 (38.6%) and 5 (8.7%) positive cases respectively ($P < 0.001$, $\chi^2$ test). Photomicrographs demonstrating different intensities and patterns of IGF-I expression are depicted in Fig. 2A–D.
Correlation of IGF-IR and IGF-I expression in sporadic and BRCA1/2-mutated breast cancers and association with clinicopathological characteristics

We then investigated potential correlations between stromal and epithelial IGF-I, IGF-IR expression, and other tumor biological parameters in patients with sporadic and BRCA mutations (Table 3). In BRCA mutation carriers, epithelial IGF-I was clearly co-expressed with stromal IGF-I \( (P < 0.0001, r = 0.51) \). In addition, intratumoral epithelial IGF-IR was correlated with the presence of epithelial and stromal IGF-I \( (P = 0.003, r = 0.39 \text{ and } P = 0.02, r = 0.33; \text{Spearman’s correlation respectively}) \). Epithelial IGF-I was negatively correlated with p53 status \( (P = 0.01, r = -0.32; \chi^2 \text{ test, Spearman’s correlation}) \), while stromal IGF-I was positively correlated with the presence of Her-2 \( (P = 0.007, r = 0.35; \chi^2 \text{ test, Spearman’s correlation}) \).

In sporadic breast cancer specimens, epithelial IGF-IR was significantly associated with epithelial IGF-I protein expression \( (P = 0.03, r = 0.22; \text{Spearman’s correlation}) \). In addition, epithelial IGF-I significantly correlated with stromal IGF-I \( (P = 0.001, r = 0.45; \text{Spearman’s correlation}) \). The only other associations between IGF family member expression and clinicopathological characteristics were a weak correlation between stromal IGF-I protein expression and ER status \( (P = 0.02, r = 0.22) \) and a correlation between stromal IGF-I and tumor grading \( (P = 0.03, r = 0.21, \text{Table 3}) \).

We finally compared the expression levels of epithelial and stromal IGF system components within the group of BRCA mutation carriers. Apart from

Figure 1: siRNA-mediated downregulation of BRCA1 in primary human mammary gland cells triggers upregulation of endogenous intracellular IGF-I. (A) To control the specificity of the used anti-BRCA1 antibody, logarithmically growing HEK293 cells were transiently transfected with pcDNA3, empty as a vector control, or harboring full-length wild-type human BRCA1. BRCA1 protein overexpression was detected by western blot analysis using the anti-BRCA1 antibody. Ponceau S staining is presented to prove equal gel loading. (B) To control the specificity of the used anti-IGF-I antibody, human recombinant IGF-I was loaded onto a 15% standard SDS–polyacrylamide gel and detected by western blot analysis using the anti-IGF-I antibody. The recombinant IGF-I used here is a tagged fusion protein, purified from bacterial lysates, with a molecular mass of about 35 kDa. (C) CRL-7347 cells were treated with siRNA specific for BRCA1. Downregulation of endogenous BRCA1 protein levels was proved by western blot analysis. Ponceau S staining is presented to prove equal gel loading. (D) CRL-7347 cells treated with siRNA specific for BRCA1 were analyzed for intracellular endogenous IGF-I protein levels by western blot analysis. Non-specific bands (NB) are shown to prove equal gel loading.
significantly elevated estrogen receptor expression levels in BRCA2-mutated specimens (14.9% vs 30%, \( P = 0.03; \chi^2 \) test), no significant differences could be observed between IGF-IR and IGF-I expression or clinicopathological parameters when comparing tumors from BRCA1 and BRCA2 mutation carriers (data not shown).

### Discussion

Over the past years, IGF family members have been identified to play a central role in breast carcinogenesis (Werner et al. 2000). IGF overexpression has been associated with phenotypic changes that are associated with malignant progression (Cocca et al. 2004, Helle 2004). Although the majority of these proteins
are produced by the liver, tumor-derived IGFs are also thought to exert their mitogenic action in an autocrine and/or paracrine fashion (Singer et al. 1995, 2000). The expression of IGF-I, the major target receptor of these proteins, varies between 40 and 80% in breast carcinomas (Happerfield et al. 1997, Eppler et al. 2002, Kostler et al. 2006). IGF-IR has been shown to be primarily regulated at the level of transcription (Baserga et al. 1997). Within this, IGF-IR has been found to be a downstream target of the wild-type BRCA1 (Abramovitch et al. 2003). Consistent with its function in the repair of DNA damage and suppression of cell growth, BRCA1 has been demonstrated to act as an inhibitor of IGF-IR

Table 3 Correlation between insulin-like growth factor (IGF)-system components in epithelial and stromal components and clinicopathological parameters in BRCA1/2-mutated breast cancer and sporadic breast cancer

<table>
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<tr>
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<th>Epithelial IGF-I</th>
<th>Stromal IGF-I</th>
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<td>BRCA1/2-mutated breast cancer</td>
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<tr>
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<td>( P=0.02, r=0.33 )</td>
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<td>Epithelial IGF-I</td>
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<tr>
<td>Stromal IGF-I</td>
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<td>( P&lt;0.001, r=0.51 )</td>
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<tr>
<td>Menopausal status</td>
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<tr>
<td>ER status</td>
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<tr>
<td>PR status</td>
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<tr>
<td>Her-2/neu expression</td>
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<td>( P=0.007, r=0.35 )</td>
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<td>p53 status</td>
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<tr>
<td>Histologic type</td>
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<tr>
<td>Grading</td>
<td></td>
<td>( P=0.03, r=0.21 )</td>
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gene transcription (Abramovitch & Werner 2003, Abramovitch et al. 2003). This has lead to the suggestion that loss of BRCA function in familial breast cancer may result in de-repression or loss of inhibition of the IGF-IR gene transcription, thereby leading to constitutive growth stimulation through the IGF axis (Abramovitch et al. 2003).

However, in the present analysis, tumoral IGF-IR expression was found to be slightly increased in specimen from patients with a BRCA mutation when compared with sporadic cases (52.6% vs 39.2%), but lacked statistical significance. By contrast, comparison of epithelial and stromal IGF-I between the two groups revealed significantly increased expression of IGF-I in both epithelial and stromal tissue compartments of BRCA1/2 mutation carriers (87.7% vs 61.8%, \(P=0.01\); 73.7% vs 34.3%, \(P<0.001\)). In addition, epithelial IGF-IR was significantly correlated with the expression of stromal and epithelial IGF-I in BRCA1-mutated specimens (\(P=0.02\), \(P=0.003\) respectively), suggesting that tumoral IGFs exert their biological effects through autocrine and paracrine mechanisms. Although higher expression levels of IGF-IR would be expected in familial breast cancer based on the disability of mutated BRCA1 to repress IGF-IR gene activation, we did not observe such a phenomenon in the present analysis. One possible reason for the insignificant difference of IGF-IR expression between mutated and sporadic tumor samples could be the higher number of grade 3 tumors in the mutated cohort (61.4% vs 41.2%), because increased dedifferentiation of tumor cells has been shown to be accompanied by downregulation of IGF-IR (Schnarr et al. 2000).

To date, numerous studies showed that high circulating levels of IGF-I are associated with an increased premenopausal breast cancer risk (Hankinson et al. 1998, Toniolo et al. 2000, Yu et al. 2002, Hankinson & Schernhammer 2003, Renéhan et al. 2004), and this correlation has been suggested to be even more pronounced in familial compared with non-familial breast cancer patients (Lukanova et al. 2001). The present analysis demonstrates, for the first time, that this difference can also be detected in tumoral tissues from patients with BRCA1/2-mutated disease. This is in contrast to the only available study performed on this field by Voskuil et al. (2004). They compared tumoral mRNA levels for several IGF family members including IGF-I in 25 sporadic and 27 familial breast cancer patients, but did not observe a difference in IGF-I mRNA between the two groups. It should be noted that apart from possible post-translational modifications of IGF-I mRNA, which do not translate into differences in protein levels, only 83% of this relatively small cohort of breast cancer patients with a strong family history of breast cancer were tested as BRCA1 or BRCA2 carriers, which could further explain the insignificant difference of IGF-I mRNA expression between the two groups in the study.

Taken together, the results of the present analysis strongly support a link between high breast cancer risk, BRCA1/2 germ line mutations, and higher tumoral IGF-I levels. Decreasing of IGF-I levels by exercise, weight control, and dietary interventions, as well as inhibiting signaling through specific antibodies or IGF-IR inhibitors might thus represent a therapeutic option for prevention and treatment of breast cancer in BRCA1/2 mutation carriers (Haluska et al. 2006, Sachdev et al. 2006). We suggest that mutated BRCA1/2 possibly influences the IGF-I gene status leading to increased tissular IGF-I expression and promotion of cell proliferation in BRCA1/2 mutation carriers with ensuing early development of malignant breast tumors.

Acknowledgments
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