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Advances in Brief

Insulin-like Growth Factor-I Receptor Overexpression Mediates Cellular Radioresistance and Local Breast Cancer Recurrence after Lumpectomy and Radiation[1]

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Abstract

The insulin-like growth factor-I receptor (IGF-IR) plays a critical role in cell growth regulation and transformation. The radiosensitivity of NIH 3T3 fibroblasts overexpressing either wild-type or mutant IGF-IR was examined. High levels of wild-type IGF-IR conferred radioresistance, and mutational analysis revealed that this effect correlated with the transforming capacity but not the mitogenic activity of the receptor. The radioresistant phenotype was reversed when the cells were incubated with antisense oligonucleotides targeted to IGF-IR mRNA, demonstrating that IGF-IR directly influences radioresistance. The clinical significance of these findings was examined in an immunohistochemical analysis of primary breast tumors, revealing that high levels of IGF-IR in tumor samples were highly correlated with ipsilateral breast tumor recurrence (IBTR) following lumpectomy and radiation therapy ($P = 0.001$). Subgroup analysis revealed that, for early breast tumor relapses (within 4 years of initial breast tumor diagnosis), elevated levels of IGF-IR were strongly associated with IBTR ($P = 0.004$) but IGF-IR expression was not prognostic for IBTR from breast cancer patients with late relapses (P was not significant). These studies provide evidence for the influence of IGF-IR on cellular radioresistance and response to therapy and raise the possibility that the radiocurability of selected tumors may be improved by pharmaceutical strategies directed toward the IGF-IR.

Introduction

Evidence is accumulating that the IGF-IR[3] plays a fundamental role in cell growth control and malignant transformation and is an important inhibitor of apoptosis (1, 2). IGF-IR is a membrane-bound heterotetramer receptor with intrinsic tyrosine kinase activity. It is highly expressed in a variety of human tumors, and its expression is required for transformation by SV40 T antigen, bovine papillomavirus, activated ras, raf, and v-src (3). Experiments using dominant negative mutants of IGF-IR, antibodies to IGF-IR, or antisense strategies directed against IGF-IR mRNA have shown that decreased or aberrant receptor expression is associated with a reversal of the transformed phenotype and with induction of apoptosis (4–6). Cells lacking IGF-IR have prolonged cell cycle kinetics, and IGF-IR-deficient mice show severely diminished growth (7). Conversely, mouse fibroblasts that overexpress IGF-IR have reduced growth factor requirements *in vitro* and demonstrate decreased susceptibility to apoptosis (8). Mutational analyses have revealed an important role for specific tyrosine residues in IGF-IR function and have allowed separation of the mitogenic from the transforming phenotype of the receptor (9). When activated, IGF-IR transmits a signal to at least four major substrates: IRS1, IRS2, Grb10, and Shc (10, 11). Other downstream effectors include ras, raf-1, and mitogen-activated protein kinase, although evidence also exists for a ras-independent pathway initiated by IGF-IR (3). The IGF-IR has been shown to be overexpressed in human breast cancer, but the importance of IGF-IR overexpression with respect to tumor control, survival, and response to therapy in breast cancer is unclear (12–16).

Here, we report the results of gene transfer and antisense experiments showing that overexpression of IGF-IR promotes radioresistance in mammalian cells. We also present an analysis of selected mutant receptors, revealing that the ability to mediate radioresistance correlates with the transforming activity of the receptor but is independent of its mitogenic activity. Prompted by results showing that IGF-IR influences cellular radiation response, we examined the clinical significance of IGF-IR expression with respect to its prognostic value in determining response to radiation therapy for breast cancer. We hypothesized that tumors that had recurred in the irradiated breast following lumpectomy and ionizing radiation would represent an especially radioresistant subgroup. We present data demonstrating a highly significant correlation of breast tumor recurrence in the irradiated breast with overexpression of IGF-IR in primary breast tumor samples.

Materials and Methods

Expression Vectors, Receptor Mutants, and Mouse Cell Lines. The CVN-IGF-IR plasmid was used to express the human IGF-IR cDNA from the SV40 early promoter, as described previously (17). The receptor gene mutants were generated using synthetic oligonucleotides designed to alter the tyrosines at residues 1250 and 1251 to phenylalanine, after subcloning of the IGF-IR cDNA into the vector pGEM11Zf(−), as described (18). Mutant cDNAs were subcloned into the expression vector pBPV under the control of the mouse metallothionein promoter and the murine sarcoma virus enhancer (6).

BALB/c3T3 cells were transfected with expression vectors carrying either the WT human IGF-IR cDNA or mutant variants using standard liposome-mediated gene transfer techniques. Control 3T3 cells were transfected with the pBPV expression vector without any insert. Cells were grown in modified essential medium supplemented with 10% fetal bovine serum (Life Technologies, Inc., Bethesda, MD). Cell lines were analyzed for receptor expression by Western blotting using a mouse monoclonal antibody directed against the IGF-1R β subunit (Oncogene Science, Uniondale, NY) and by Scatchard analysis, as described previously (19).

Clonogenic Survival Assays and Antisense Experiments. Clonogenic survival assays were carried out in duplicate on subconfluent cells, as de-

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[3] The abbreviations used are: IGF-IR, insulin-like growth factor-I receptor; WT, wild type; ODN, oligodeoxynucleotide; IBTR, ipsilateral breast tumor recurrence; DDFS, distant disease-free survival; OS, overall survival; ER, estrogen receptor; PR, progesterone receptor.

scribed (20). Irradiation was performed with a ^{137}Cs irradiator source, with a dose rate of 225 rad/min. Survival curves were plotted as the logarithm of the surviving cells *versus* the radiation dose. Only colonies containing ≥50 cells were scored. For antisense experiments, cells were treated with a concentration of 0.5 μM phosphorothioate antisense and sense ODNs corresponding to codons 21–26 of the *IGF-IR* gene (21). Twenty-four h later, cells were irradiated with a dose of 600 rad, and surviving colonies were enumerated 14 days later.

Patient Population. The breast cancer data base has a median follow-up of 13 years and a minimum follow-up of 4 years. All patients were treated by lumpectomy, with or without axillary dissection, followed by full-course radiation therapy to the intact breast (median dose of 48 Gy), with a boost dose directed at the lumpectomy site to a total median dose of 64 Gy. IBTR was defined as histologically confirmed recurrent invasive breast carcinoma occurring in the ipsilateral breast within the previously irradiated field. The paraffin blocks from the primary breast tumors of 47 index patients with a diagnosis of invasive ductal carcinoma who underwent lumpectomy and radiation therapy and who subsequently had IBTR as the first site of failure were identified (Table 1). From our breast cancer data base, 47 matching control patients treated with lumpectomy and radiation therapy who did not have IBTR were also identified. The index and control groups were evenly matched with similar age, tumor histology, tumor size, stage distribution, axillary lymph node status, date of radiation therapy treatment, menopausal status, type of adjuvant systemic treatment, length of follow-up, DDFS, and OS, as determined by χ^2 analyses (Table 1).

Twenty-three patients had a local breast tumor relapse that occurred ≤4 years from the date of initial diagnosis and were considered to have had an early tumor relapse (22). An additional 24 patients had late tumor relapse that occurred >4 years after diagnosis of the primary breast cancer. A protocol for the study was approved by the Human Investigations Committee at the Yale University School of Medicine.

Immunohistochemistry. The 5-μm-thick sections containing invasive ductal carcinoma were deparaffinized in xylene, dehydrated in ethanol, rehydrated with water, and washed in 1% PBS-BSA. IGF-IR status was determined by using a polyclonal IGF-IR β subunit antibody (Santa Cruz Biotechnology, Santa Cruz, CA) at a 1:1000 dilution. To detect reaction, the slides were incubated for 30 min with goat antirabbit biotinylated antibody (1:500) and peroxidase-conjugated streptavidin. The immune complex was visualized with the chromogenic substrate diaminobenzidine tetrahydrochloride. Overexpression of IGF-IR was determined by membranous/cytoplasmic staining in the invasive cancer component. All samples were also stained with positive and negative controls that included rabbit antisera and polyclonal anti-IGF-IR antibody preabsorbed with IGF-IR peptide.

The pathologist processing, staining, and grading the specimens was blinded as to the clinical histories of the patients. The staining on each slide was rated on a 4-point scale: 0, none; 1+, light; 2+, moderate; 3+, heavy; and 4+, intense. A value of ≥2+ intensity was considered positive, and an H-score (defined as the product of intensity and distribution) of ≥75 was used as a cutoff for IGF-IR, ER, and PR positivity. ER/PR receptor status were determined by immunohistochemistry using the previously described ERICA method (Abbott Laboratories, Chicago, IL; Ref. 23).

Statistics. All patient data, including clinical, pathological, and outcome measures, were entered into a computerized data base employing the PRODAS data base management system (Conceptual Software Inc., Houston, TX). Differences in categorical variables between the index and control population were compared using standard χ^2 analysis, as well as the McNemar statistical test for a matched case-control study.

Table 1 *IGF-IR levels in transfected cell lines*[a]

Cell line	No. of receptors/cell	K_d (nM)
3T3	0.2×10^5	2.0
3T3-IGF-IR (WT)	1.3×10^5	1.3
3T3-Y1250F	3.8×10^5	1.0
3T3-Y1251F	0.8×10^5	1.8

[a] Receptor levels were determined by Scatchard analysis. BALB/c3T3 cells were transfected with expression vectors carrying either the WT human IGF-IR cDNA or mutant variants, and stable cell lines were established by selection for G418 resistance based on the presence of the *neo* gene in the vector.

Results

BALB/c3T3 mouse fibroblasts were transfected with vectors expressing either the human WT IGF-IR cDNA or mutant derivatives (19). Using site-directed mutagenesis, mutations resulting in substitution of phenylalanine for tyrosine at positions 1250 (Y1250F) and 1251 (Y1251F) were introduced into the cloned IGF-IR cDNA to better define the IGF-IR signal transduction pathways (9). Control 3T3 fibroblasts consisted of cells transfected with a vector lacking IGF-IR sequences.

Scatchard analyses were performed to quantitate receptor levels (Table 1). The control 3T3 fibroblasts had 20,000 receptors per cell, whereas the WT IGF-IR, Y1250F, and Y1251F cells showed receptor overexpression, with 130,000, 380,000, and 80,000 receptors, respectively. The overexpressed mutant receptors showed IGF-I binding affinities similar to the WT, as determined by Scatchard analysis.

Clonogenic survival assays were performed to determine the radiation sensitivity of the cell lines (Fig. 1*A*). The WT IGF-IR cells were relatively radioresistant compared to the parental 3T3 cells. This difference has been reproduced in four independent experiments and with two independent WT IGF-IR clones (p6 and J-WT). The Y1250F cell line was also found to be radioresistant, but overexpression of the Y1251F receptor did not alter cellular radiosensitivity relative to the 3T3 cells. Although the Y1251F cells showed 4–5-fold fewer receptors than the Y1250F cells, this difference does not account for the lack of an effect on radioresistance because the Y1251F and WT IGF-IR cells show similar levels of receptor overexpression. The Y1251F mutant cell line has IGF-IR levels in the range of the WT IGF-IR clone but does not demonstrate a radioresistance phenotype. The Y1251F IGF-IR mutant has been previously shown to lack the normal capacity of the receptor to mediate transformation. It does, however, retain IGF-I-mediated mitogenic activity. In contrast, neither receptor-mediated transformation nor mitogenesis are disrupted by the Y1250F mutation. Hence, the ability of IGF-IR to confer radioresistance correlates with its capacity to mediate transformation but is independent of the mitogenic activity of the receptor.

To further test the specificity of the IGF-IR-related cellular radioresistance, antisense ODNs directed against IGF-IR mRNA were tested for their ability to alter the radiation response (Fig. 1*B*). Pretreatment of the WT IGF-IR cells with antisense ODNs resulted in an almost 5-fold reduction in survival, as compared to cells without ODN treatment, following a single fraction of 600 rad. There was a minimal reduction in 600-rad survival when the WT IGF-IR cells were treated with sense ODNs prior to irradiation. Likewise, a similar reversal in radioresistance was seen in Y1250F IGF-IR mutants when treated with the antisense ODNs (data not shown).

To determine the clinical significance of IGF-IR expression, we examined protein levels by immunohistochemical techniques in tumor specimens from patients with early stage breast cancer in a matched case-control study. We identified a set of 47 index patients with a diagnosis of invasive ductal carcinoma who underwent lumpectomy and radiation therapy and who subsequently had IBTR as the first site of failure (Table 2). From our breast cancer data base, 47 matching control patients who did not have IBTR were also identified. The index and control groups had similar age, tumor histology, tumor size, stage distribution, axillary lymph node status, menopausal status, type of adjuvant systemic treatment, length of follow-up, DDFS, and OS, as determined by χ^2 analyses (Table 2).

In the breast cancer specimens showing overexpression of IGF-IR, staining with the polyclonal IGF-IR β subunit antibody was predominantly membranous and cytoplasmic (Fig. 2*A*). This specific staining pattern was not visualized either when breast cancer sections were stained with the secondary rabbit antibody alone (Fig. 2*B*) or when the

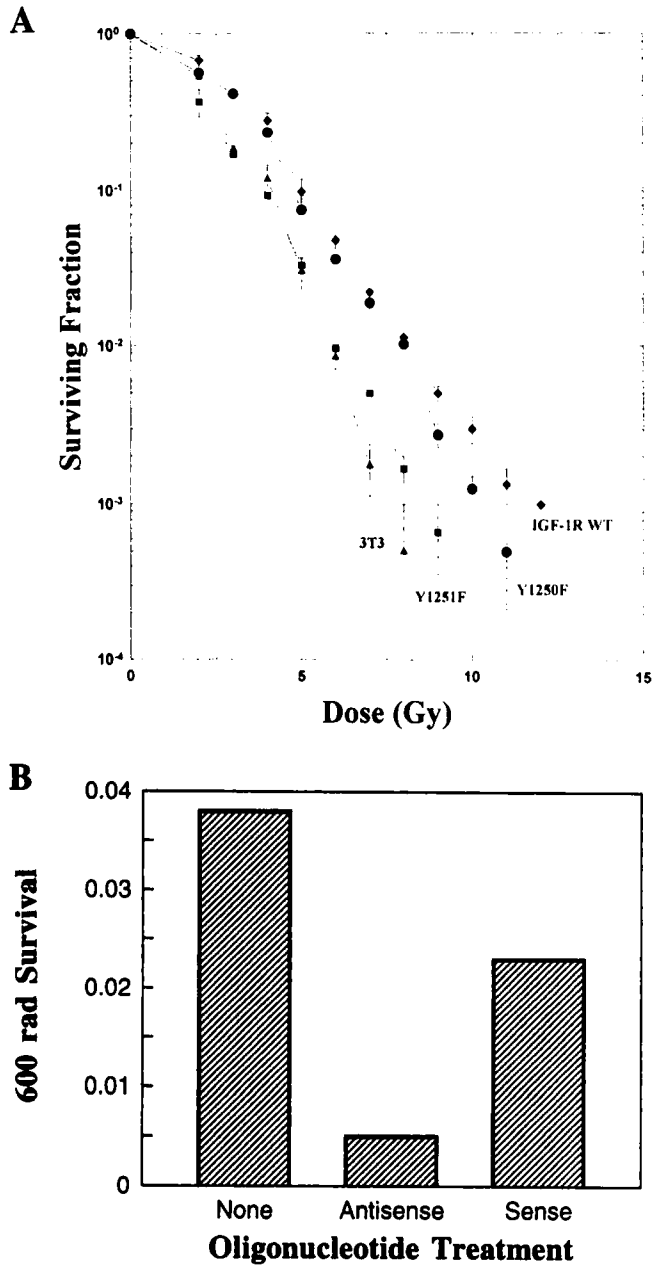

Fig. 1. IGF-IR overexpression mediates radioresistance of mouse fibroblasts. *A*, radiation sensitivity of parental BALB/c3T3 mouse fibroblasts (▲) and transfected 3T3 cell lines overexpressing either WT or mutant IGF-I receptors [WT IGF-IR (◆), Y1250F (●), and Y1251F (■)]. Clonogenic survival assays were carried out in duplicate. *Data points*, mean results of four independent experiments; bars, SEs. *B*, antisense ODN suppression of IGF-IR-mediated radioresistance. Mouse 3T3 cells overexpressing IGF-IR were pretreated with either sense or antisense ODNs directed against IGF-IR mRNA and then irradiated with a dose of 600 rad. Control cells received no ODN treatment.

IGF-IR antibody was preincubated with IGF-IR β subunit peptide prior to use (Fig. 2*C*).

Overall, there was a highly statistically significant difference in IGF-IR overexpression, as determined by H-score (intensity × distribution) between index and control cases (Table 3). We found 20 index cases (43%) had an H-score ≥75, as compared to only 8 control cases (12%; $P = 0.001$), indicating that IGF-IR overexpression may be correlated with clinical radioresistance and with consequent disease recurrence following radiation therapy. We also analyzed our results with the McNemar statistical test, which is a more stringent test for case control studies, and we found that the data retained statistical significance ($P = 0.003$).

We analyzed levels of IGF-IR in the subset of breast cancer patients with early breast tumor relapses (*i.e.*, within 4 years of initial diagnosis) and found that 52% (12 cases) had IGF-IR overexpression as compared to 13% (3 cases) of control breast cancer patients ($P = 0.004$; Table 2). However, in breast cancer patients with late relapses, IGF-IR overexpression was observed in 29% (seven cases) of index cases as compared to 21% (five cases) of late control cases (*P* was not significant). Elevated levels of IGF-IR, therefore, appear to be strongly associated only with early breast tumor relapses. Such relapses are thought to be true recurrences, in contrast to the later relapses, which may represent *de novo* primary breast cancers.

There was no association between IGF-IR overexpression and ER/PR status, OS, DDFS, or axillary lymph node involvement with breast cancer using χ^2 analysis (data not shown).

Discussion

These studies provide both *in vitro* and *in vivo* evidence that overexpression of human IGF-IR confers cellular radioresistance. Using defined IGF-IR mutants to probe IGF-IR-related pathways, we found that tyrosine 1251 is critical in mediating not only transformation, as reported previously (9), but also radioresistance. Experiments using antisense oligonucleotides directed against IGF-IR mRNA further demonstrate the specificity of this effect. Our finding of IGF-IR-mediated radioresistance is in keeping with previous work that demonstrated the antiapoptotic action of the receptor pathway (24, 25). It is not yet clear whether the IGF-IR-mediated effect on radiation response involves any of the known apoptotic regulators, such as the Bcl-2 family member proteins or the CPP32 serine-cysteine proteases.

Several other growth-regulatory genes have also been shown to influence radiation response. An activated *ras* oncogene has been found to promote radioresistance in mouse NIH 3T3 cells, and *c-raf-1* has been correlated with radioresistance in a human squamous cell carcinoma cell line (26, 27). Certain genes, including c-*myc* and c-Ha-*ras*, can act synergistically in promoting transformation and radiation resistance in primary rat fibroblasts (28). Many of these factors act downstream in IGF-IR-initiated pathways, further supporting a fundamental role for IGF-IR in modulating cellular radiation response.

Conservative management of early-stage breast cancer by lumpectomy followed by radiation therapy to the intact breast has now gained widespread acceptance as the preferred method of treatment for the

Table 2 *Characteristics of index and control breast cancer cases*[a]

Data	Index	Control	*P*
Total no. of patients	47	47	NS[b]
Infiltrating ductal cancer	47 (100%)	47 (100%)	NS[b]
Median follow-up (yr)	13	13	NS[b]
Stage I/II	47 (100%)	47 (100%)	NS[b]
Mean pathologic size (cm)	1.8	1.9	NS[b]
Axillary dissection	22 (47%)	22 (47%)	NS[b]
Positive lymph nodes	4 (9%)	6 (13%)	NS[b]
Metastatic disease	7 (16%)	12 (28%)	NS[b]
Median survival (yr)	6.8	6.6	NS[b]
Adjuvant chemotherapy	7 (16%)	5 (12%)	NS[b]
Adjuvant tamoxifen	10 (21%)	12 (26%)	NS[b]
10-year breast relapse-free survival	0 (0%)	47 (100%)	<0.0001
10-year DDFS	61%	63%	NS[b]

[a] The population base for this study was comprised of 1100 patients with stage I or II breast cancer who were treated with lumpectomy and radiation therapy to the intact breast at Yale-New Haven Hospital between 1973 and 1993. A set of 93 patients with IBTR as the first site of disease recurrence after conservative surgery and radiation therapy were identified, and paraffin-embedded blocks of tissue from 51 of these were obtained. The final set comprised 47 patients with confirmed invasive ductal carcinoma, serving as the index cases for this study. Following the identification of the 47 patients with IBTR, the data base was searched for 47 matching control early-stage breast cancer patients who did not have an IBTR.

[b] NS, not significant.

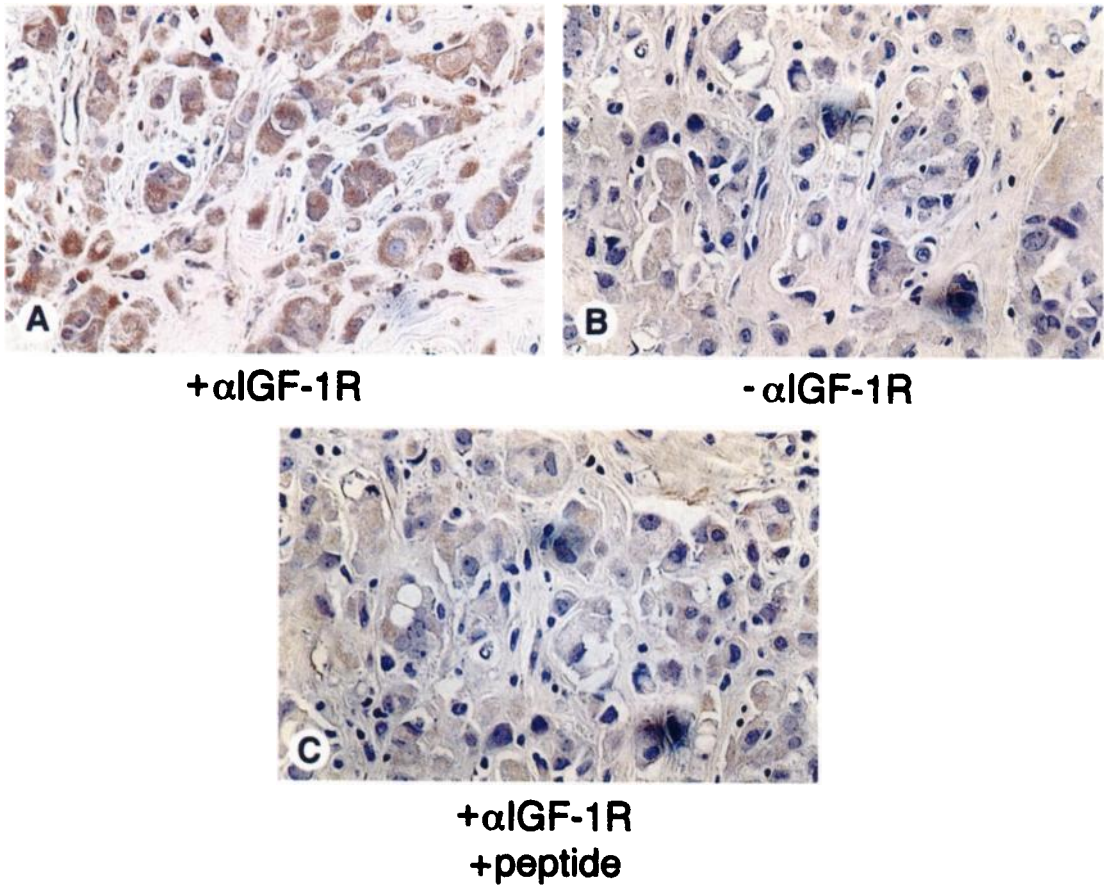

Fig. 2. IGF-IR expression in human breast cancer. IGF-IR protein expression was detected in breast carcinoma specimens by immunohistochemistry. *A*, index case stained with anti-IGF-IR antibody. *B*, index case stained with rabbit antisera at the same dilution. *C*, index case stained with IGF-IR polyclonal antibody preincubated with IGF-IR peptide.

majority of patients (29). It is clear that some apparently localized tumors still recur after standard doses of radiation. Long-term follow-up reveals an actuarial local recurrence rate of 1–2% per year (30). Approximately 20–40% of patients with IBTR will develop metastatic disease and succumb to disease (31). The importance of being able to determine those patients at risk for IBTR is therefore clear. Studies have revealed that young patient age, extensive intraductal component, presence of tumor necrosis, positive surgical margins, and peritumoral lymphatic infiltration can help to predict the likelihood of local recurrence. Recently, there has been growing interest in identifying genetic factors that may influence the natural history of the disease and may determine response to treatment. A previous report from our group suggested that elevated levels of HER-2/neu oncoprotein may be associated with IBTR after lumpectomy and radiation therapy, but the sample size was small (32). The work presented here based on a more extensive analysis identifies the IGF-IR protein as an important new molecular marker that appears to be highly predictive of IBTR following lumpectomy and radiation, with elevated levels of IGF-IR associated with local breast tumor recurrence.

Table 3 *Correlation of IGF-IR levels with IBTR*[a]

Protein expression	Index cases	Control cases
Overall:IBTR[b]		
IGF-IR+	20/47 (43%)	8/47 (12%)
IGF-IR−	27/47 (57%)	39/47 (88%)
Early IBTR[c]		
IGF-IR+	12/23 (52%)	3/23 (13%)
IGF-IR−	11/23 (48%)	20/23 (87%)
Late:IBTR[d]		
IGF-IR+	7/24 (29%)	5/24 (21%)
IGF-IR−	17/24 (71%)	19/24 (79%)

[a] The overexpression of IGF-IR in breast cancer lumpectomy specimens was determined by immunohistochemistry.
[b] $P = 0.001$.
[c] $P = 0.004$.
[d] P was not significant.

The importance of IGF-IR expression in breast cancer has been previously examined in several studies (12–16). However, the results have been conflicting, and no consensus has yet emerged. One study found that tumors with amplification of the *IGF-IR* gene and consequent receptor overexpression are associated with decreased relapse-free survival (16). Other reports, however, concluded that IGF-IR overexpression was correlated with improved relapse-free survival and OS. In these studies, the cases were classified as either positive or essentially negative for IGF-IR, without distinguishing between high and moderate levels of expression. Also, IGF-IR levels were estimated by measuring binding of radiolabeled IGF-I to protein extracts from tumor biopsy specimens. In this method, it is not possible to rule out the contribution of other IGF-I-binding proteins, and the distribution of the binding activity in the malignant and nonmalignant portions of the specimen cannot be determined. In our work, we evaluated IGF-IR status by immunohistochemistry so that we could carefully restrict the analysis to the cancer cells and assess both the intensity and distribution of the positive staining for the receptor. More importantly, none of the other IGF-IR studies specifically looked at the issue of local breast tumor recurrence. We deliberately restricted our analysis to patients with IBTR and to their matched controls to focus on one specific aspect of breast cancer biology, *i.e.*,

the response to radiation therapy. Furthermore, we examined a homogeneous patient population treated in a uniform fashion with long-term follow-up.

We have found that IGF-IR has prognostic significance with respect to IBTR, but we did not find any association between IGF-IR levels and OS or DDFS. Because a high proportion of local recurrences in the irradiated breast can still be cured by mastectomy, it is not surprising that we did not find such an association. It is possible, however, that a correlation between IGF-IR levels and survival might be found upon analysis of a larger group of patients. The subset analysis showing a strong association of low IGF-IR levels with early breast cancer recurrence is of particular importance because breast tumor relapses occurring within 4 years of primary tumor diagnosis are more likely to be clonogenic recurrences. The influence of IGF-IR is clearly evident among this subgroup, suggesting that elevated levels of IGF-IR predispose to early relapses after radiation therapy. The late recurring tumors are more likely to represent new primaries, and as such, their occurrence would not be expected to correlate with molecular markers of radioresistance in the initial tumors.

The work presented here demonstrates that IGF-IR can influence radiosensitivity in mammalian cells in culture and that these proteins may serve as important prognostic factors in predicting disease recurrence and response to treatment in human breast cancer. Furthermore, our findings may have important therapeutic and prognostic implications because they identify IGF-IR as a potential new target for pharmaceutical strategies seeking to enhance tumor radiocurability.

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