Insulin-like Growth Factor-I Receptors Are Overexpressed and Predict a Low Risk in Human Breast Cancer

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ABSTRACT

IGF-I receptor (IGFR) content and its prognostic significance were evaluated in human breast cancer specimens using a sensitive and specific radioimmunoassay (V. Pezzino et al., Metabolism, 40: 861, 1991). The prognostic significance of IGFR expression was investigated by two different approaches: (a) detectable IGFR content was measured in 82% of specimens in a consecutive series of 184 human breast cancers and in 32% of 19 normal breast tissues. The average IGFR content in breast cancer was nearly 10-fold higher than the value observed in normal breast tissue (7.6 ± 0.8 versus 0.8 ± 0.1 ng/ml protein, mean ± SEM; P < 0.001). IGFR content was positively correlated with estrogen (ER) and insulin receptor content (r = 0.269 and 0.515, respectively, Pearson correlation) but not with progesterone receptors (PR). No significant correlation was observed between IGFR content and a variety of tumor parameters (tumor size, lymph node involvement, grade) and host characteristics (age, body mass index, menopausal status); (b) IGFR content was measured in a noncontinuous series of 265 primary breast cancer specimens subdivided into 136 high-risk and 129 low-risk specimens on the basis of being either negative (ER-/PR-/aneuploid/high S-phase) or positive (ER+/PR+/diploid/low S-phase) for four well-established prognostic factors. IGFR levels were significantly higher in the low-risk group (6.4 ± 0.4 ng/ml protein, mean ± SEM) than in the high-risk group (3.6 ± 0.5; P < 0.0001, Wilcoxon sum rank test). In summary, our data indicate that there is an elevated IGFR content in most human breast cancers compared with normal breast tissue and that an elevated IGFR content is a favorable prognostic indicator.

INTRODUCTION

IGF-I4 and IGF-II types I and II, are members of a family of polypeptide hormones having approximately 50% homology to insulin (1, 2). They exert a broad range of metabolic and mitogenic actions through either endocrine, paracrine, or autocrine mechanisms. IGF-I and IGF-II are potent regulators of breast cancer cells in culture (3, 4). Recent evidence suggests that IGF-I is produced by the stromal cells of human breast cancers, whereas IGF-II is secreted by the epithelial cells (4–6).

The biological activities of both IGF-I and IGF-II are mediated through the IGF-I receptor (7). This receptor is similar to, but distinct from, the insulin receptor. Like the insulin receptor, the IGF-I receptor is a membrane glycoprotein of approximately M, 350,000. This receptor consists of two M, 130,000 α-subunits and two M, 90,000 β-subunits (7, 8). These four subunits are disulfide-linked in an α2β2-tetrameric structure. The α-subunit is extracellular, while the β-subunit is a transmembrane protein that has cytoplasmic tyrosine kinase activity. IGF-I binding to the extracellular domain stimulates the tyrosine kinase activity, which, in turn, phosphorylates cytoplasmic components of an IGF-I-specific signal cascade leading to several biological effects, including cell growth.

IGFR are present in cultured breast cancer cells (9, 10), and a monoclonal antibody against the IGF-I blocks the mitogenic effect of IGFs both in vitro, in breast cancer cells, and in vivo, on the growth of breast cancer cells in the nude mouse (11, 12). IGFR are also present in most (50–93%) human primary breast tumors (6, 13–15). In one study (13), IGFR were found to be less frequently present or undetectable in benign breast diseases when compared with breast cancer tissues. In several studies (16–18), the IGFR content was measured in breast cancer specimens and evaluated as a prognostic factor for the malignant disease evolution. However, those studies were not able to firmly establish the prognostic value of IGFR content in breast cancer since different studies yielded conflicting results, indicating either positive, negative, or no relationship between IGFR content and survival (16–18). One reason for these conflicting results may be the technique used for IGFR measurement. In these studies IGFR were measured by 125I-IGF-I binding, an assay that has several potential drawbacks: (a) only accessible and unoccupied receptors can be detected, and Scatchard plots are often curvilinear, which makes the determination of the receptor number difficult; (b) IGF-binding proteins can be coexpressed with the IGFR (19) and interfere with the binding assay by competing with the receptor for both labeled and unlabeled IGF-I.

We have recently developed a radioimmunoassay for the IGFR using a rabbit polyclonal anti-IGF-I antibody and highly purified human placental IGFR (20). This assay is sensitive and specific, reacting negligibly with the insulin receptor and being unaffected by IGF-I and IGF-binding proteins. In the present study, we used this method to measure IGFR content in 449 breast cancer specimens. We first measured IGFR content in a consecutive series of 26 nonmalignant breast tissue and 184 breast cancer specimens and correlated it with several host and tumor variables. To evaluate the potential prognostic significance of IGFR content in breast cancer, we then measured this receptor protein in a second series of 265 primary breast cancer specimens divided into high-risk (n = 136) and low-risk (n = 129) groups on the basis of 4 well-established prognostic factors. The results obtained indicate that the presence of IGFR is a favorable prognostic factor in breast cancer.

PATIENTS AND METHODS

Materials

The following materials were purchased: bovine serum albumin, bacitracin, phenylmethylsulfonyl fluoride, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, Triton X-100, charcoal, and diethylstilbestrol from Sigma Chemical Company (St. Louis, MO); tritiated estradiol, tritiated ORG 2058, and unlabeled ORG 2058 from Amersham (Amersham, United Kingdom); dextran T 70 from Pharmacia Fine Chemicals (Uppsala, Sweden). All the reagents used for IGFR radioimmunoassay were prepared as previously described (20). All other reagents were of analytical grade.

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3 Deceased.
4 The abbreviations used are: IGF, insulin-like growth factor; IR, insulin receptor; IGFR, IGF-I receptor(s); ER, estrogen receptor; PR, progesterone receptor.

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Patients

Two separate series of breast cancer specimens were studied.

Series 1. One hundred, eighty-four breast cancer specimens were obtained as a consecutive series from breast cancers sent to the Cattedra di Endocrinologia at the University of Catania, Italy, for estrogen and progesterone receptor measurement. Histological sections of these 184 breast tumors were classified according to the World Health Organization criteria (21). The grading of infiltrating ductal carcinomas was assessed on a scale of 1 (well differentiated) to 3 (poorly differentiated), taking into account the degree of the tubular differentiation, the nuclear pleomorphism, and the mitotic activity according to the procedure of Bloom and Richardson (22). On the basis of tumor size, breast cancers were subdivided into three groups: diameter < 2 cm, diameter between 2 and 5 cm, and diameter > 5 cm. The characteristics of both tumors and host patients are summarized in Table 1. In addition to these 184 breast cancer specimens, 26 nonmalignant mammary samples (7 fibroadenomas and 19 normal breast tissues) were also analyzed. The normal breast tissues specimens included 15 normal breast tissue specimens obtained during total mastectomy from patients with breast carcinoma and 4 normal breast tissues from patients undergoing reduction mammoplasty.

Series 2. This was a nonconsecutive series of 265 specimens from the San Antonio Tumor Bank at the University of Texas Health Science Center at San Antonio. All specimens had been previously analyzed for the presence of four different well-established prognostic markers: ER and PR content, DNA ploidy, and S-phase. All four indexes were negative in 136 specimens (ER positive/PR positive/diploid/low S-phase) and, therefore, the patients from whom these specimens were obtained were considered at low risk for disease recurrence and death. In contrast, all four indexes were positive in 129 specimens (ER negative/PR negative/aneuploid/high S-phase) and, therefore, the patients from whom these specimens were obtained were considered at high risk. No tissue having other combinations of the four markers was included in this study.

Collection of Tumors and Tissue Processing

Specimens were collected at the time of surgery and were dissected free of fat and connective tissue. Samples were then divided into several parts and frozen. To prepare solubilized receptors for assay, we used procedures used previously to maximize receptor recovery (20). In brief, all tissue specimens were weighed, minced, suspended at 0.1-0.2 g/ml in 50 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid buffer, pH 7.6, containing 1 mg/ml bacitracin and 1 mM phenylmethylsulfonyl fluoride, and homogenized with a Polytron homogenizer (Kinematica, Lucern, Switzerland) at 4°C for 15 s at a setting of 6. The homogenized material was then incubated with Triton X-100 (1% final concentration) for 60 min at 4°C under continuous shaking. The solubilized material was then centrifuged at 10,000 X g for 10 min at 4°C, and the supernatant was frozen at -80°C until assayed.

Table 1 Characteristics of patients and tumors (series 1)

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>57±*</td>
</tr>
<tr>
<td>Median</td>
<td>59±*</td>
</tr>
<tr>
<td>Range</td>
<td>29-84±*</td>
</tr>
<tr>
<td>Menopausal status</td>
<td></td>
</tr>
<tr>
<td>Premenopausal</td>
<td>52</td>
</tr>
<tr>
<td>Postmenopausal</td>
<td>132</td>
</tr>
<tr>
<td>Tumors</td>
<td></td>
</tr>
<tr>
<td>Size (diameter)</td>
<td></td>
</tr>
<tr>
<td>&lt;2 cm</td>
<td>97</td>
</tr>
<tr>
<td>2-5 cm</td>
<td>67</td>
</tr>
<tr>
<td>&gt;5 cm</td>
<td>20</td>
</tr>
<tr>
<td>Grading</td>
<td></td>
</tr>
<tr>
<td>G1</td>
<td>7</td>
</tr>
<tr>
<td>G2</td>
<td>90</td>
</tr>
<tr>
<td>G3</td>
<td>44</td>
</tr>
<tr>
<td>Nodal status</td>
<td></td>
</tr>
<tr>
<td>N-</td>
<td>82</td>
</tr>
<tr>
<td>N+</td>
<td>102</td>
</tr>
<tr>
<td>Histo logical type</td>
<td></td>
</tr>
<tr>
<td>Ductal</td>
<td>141</td>
</tr>
<tr>
<td>Lobular</td>
<td>28</td>
</tr>
<tr>
<td>Others</td>
<td>15</td>
</tr>
</tbody>
</table>

* Years.

IGF-I Receptor Radioimmunoassay

The IGF-I radioimmunoassay was performed as previously described (20). The sensitivity of the assay is 0.7 ng, the cross-reactivity with the insulin receptor is <5%, and IGF-I- and IGF-I-binding proteins do not cross-react in the assay (data not shown). Receptor content in tissue specimens was expressed as ng of IGF-I receptor/0.1 mg of protein or DNA.

Estrogen and Progesterone Receptor Measurements

ER and PR measurements were performed as recommended by the EORTC (23) for the first series and as described by Dressler et al. (24) in the second series. In both series, the receptor content was expressed as fmol of receptor/mg of protein. Values >10 fmol/mg of protein were considered positive for both receptors in the first series (23). ER values > 33 fmol/mg of protein and PR values > 5 fmol/mg of protein were considered positive in the second series.

DNA Flow Cytometry

DNA flow cytometry was carried out as described elsewhere (25). DNA content was defined as aneuploid if two discrete G0/G1 peaks could be confirmed after the addition of human peripheral blood lymphocytes. In addition, the aneuploid G0/G1 peak had to contain at least 10% (20%) in the tetraploid region of the 50,000 sample events collected and have a corresponding identifiable G2/M peak. When ploidy status could not be determined because of the poor sample quality or insufficient resolution to distinguish two peaks (coefficients of variation >6%), the histograms were considered interpretable for ploidy status. The MODFIT program (Verity Software House, Inc., Topsham, ME) was used for cell cycle analysis. Specimens with less than 3.0 or more than 12.0% of cells in S-phase were considered low or high S-phase tumors, respectively.

Other Methods

Insulin receptor radioimmunoassay was performed as previously described (26). Proteins were measured by the method of Lowry et al. (27), and DNA was measured according to the method of Labarca and Paigen (28).

Statistical Analysis

Nonparametric Wilcoxon rank sum tests were used to compare distributions of IGFR values between various groups of patients. Pearson product-moment correlation was computed to assess the relationship between IGFR content and each single tumor or host prognostic factor. Since some of these factors did not follow normal distributions, either logarithmic (IGFR, IR, and PR) or square root (ER) transformation was applied to these data prior to the analysis. Multiple linear regression analysis was also performed to predict IGFR content on the basis of other variables.

RESULTS

IGF-I Receptor Content in Human Breast Cancer

We first evaluated the reactivity of breast cancer tissues in the IGF-I radioimmunoassay. As previously reported (20), progressive dilutions of tissues generated competition-inhibition curves that were parallel to the placental purified IGFR standard (data not shown), suggesting that both breast cancer and placental IGFR reacted in a similar way in the assay.

We then evaluated the IGFR content in the two separate series of breast cancer patients, obtained as described in "Patients and Methods."

In the first consecutive series of 184 primary breast cancer specimens, 19 normal breast tissue specimens and 7 fibroadenoma specimens, a detectable IGFR content was found in 150 of 184 (81.5%) breast cancer tissues, in 7 of 7 fibroadenomas, but only in 6 of 19 (31.6%) normal breast specimens. When the specimen IGFR content was lower than the smallest amount detectable in the assay, a value of 0.5 ng/0.1 mg protein was assigned to undetectable specimens for the
calculation of average IGFR content. The average IGFR content in breast cancer tissues was 7.56 ± 0.84 ng/0.1 mg protein (mean ± SEM; median, 4.01; range 60.40) (Fig. 1). This value in breast cancer specimens was nearly 10-fold higher (P < 0.001) than the mean value observed in normal breast tissues (0.79 ± 0.12 ng IGFR/0.1 mg protein; median, 0.50; range, 1.70). This significant difference was also present when in the last 57 specimens of our continuous series (45 breast cancers and 12 normal breast tissues) IGFR content was normalized per DNA content. In this series, a nearly 10-fold IGFR content increase was observed (1242 ± 132 ng IGFR/0.1 mg DNA in breast cancer specimens versus 131 ± 17 ng IGFR/0.1 mg DNA in normal breast specimens, mean ± SEM; P < 0.001). A difference between IGFR content in cancer tissue versus normal tissue was also present when 15 paired specimens of breast cancer and adjacent normal breast tissue were compared (3.70 ± 0.21 versus 0.71 ± 0.10 ng/0.1 mg protein, mean ± SEM, respectively; P < 0.001). The average IGFR content in fibroadenoma specimens was higher than that observed in normal breast tissue (2.29 ± 1.60 ng IGFR/0.1 mg protein, mean ± SEM; median, 2.15; range, 4.54), but the difference was not statistically significant. These data indicate, therefore, that breast cancer tissues contain higher levels of IGFR than normal breast tissues.

The second series included 265 primary breast cancers already analyzed for four markers of risk of death and/or recurrence. These included two markers of differentiation, ER and PR, and two indexes of proliferation, DNA ploidy and the percentage of cells in the S-phase fraction. In the 129 low-risk tumors (positive ER and PR, diploid, and low S-phase) IGFR levels were significantly higher (6.34 ± 0.43 ng IGFR/0.1 mg protein, mean ± SEM; median, 5.14) than in the 136 high-risk tumors (3.61 ± 0.48 ng IGFR/0.1 mg protein; median, 2.04; P < 0.0001, Wilcoxon rank sum test) (Fig. 2). A similar difference between the two groups was also observed when data were normalized for DNA content (2848 ± 321 ng IGFR/0.1 mg DNA, mean ± SEM; median, 2252 in the low-risk group versus 1178 ± 104 ng IGFR/0.1 mg DNA; median, 828 in the high-risk group; P < 0.0001). An undetectable IGFR content was found in 5 of 129 (3.9%) low-risk and in 15 of 136 (11.0%) high-risk tumors.

Correlations

We then tried to address the question of whether in the first continuous series of 184 breast cancer specimens the IGFR content was correlated with other characteristics of the tumor. Some data from each group were first transformed in their natural logarithms (IGFR, IR, and PR) or square roots (ER) to produce a normal-shaped distribution. Then, the Pearson product-moment correlation was calculated, and the multiple linear regression analysis was carried out between IGFR content and other variables. These variables were grouped into three categories: host variables, tumor variables other than receptors, and hormone receptors. These data are summarized in Table 2.

**Fig. 2.** Distribution of IGF-I receptor content in two groups of breast cancer specimens. Breast cancers were considered at high risk (n = 136) or low risk (n = 129) on the basis of having a homogenous positivity or negativity for 4 well-established prognostic factors, such as ER, PR, DNA ploidy, and S-phase. High risk, ER-/PR-/aneuploid/high S-phase. Low risk, ER+/PR+/diploid/low S-phase. Shaded area, IGF-I receptor values lower than assay detectability.

<table>
<thead>
<tr>
<th>Variable Correlation</th>
<th>Host</th>
<th>0.055</th>
<th>Menopausal status</th>
<th>-0.052</th>
<th>Body mass index</th>
<th>0.087</th>
</tr>
</thead>
<tbody>
<tr>
<td>tumor</td>
<td>Size</td>
<td>-0.028</td>
<td>Axillary node</td>
<td>0.059</td>
<td>Tumor grade</td>
<td>-0.008</td>
</tr>
<tr>
<td>Hormone receptors</td>
<td>Square root of ER</td>
<td>0.269</td>
<td>Log PR</td>
<td>0.162</td>
<td>Log IR</td>
<td>0.515</td>
</tr>
</tbody>
</table>

**Host Variables.** Three host variables were considered: age, menopausal status, and body weight (expressed as body mass index). None of these variables significantly correlated with IGFR content of the tumors.

**Tumor Variables Other Than Receptors.** Histological type, tumor size, grade of differentiation, and axillary node involvement were analyzed. None of these variables was significantly correlated with the IGFR content of the tumor.

**Hormone Receptors.** Three hormone receptors were studied in addition to IGFR: ER, PR, and IR. Log IGFR was highly correlated with log IR (Pearson correlation, r = 0.515). However, a number of specimens with no relationship between the two receptor contents (i.e., high IGFR and low IR content and vice versa) were also identified. Log IGFR was significantly correlated with the square root of ER (r = 0.269) but not with log PR. When multiple regression analysis was used, only two factors, log IR and square root of ER, were able to give a good prediction of log IGFR. The multiple R² for this correlation was 0.29 (i.e., 29% of the variation in log IGFR was explained by variation in log IR and square root of ER).

**DISCUSSION**

In the present study, we used a specific and sensitive radioimmunoassay to measure IGFR content in 449 samples of breast carcinomas. The measurement of IGFR by radioimmunoassay has several advantages over classical binding studies, including the ability to detect both occupied and unoccupied receptors, to give a direct quantification of the receptor protein, and to avoid the interference of IGF-binding proteins. Additional advantages are that a very small amount of tissue is needed for the radioimmunoassay (approximately
0.1–0.2 mg of tissue protein), and multiple specimens may be stored frozen and analyzed in a single assay, making this assay clinically accessible.

In agreement with previous reports (6, 13–15), using our radioimmunoassay, we find that most breast tumors (88%) contain a measurable amount of IGFR and, therefore, may be able to respond to IGF-I (or IGF-II) through either endocrine, autocrine, or paracrine pathways. IGFR content values were widely distributed in different breast cancer specimens, indicating that IGFR expression is highly variable in different breast tumors.

The mean IGFR content in malignant breast tissue was approximately 10-fold higher than in normal breast tissue. However, there was a certain degree of overlap in IGFR content between normal and cancer tissues. In fact, specimens with undetectable IGFR levels were identified in both normal and malignant tissues: this was the case, however, in <20% of breast cancer specimens and in nearly 70% of normal breast specimens. Since surgical specimens may be a mixture of malignant and normal epithelial cells, and also stromal cells, the malignant to normal cell ratio as well as the stromal to epithelial cell ratio may change in different specimens. This potential problem, which is also present for clinically accepted procedures such as estrogen and progesterone receptor measurement, was addressed by normalizing IGFR content to DNA content, the latter being a more direct expression of cell number. Our data, when normalized for either protein or DNA content, indicated that breast cancer specimens had an IGFR content approximately 10-fold higher than normal breast tissue, confirming that overexpression of the IGFR is a characteristic feature of most breast cancers. The mechanism of IGFR overexpression is unknown. Gene amplification is not a likely cause of IGFR protein overexpression in breast cancer, since it has been recently reported that only 2% of these tumors have an increased IGFR gene copy number (18). Other mechanisms, therefore, such as enhanced protein synthesis or decreased protein turnover, must be involved in the increased IGFR protein expression in most breast cancers.

The second issue addressed in the present study was the prognostic value of IGFR content in breast cancer. Conflicting results have been published. Bonneterre et al. (17) described a longer disease-free survival in patients with tumors having high IGFR levels, whereas Foekens et al. (16) found no relationship between IGFR levels and survival. The latter group recently reported that overall survival is longer in patients having tumors with a low IGFR gene copy number compared with patients having tumors with high IGFR copy number (18). IGFR protein content, however, was not measured in all of the tumors included in this study (18).

To address the problem of the prognostic value of IGFR content in breast cancers, we studied two separate series of patients. We measured IGFR content in a selected series of specimens collected from primary breast cancer patients, previously classified as being at low or high risk for recurrence and/or progression on the basis of their steroid receptor status, DNA content, and percentage of cells in S-phase. Specimens with the worst combination of all four markers (i.e., ER and PR negative, aneuploid, and high S-phase fraction) and, therefore, considered at high risk had a significantly lower average IGFR content when compared with specimens considered at low risk because of having all four markers in the favorable combination. These results suggest, therefore, that IGFR may be an additional positive prognostic marker in primary breast cancer patients. However, a large overlap of IGFR content values was present in these two groups, indicating that other factors influence the biology of breast tumors.

In order to acquire further information concerning the prognostic role of IGFR content in breast cancer, IGFR content was correlated with several tumor and patient characteristics known to influence the malignancy outcome in a separate consecutive series of 184 specimens. No significant correlation was found between IGFR content and various tumor (tumor size, lymph node involvement, tumor grade) or host (age, body mass index, menopausal status) characteristics except for a positive relationship between IGFR and ER content, an observation that is consistent with previous reports (6, 13–17).

The IGFR, like the IR, is a member of the tyrosine kinase receptor family (29). Overexpression by transfection of either of these two receptors can induce malignant changes but only in the presence of the ligand (30, 31). Therefore, an apparent paradox appears to exist between in vitro data, suggesting that IGFR overexpression plays a role in tumorigenesis, and in vivo data, suggesting that IGFR overexpression in breast cancer cells is associated with a more favorable clinical outcome. In in vitro studies, the cells used (Chinese hamster ovary, NIH 3T3) have low numbers of endogenous receptors which are markedly increased (>100-fold) with transfection, and therefore, the transfection of a high number of receptors renders the cells very sensitive to growth stimulatory effects of small concentrations of ligand. IGFR are present in normal breast epithelial cells, where, most likely, they play a role in normal cell metabolism when activated by IGFs. Therefore, the presence of IGFR in breast cancer cells may reflect a more differentiated breast cancer cell phenotype and, together with other biological characteristics of the tumor, a better clinical outcome.

The IGFR situation in breast cancer, therefore, appears to be similar to that occurring for estrogen receptors. Estrogen stimulates the growth of breast cancer cells, but the presence of estrogen receptors in breast cancer cells indicates a more favorable clinical outcome, since their presence suggests a more differentiated phenotype. In fact, we have observed a correlation between IGFR and ER content. Accordingly, IGFR content, along with ER content, may be a useful marker of breast cancer differentiation and the clinical course of patients with this disease.

It has been reported that plasma IGF-I concentrations are higher in primary breast cancer patients when compared with control women (32). Even if there is no direct evidence that the elevated plasma levels of IGF-I reflect elevated levels of this growth factor at the tumor level, the possibility exists that increased levels of circulating IGF-I may contribute to breast tumor growth. Thus, treatment of patients with agents that decrease plasma levels of IGF-I, such as tamoxifen (33) or somatostatin (34), might induce a favorable change in the biology of the tumor by removing the stimulatory effects of this growth factor.

One additional finding of the present study is the correlation between IGFR and IR content in breast cancer. We previously reported that insulin receptors are increased in human breast cancer (35). We (36) and others (37, 38) also demonstrated that these receptors are functional in human breast cancer. Since IGFR and IR are closely related proteins and both are elevated in breast cancer, it would be expected that both receptors are expressed in a similar way in the same specimen. In fact, a significant positive correlation between IGFR and IR was found in our study. However, a continuous spectrum of IGFR and IR content ratio was found with subsets of tumors having high IGFR and low IR protein contents or vice versa. Further studies are needed to determine the clinical significance of these observations and their possible relationship with peculiar biological and clinical characteristics of the tumors.

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