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Membrane localization of insulin receptor substrate-2 (IRS-2) is associated with decreased overall survival in breast cancer

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Abstract

Recent studies have identified a role for insulin receptor substrate-2 (IRS-2) in promoting motility and metastasis in breast cancer. However, no published studies to date have examined IRS-2 expression in human breast tumors. We examined IRS-2 expression by immunohistochemistry (IHC) in normal breast tissue, benign breast lesions, and malignant breast tumors from the institutional pathology archives and a tumor microarray from a separate institution. Three distinct IRS-2 staining patterns were noted: diffusely cytoplasmic, punctate cytoplasmic, and localized to the cell membrane. The individual and pooled datasets were analyzed for associations of IRS-2 staining pattern with core clinical parameters and clinical outcomes. Univariate analysis revealed a trend toward decreased overall survival (OS) with IRS-2 membrane staining, and this association became significant upon multivariate analysis ($P = 0.01$). In progesterone receptor negative (PR-) tumors, in particular, IRS-2 staining at the membrane correlated with significantly worse OS than other IRS-2 staining patterns ($P < 0.001$). When PR status and IRS-2 staining pattern were evaluated in combination, PR- tumors with IRS-2 at the membrane were associated with a significantly decreased OS when compared with all other combinations ($P = 0.002$). Evaluation of

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IRS-2 staining patterns could potentially be used to identify patients with PR- tumors who would most benefit from aggressive treatment.

Keywords

IRS proteins; Breast cancer; Progesterone receptor; IGF-1 receptor

Introduction

Insulin receptor substrate-1 (IRS-1) and IRS-2 adaptor proteins are downstream signaling intermediates of several receptors that transmit signals to impact breast carcinoma cell function [1–8]. The most widely studied of these receptors is the insulin-like growth factor-1 receptor (IGF-1R), which has been implicated in breast cancer progression and response to therapy [1–4]. Despite a high level of sequence homology, IRS-1 and -2 play divergent roles in breast cancer [9]. Signaling through IRS-1 promotes breast carcinoma cell proliferation; whereas signals transmitted through IRS-2 regulate breast carcinoma cell motility and invasion, as well as glycolysis [10–17]. Studies from our own lab have demonstrated that IRS-2 acts as a positive regulator of metastasis, while IRS-1 cannot compensate for this function and may negatively regulate metastasis [10,11]. Taken together, the results from these studies support that IRS-1 and -2 can markedly influence the outcome of signaling through their upstream receptors, and the expression of these proteins is likely to impact the biology of breast tumors.

Although IRS-1 and -2 are expressed relatively ubiquitously in human tissues, differences in their expression patterns have been reported [18]. In the developing mouse mammary gland, IRS-1 is expressed in a subset of luminal mammary epithelial cells in mature ducts and also in the body of the terminal end bud (TEB) [19]. In contrast, IRS-2 is expressed homogeneously in the ductal luminal epithelial cells and also throughout the TEB, including the cap cell layer [19]. These differential expression patterns likely reflect differences in the regulatory mechanisms that control expression of their genes. *IRS-1* is an estrogen-regulated gene, and its expression correlates positively with estrogen receptor (ER) expression in human breast tumors [20–24]. IRS-1 expression is the highest in more well-differentiated, ER-positive (ER+) cell lines and tumors, and its expression may decrease with disease progression, as expression or function of the ER is lost [25]. *IRS-2* is a progesterone-responsive gene, and it is expressed at higher levels in more poorly differentiated, ER-negative (ER-) breast carcinoma cells [10,26]. Given that breast carcinoma cells that express IRS-2 often lack expression of the progesterone receptor (PR), alternative mechanisms are likely to play a more dominant role in regulating IRS-2 expression in breast tumors. In this regard, epidermal growth factor (EGF) signaling and hypoxia positively regulate the expression of *IRS-2* [27,28].

In addition to differential regulation of *IRS-1* and -2 at the level of gene transcription, intracellular localization is also likely to play an important role in the divergent functions of these adaptor proteins. Although many tumors exhibit diffuse, cytoplasmic expression of IRS-1, IRS-1 can also localize to the nucleus where it can interact with ER- α and modulate its transcriptional activity [20,25,29,30]. An association with increased tamoxifen response and survival for patients with tumors expressing IRS-1 in the nucleus has been reported [30]. The authors of that study suggested that nuclear IRS-1 reflects an upregulation of ER signaling, which would render a tumor more sensitive to an ER antagonist such as tamoxifen [30]. To date, there are no published studies examining IRS-2 expression in human breast tumors. In this study, we evaluated IRS-2 expression in invasive ductal carcinomas and

identified distinct IRS-2 expression patterns that have significance for overall patient survival outcomes.

Materials and methods

Tumor sections

Formalin-fixed, paraffin-embedded tumor sections were obtained from the Pathology Department archives and tumor bank at the University of Massachusetts Medical School. Institutional Review Board (IRB) approval was obtained for this study, and informed consent was obtained for all participating subjects. The retrospective study population consisted of patients diagnosed between the years of 1997 and 2007 with breast cancer of any stage. Complete data on tumor size, tumor grade, node status, and receptor status were available. For many patients, follow-up data on recurrence-free survival, overall survival (OS), metastases, therapy, and co-morbid conditions were also available. Median follow-up was 69.5 months for recurrence-free survival and 72.5 months for OS.

Tissue microarray

A tissue microarray was constructed from 154 cases from the surgical pathology files at the University of Michigan Health System [31]. Three 0.6-mm diameter cores were taken from each formalin-fixed, paraffin-embedded block. Cases which did not contain cores with tumor cells were excluded from analysis, resulting in 130 final cases. The study was approved by the IRB, and informed consent was obtained for all subjects. The retrospective study population consisted of patients diagnosed between the years of 1987 and 1991 with invasive breast carcinoma. Complete data on tumor size, tumor grade, node status, receptor status, and other clinicopathologic variables, as well as recurrence-free survival, OS, and therapy were available. Median follow-up time was 80.0 months for recurrence-free survival and 104.4 months for OS.

Immunohistochemistry

Immunohistochemical studies were performed on 5- μ m sections. Archived blocks were stored in a climate-controlled environment. All staining was performed immediately after sectioning to maintain maximum antigenicity for detection. Tissue sections were deparaffinized and rehydrated, and antigen retrieval was carried out with 0.01 M citrate buffer, pH 6.0, for slides to be stained for IRS-1, or 0.001 M EDTA, pH 8.0, for slides to be stained for IRS-2, and heating in a 770-W microwave oven for 14 min. The slides were stained on the Dako Autostainer (Dako, Carpinteria, CA) using EnVision+ (Dako) staining reagents. Tissue sections were blocked with Dual Endogenous Block for 10 min, and then incubated for 30 min with either rabbit polyclonal IRS-1 (C20, Santa Cruz, Santa Cruz, CA) at a concentration of 1:400 or rabbit monoclonal IRS-2 (1849, Eptomics, Burlingame, CA) at a concentration of 1:400. Following a buffer wash, sections were incubated with the EnVision+ Dual Link detection reagent for 30 min, and then treated with a solution of diaminobenzidine and hydrogen peroxide for 10 min to produce the visible brown pigment. DAB Enhancer was used to enrich the final color. The tissue sections were counterstained with hematoxylin, dehydrated, and coverslipped using a permanent mounting medium.

Sections were evaluated for staining pattern using the following criteria: (1) Diffuse staining was defined as even staining throughout the cytoplasm with no clear demarcation of cell borders; (2) Punctate staining was defined as clearly demarcated puncta of staining within the cytoplasm of each cell with or without diffuse background staining of the cytoplasm; (3) Membrane staining was defined as clear demarcation of cell borders by staining with or without diffuse background staining of the cytoplasm. The individual assessing staining patterns was blinded to all prognostic and follow-up data. A second blinded individual

assessed a subset of 30 cases for IRS-2 staining patterns with 93.3% concordance. In addition, a subset of the tumors was evaluated using a different antibody that recognizes IRS-2 to confirm the observed staining patterns. The tissue microarray was evaluated using the same criteria that were used for the tumor sections. Three cores per patient were evaluated, and the membrane staining pattern was designated if it was contained in any of the three cores per patient sample.

Imaging

Stained tumor sections were viewed on an Olympus BX41 light microscope (Olympus, Center Valley, PA). Photomicrographs were obtained using an Evolution MPCColor camera (Media Cybernetics, Bethesda, MD).

Immunoblotting

Cells were solubilized at 4°C in RIPA buffer, and cell extracts containing equivalent amounts of protein were resolved by SDS-PAGE and transferred to nitrocellulose filters. For samples requiring cytoplasmic/nuclear fractionation, the NE-PER kit (Pierce, Rockford, IL) was used according to the manufacturer's instructions. The filters were blocked for 1 h with a 50 mM Tris buffer, pH 7.5, containing 0.15 M NaCl, 0.05% Tween 20, and 5% (wt/vol) dry milk, incubated overnight at 4°C in the same buffer containing primary antibodies and then incubated for 1 h in blocking buffer containing peroxidase-conjugated secondary antibodies. Proteins were detected by enhanced chemiluminescence (Pierce). The following antibodies were used for immunoblotting: IRS-1 (#C20, Santa Cruz), IRS-2 (#420293, Calbiochem, Gibbstown, NJ; #1849, Epitomics), GAPDH (#A300-642A, Bethyl, Montgomery, TX), hnRNP A1 (#4B10, Santa Cruz), peroxidase-conjugated goat anti-rabbit IgG (Jackson, West Grove, PA), peroxidase-conjugated goat anti-mouse IgG (Jackson).

Cell lines, shRNA, and transfection

The MDA-MB-231 cell line was obtained from the ATCC Cell Biology Collection. A lentiviral vector containing a small hairpin RNA (shRNA) targeting IRS-2 was obtained from Open Biosystems (Huntsville, AL). MDA-MB-231 cells were infected with virus, and stably expressing cells were selected by the addition of 2 µg/ml puromycin. For IHC analysis, cells were pelleted and fixed in 10% zinc formalin before embedding in paraffin blocks.

Statistical analysis

Overall survival was measured from the date of first cancer diagnosis to the date of death from any cause and was censored from the date of last follow-up for survivors. Data for age, tumor size, node status, grade, ER status, PR status, HER2 status, and therapy were obtained as baseline variables. Therapy was defined as any combination of chemotherapy, radiation, or tamoxifen. As most samples were surgical specimens, surgery was not included. OS was estimated by the Kaplan–Meier method and assessed by the use of log-rank test for univariate analysis. We used the Cox proportional-hazard model to assess and control the simultaneous contribution of baseline covariates in multivariable analyses. First, we estimated the effect of IRS-2 within each individual dataset in the multivariable analysis. We then combined the two datasets in a proportional hazards model that tested for heterogeneity in the effect estimates from the two datasets by the inclusion of multiplicative terms involving the study indicator. The pooled results from the two datasets were presented if there was no significant heterogeneity of the effects for IRS-2 variables. A two-sided *P*-value of <0.05 was considered to indicate statistical significance. The REMARK criteria were used for this study [32].

Results

IRS staining in normal breast tissue and benign breast disease

Twenty cases from the pathology archives were reviewed to identify all normal and pathological findings. Five cases were found to contain normal ducts. In total, 22 benign lesions were identified in the tissue set. The tissue set was evaluated by IHC for IRS-1 and -2 expression. The IRS-1 antibody used in this study has been characterized in previous studies and stained pancreatic islet cells as expected [25,33,34] (Fig. 1c). We evaluated the specificity of the IRS-2 antibody by staining MDA-MB-231 cells that expressed an IRS-2-specific shRNA to suppress IRS-2 expression. Trypsinized cells were spun down, and the cell pellets fixed in zinc formalin, embedded in paraffin, and stained using the same IHC protocol that was used for staining the tissue sections. Parental MDA-MB-231 cells stained positive for IRS-2, and this staining was diminished significantly when IRS-2 expression was suppressed (Fig. 1a). The antibody also exhibited specificity for IRS-2 by immunoblot (Fig. 1b). As a positive control, pancreatic islets stained positive for IRS-2 using this antibody (Fig. 1c).

All five cases containing normal ducts exhibited nuclear IRS-1 staining in the luminal epithelium (Fig. 2). Four of five cases also exhibited fine, punctate IRS-1 staining in the cytoplasm in the myoepithelial (basal) cells. IRS-2 was expressed strongly in the myoepithelial cells of the normal ducts, and in 4 of 5 cases it was also expressed diffusely in the cytoplasm of the luminal epithelium (Fig. 2). However, one case exhibited punctate cytoplasmic staining for IRS-2 in the luminal epithelium, and one case exhibited staining at the membrane in addition to diffuse cytoplasmic staining. IRS-2 was not expressed in the nuclei of either the myoepithelial or luminal epithelial cells of normal ducts.

Twenty-two benign lesions were examined consisting of the following: 6 fibroadenomas, 7 cases of ductal hyperplasia (6 of the usual type, 1 of the atypical type), 7 cases of sclerosing adenosis, and 2 intraductal papillomas. Representative images of each type are shown in Fig. 2. Two cases were negative altogether for IRS-1 staining, including one case of sclerosing adenosis and the atypical ductal hyperplasia. The majority of the benign lesions (77.3%) demonstrated positive nuclear staining for IRS-1 (Fig. 2), and 20 of 22 cases also exhibited punctate IRS-1 staining of the myoepithelial cells. All of the benign lesions exhibited diffuse cytoplasmic IRS-2 expression in the luminal epithelium, with two cases each demonstrating additional membrane or punctate staining of luminal cells. Strong cytoplasmic IRS-2 staining was also observed in the myoepithelial cells of the benign lesions.

IRS expression patterns in invasive ductal carcinoma

IRS expression was evaluated in 157 invasive ductal carcinoma tumor sections from the pathology archives. This tumor set consisted of the following: grade 1 (21 tumors), grade 2 (39 tumors), and grade 3 (97 tumors). Detailed clinical information was available for a subset of these tumors (128 tumors), and the clinical characteristics of this tumor subset are shown in Table 1 (Set 1). Consistent with our findings in normal and benign breast tissue and previously published results, IRS-1 was expressed in the nuclei and cytoplasm of invasive tumors (Fig. 3a) [20,25,30]. IRS-2 was not expressed in the nucleus in any of the invasive tumors (Fig. 3a). Analysis of SUM-159PT and MDA-MB-231 breast carcinoma cells after fractionation into cytoplasmic and nuclear fractions confirmed the nuclear localization patterns of IRS-1 and -2 in the human tumors. IRS-1 was localized in both the nucleus and cytoplasm in both cell lines, while IRS-2 was localized only in the cytoplasm (Fig. 3b).

Upon further analysis of the IRS-2 staining in the invasive tumors, three distinct staining patterns were observed: diffuse cytoplasmic staining (diffuse; Fig. 4a), punctate cytoplasmic

staining (punctate; Fig. 4b), and membrane staining (membrane; Fig. 4c). While diffuse cytoplasmic staining was the dominant IRS-2 staining pattern in normal ducts and benign lesions, punctate and membrane staining patterns increased in tumors. This alteration in IRS-2 staining patterns during malignant progression was demonstrated in one case in which normal ducts, ductal carcinoma in situ (DCIS), and invasive tumor were all present. IRS-2 staining of the normal ductal epithelium was diffusely cytoplasmic, whereas membrane staining was observed in the adjacent DCIS and invasive tumor (Fig. 4j).

To confirm the IRS-2 staining patterns in a second tumor set, we evaluated a tissue microarray from a separate institution containing 130 new patient cases. The clinical characteristics of this tumor subset are summarized in Table 1 (Set 2). The microarray was stained for IRS-2 by IHC and evaluated by the same individual who evaluated Set 1. All three IRS-2 staining patterns were observed (Fig. 4d–i), although in different percentages than were observed for Set 1. This may reflect the differences in the distribution of tumors by grade in the two individual datasets (Table 1).

Correlations with clinical and pathological characteristics

The two datasets were analyzed individually and then pooled for the association of IRS-2 staining patterns with survival outcomes. Clinical parameters and OS trends for the pooled dataset are presented in Table 2. Initially, survival data were analyzed for the tumor subset defined as Set 1 in Table 1, and correlations were drawn to IRS-2 staining pattern. Although not statistically significant, a trend toward decreased OS with IRS-2 membrane staining was noted on the initial univariate analysis. This trend became statistically significant in the follow-up multivariate analysis when the dataset was adjusted for confounders such as grade, tumor size, node status, receptor status, and therapy (HR = 4.61, 95% CI 1.35–15.71, $P = 0.02$) (Table 3 and Fig. 5a). No statistically significant associations with OS were observed for the diffuse or punctate staining patterns in either the univariate or multivariate analysis (Supplemental Table 1, Supplemental Figs. 1a and 2a). In the analysis of recurrence-free survival, the effect estimates for IRS-2 staining patterns were similar in direction and magnitude as those found in the analysis of OS.

The effect of IRS-2 membrane pattern on OS was analyzed within each clinical parameter individually. From this analysis, the only clinical parameter to show a significant association with IRS-2 localization at the membrane was PR negative (PR[−]) status. Univariate analysis revealed that patients with tumors that were PR[−] and stained positive for IRS-2 at the membrane had significantly worse survival outcomes when compared with patients with tumors that were PR[−] but did not have IRS-2 membrane staining ($P = 0.04$) (Table 3). To examine further the association of IRS-2 membrane staining and PR expression with regard to patient survival, the data were analyzed by PR status together with IRS-2 membrane staining status. As shown in Fig. 5b, decreased survival was observed only in the PR[−] tumors that exhibited IRS-2 membrane staining ($P = 0.03$). Expression of IRS-2 at the membrane did not confer decreased survival in patients with tumors that expressed PR ($P = 0.40$).

Statistical analysis of the tissue microarray dataset (Set 2) produced results consistent with the findings from Set 1 (Table 3, Fig. 5c, d). Though not statistically significant, IRS-2 at the membrane nearly doubled risk of death. Among PR[−] tumors, membrane staining conferred a statistically significant, nearly 6-fold decrease in OS on multivariate analysis ($P = 0.01$). When PR status and membrane staining were evaluated in combination, PR[−] tumors with membrane IRS-2 exhibited a decrease in OS compared to all other subtypes ($P = 0.021$), as we had observed for the original tumor dataset (Set 1).

The effect estimates for IRS-2 membrane staining were not statistically different between the two datasets: *P*-value from the heterogeneity test was 0.47 among all cases and 0.74 among PR[−] cases. When Sets 1 and 2 were analyzed as a pooled dataset with a total tumor number of 258, the observed trends from the individual datasets became even stronger (Table 3, PFig. 5e, f). On multivariate analysis of the pooled dataset, tumors with IRS-2 at the membrane exhibited a significant decrease in OS (*P* = 0.01), and this trend was even more highly significant among PR[−] tumors (*P* < 0.001). As with each independent set, PR negative status in combination with the IRS-2 membrane staining pattern was associated with a significant decrease in OS compared to all other subtypes in the pooled dataset (HR = 3.36, 95% CI 1.58–7.16, *P* = 0.002, as compared to PR[−]/memb[−]).

The individual and pooled datasets were also analyzed for the effect of diffuse and punctate IRS-2 staining on OS of patients with PR[−] tumors (Supplemental Table 1, Supplemental Figs. 1 and 2). On multivariate analysis, the diffuse staining pattern improved survival in patients with PR[−] tumors in the individual and pooled datasets, although not to the extent that PR⁺ status improved survival. These results were statistically significant only in Set 2 and the pooled dataset (*P* = 0.03 and 0.02, respectively). In PR⁺ tumors, there was no further benefit of the diffuse staining pattern on OS rates. A significant effect on survival of combined punctate staining and PR status was observed upon univariate analysis in the pooled dataset only (*P* = 0.02). However, on multivariate analysis, this effect appears to be due to PR status alone.

Discussion

In this study, we present the first report of IRS-2 expression in normal human breast and breast tumors. In the normal breast, IRS-2 is expressed strongly in the myoepithelial cell layer, with a lower level of diffuse cytoplasmic staining in the luminal epithelial cells. This expression pattern persists in benign breast disease. In invasive breast tumors, IRS-2 is localized in one of three staining patterns: diffusely cytoplasmic, punctate in the cytoplasm, and at the plasma membrane. IRS-2 is absent from the nucleus in both normal and tumor tissue. With regard to clinical relevance, IRS-2 membrane staining is associated with decreased OS of breast cancer patients. In addition, IRS-2 membrane staining identifies a sub-population of patients with PR[−] tumors that have significantly worse OS outcomes. Taken together, our results demonstrate that IRS-1 and -2 have distinct intracellular localization patterns in human breast tumors, and they reveal a potential role for IRS-2 in the aggressive biology of PR[−] breast tumors.

Our observation that IRS-1 and -2 are expressed in distinct intracellular compartments reveals a potential mechanism for their divergent roles in breast cancer [12]. The targeting of IRS-1 and -2 to unique intracellular compartments would localize the signals that are generated and determine access of these adaptor proteins to distinct subsets of downstream effectors. As a result, different functional outcomes would occur. In the nucleus, IRS-1 interacts with ER- α and regulates its transcriptional activity [29]. IRS-1 can also interact with β -catenin, the androgen receptor, and upstream binding factor-1 to positively regulate target gene expression [35,36]. Regulation of genes such as Cyclin D and c-Myc is likely to contribute to the IRS-1-dependent stimulation of proliferation [35,37]. In contrast, IRS-2 is excluded from the nucleus and instead can be found in the cytoplasm or at the cell membrane in many invasive breast carcinomas. The localization of IRS-2 at or near the cell membrane would provide access to downstream effectors that are involved in regulating dynamic adhesive and cytoskeletal rearrangements that are required for cell movement [38]. Membrane recruitment of IRS-2 would also localize its signaling to regulate the surface expression of glucose transporter 1 (GLUT1) to promote aerobic glycolysis [17].

Our finding that membrane localization of IRS-2 is associated with poor prognosis supports the hypothesis that IRS-2-mediated signaling promotes tumor progression and metastasis [10,11,13,14]. Upon ligand stimulation, the IRS proteins are recruited to upstream receptors where they are phosphorylated on tyrosine residues and initiate signaling [8]. We hypothesize that IRS-2 at the cell membrane is more likely to be tyrosine phosphorylated and actively signaling than the population of IRS-2 that is diffusely expressed in the cytoplasm because the upstream activating receptors are present at the cell membrane. In support of this hypothesis, diffuse localization of IRS-2 was associated with improved survival outcomes in patients with PR[−] tumors in our study. The population of punctate IRS-2 may result from internalization of the adaptor proteins with surface receptors [39,40]. However, the question of whether receptor internalization would enhance or attenuate signaling remains to be determined. Generation of phosphospecific antibodies that can distinguish “active” from “inactive” IRS-2 will be necessary for future studies to confirm the functional status of the membrane, diffuse cytoplasmic, and punctate populations of IRS-2. It will also be important to investigate the expression and activity of upstream regulatory receptors, such as the IGF-1R or insulin receptor, to establish their connection with IRS-2 localization.

For tumors exhibiting membrane staining, those that are PR[−] demonstrate the worst OS outcomes. Loss of PR expression during disease progression, especially following endocrine therapy, is associated with decreased survival [41–44]. In general, loss of PR expression is indicative of a more aggressive tumor behavior. To date, a biological explanation for the increased aggressiveness associated with PR[−] tumors has not been fully elucidated. The loss of PR expression is thought to represent a down-regulation of ER signaling, a pathway that positively regulates IRS-1 [45]. Previous studies in our lab have shown that loss of IRS-1 results in upregulation of IRS-2 expression and function and promotes tumor progression [12]. In PR[−] tumors, downregulation of IRS-1 function due to absent ER activity would enhance IRS-2 signaling, leading to increased metastatic potential and risk of death. The fact that we did not observe significant correlations between ER expression, IRS-2 membrane staining, and survival may be due to the fact that many of the ER⁺ tumors in our dataset were PR[−], indicating that the ER pathway was not active [46]. Alternatively, loss of PR expression may be the result of enhanced IGF-1R/IRS-2 signaling through PI3K/Akt/mTOR, which can downregulate PR expression independent of ER activity [47]. Further studies are warranted to determine if specific signaling pathways, particularly PI3K/Akt/mTOR, are preferentially activated in PR[−] tumors with IRS-2 membrane localization. Interestingly, patients with PR⁺ tumors that express IRS-2 at the membrane did not exhibit significantly diminished survival outcomes in our combined dataset. This finding supports the hypothesis that ER signaling, and potentially IRS-1, may be dominant with regard to suppressing the impact of IRS-2-mediated signaling. Additional studies to address this question will be important to understand fully the cross talk between these hormone and growth factor signaling pathways.

Our study is the first report on the expression of IRS-2 in human breast cancer. The fact that the correlations of IRS-2 membrane localization and poor outcomes in PR[−] patients were observed in tumors from two separate institutions and in both whole tumor sections and a tissue array support the validity of these observations. However, to confirm IRS-2 localization as a predictive biomarker in breast cancer, these results need to be carefully validated through evaluation of a much larger cohort of patients with longer follow-up times to increase the statistical power of these findings. Ideally, a prospective study in which tumor biopsies could be taken before and after adjuvant treatment would better control for effects of therapy on IRS-2 localization. We established rules to classify the IRS-2 staining patterns in our study to address the subjectivity of determining IRS-2 localization, and two independent investigators were in greater than 90% concordance with identifying the

localization pattern. Analysis of additional cohorts of patients by independent investigators will be important to confirm that the identification of IRS-2 staining patterns is not investigator dependent. The molecular mechanisms underlying the present findings also require further investigation. The membrane IRS-2 staining pattern could represent activation of specific pathways, which promote aggressive tumor behavior. Receptor activation upstream as well as activation of downstream signaling pathways need to be evaluated and correlated to IRS-2 expression patterns. In vitro studies in cell lines are also needed to further explore the role of the localization of IRS-2 in breast carcinoma cell motility, invasion, and metabolism.

In summary, we have identified an IRS-2 staining pattern that has prognostic significance for the OS of breast cancer patients. This association was found to be even stronger for patients with PR[−] tumors. Evaluation of IRS-2 staining patterns could potentially be used to identify patients with PR[−] tumors who would most benefit from aggressive treatment.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Fig. 1. Analysis of IRS-2 antibody specificity. **a** MDA-MB-231 cells (control) and MDA-MB-231 cells expressing an IRS-2-specific shRNA (shIRS2) were fixed in formalin and embedded in paraffin. Sections were stained by IHC using an IRS-2-specific antibody (#1849, Epitomics). Magnification $\times 20$. **b** Aliquots of cell lysates from MDA-MB-231 cells (C) and MDA-MB-231 cells expressing an IRS-2-specific shRNA (shIRS2) were immunoblotted for IRS-2 (#1849, Epitomics) and IRS-1 (#C20, Santa Cruz). **c** Immunohistochemical staining of human pancreas with rabbit IgG, IRS-1 (#C20, Santa Cruz), and IRS-2 (#1849, Epitomics). Magnification $\times 40$

Fig. 2. IRS expression in normal human breast tissue and benign breast lesions. Representative images of IRS-1 and -2 staining in normal ducts, fibroadenoma, usual ductal hyperplasia, sclerosing adenosis, and intraductal papilloma. Magnification $\times 20$ for large images, $\times 40$ for insets

Fig. 3. IRS expression in human breast tumors and cell lines. **a** Representative images of IRS-1 and -2 staining in grade 1, grade 2, and grade 3 breast tumors. Magnification $\times 40$ for all images. **b** Cytoplasmic (cyto) and nuclear (nuc) fractions from SUM-159PT and MDA-MB-231 breast carcinoma cells were immunoblotted with antibodies specific for IRS-1, IRS-2, GAPDH (cytoplasmic control), and hnRNP A1 (nuclear control)

Fig. 4. IRS-2 localization in human breast tumors. **a–c** Representative images showing diffuse, punctate, and membrane IRS-2 staining patterns in breast tumors. Magnification $\times 40$ for all images. **d–i** Representative images from the tissue microarray showing diffuse, punctate, and membrane IRS-2 staining patterns in breast tumor cores. Magnification $\times 20$ (**d–f**), $\times 40$ (**g–i**). **j** IRS-2 staining in a normal duct, adjacent DCIS, and invasive tumor within the same tissue section

Fig. 5. Analysis of IRS-2 staining patterns, progesterone receptor status, and overall survival. **a, c, e** Kaplan–Meier survival curves showing overall survival (OS) in Set 1 (**a**), Set 2 (**c**), and the Pooled Set (**e**) for patients with tumors exhibiting IRS-2 staining at the membrane compared with non-membrane IRS-2 staining. P values for both univariate and multivariate analyses are shown. **b, d, f** Kaplan–Meier survival curves showing OS in tumors from Set 1 (**b**), Set 2 (**d**), and the Pooled Set (**f**) as a function of both progesterone receptor (PR) and IRS-2 membrane staining status. P values based on univariate analysis

Table 1

Clinical characteristics of tumor datasets

	Set 1 <i>n</i> = 128	Set 2 <i>n</i> = 130	<i>P</i> value
Age (years), no. (%)			
≤50	42 (34.1)	35 (26.9)	0.21
>50	81 (65.9)	95 (73.1)	
Unknown	5	–	
Median age (years)	57	60	0.75
Tumor size (stage), no. (%)			
1	72 (57.6)	69 (53.9)	0.57
2	46 (36.8)	46 (35.9)	
3	6 (4.8)	10 (7.8)	
4	1 (0.8)	3 (2.3)	
Unknown	3	2	
Node status (stage), no. (%)			
0	50 (48.1)	58 (51.8)	0.64
1	36 (34.6)	32 (28.6)	
2	9 (8.65)	14 (12.5)	
3	9 (8.65)	8 (7.1)	
Unknown	24	18	
Grade, no. (%)			
1	17 (13.7)	12 (9.8)	<0.0001
2	21 (16.9)	54 (44.3)	
3	86 (69.4)	56 (45.9)	
Unknown	4	8	
Estrogen receptor, no. (%)			
Negative	52 (41.6)	42 (34.4)	0.25
Positive	73 (58.4)	80 (65.6)	
Unknown	3	8	
Progesterone receptor, no. (%)			
Negative	40 (32.5)	56 (45.5)	0.04
Positive	83 (67.5)	67 (54.5)	
Unknown	5	7	
HER2, no. (%)			
Negative	79 (62.7)	90 (73.2)	0.08
Positive	47 (37.3)	33 (26.8)	
Unknown	2	7	
IRS-2 staining pattern			
Membrane	42 (33.9)	19 (14.7)	0.001
Diffuse	49 (39.5)	72 (55.8)	
Punctate	33 (26.6)	38 (29.5)	
Unknown	4	1	

	Set 1 <i>n</i> = 128	Set 2 <i>n</i> = 130	<i>P</i> value
Adjuvant therapy			
Any	90	108	0.23
None	9	18	
Unknown	29	4	
Median follow-up for recurrence-free survival (months)	69.5	80.0	<0.001
Median follow-up for overall survival (months)	72.5	104.4	<0.001

Table 2

Survival analysis of clinical parameters in the pooled dataset

	Univariate ^a	Multivariate ^c		
	<i>P</i> value	HR ^b	95% CI	<i>P</i> value
Tumor size (stage)				
1	<0.0001	1.00	–	<0.001
2		2.12	1.28–3.51	
3		5.78	2.66–12.56	
4		4.39	1.18–16.39	
Node status (stage)				
0	<0.0001	1.00	–	0.03
1		1.95	1.13–3.38	
2		2.67	1.30–5.49	
3		1.73	0.76–3.95	
Grade				
1	0.11	1.00	–	0.07
2		0.82	0.35–1.92	
3		1.72	0.70–4.24	
Adjuvant therapy				
Any	0.02	1.00	–	
None		2.64	1.25–5.59	0.01
Unknown		1.77	0.74–4.25	0.20
Estrogen receptor (ER)				
Negative	0.11	1.00	–	0.14
Positive		1.62	0.86–3.06	
Progesterone receptor (PR)				
Negative	0.002	1.00	–	
Positive		0.41	0.23–0.74	0.003
HER2 receptor				
Negative	0.21	1.00	–	
Positive		0.85	0.51–1.40	0.51

^aUnivariate *P* values were obtained from the log-rank test^bHazard ratios shown were adjusted for age (as a continuous variable) and study, as well as other core clinical variables (as categorical variables) shown in the table, including tumor size, node status, grade, adjuvant therapy, ER, PR, and HER2^c*P* values for tumor size, stage, and grade in multivariate analysis were obtained by treating each, in turn, as an ordered variable while adjusting for other covariates as categorical variables

Table 3

Survival analysis for IRS-2 staining patterns

	Univariate	Multivariate		
	<i>P</i> value	HR	95% CI	<i>P</i> value
<i>Set 1 only (n = 128)</i>				
IRS-2 staining pattern ^a				
Membrane	0.69	1.00	–	–
Diffuse		0.25	0.07–0.87	0.03
Punctate		0.14	0.03–0.72	0.02
IRS-2 membrane staining ^a				
Negative	0.43	1.00	–	–
Positive		4.61	1.35–15.71	0.02
Membrane staining (PR [–] only) ^{a,b}				
Negative	0.04	1.00	–	–
Positive		18.69	1.26–276.47	0.03
PR/membrane ^a				
PR [–] /membrane [–]	0.03	1.00	–	–
PR ⁺ /membrane [–]		0.97	0.20–4.70	0.97
PR [–] /membrane ⁺		4.59	0.98–21.62	0.054
PR ⁺ /membrane ⁺		4.52	0.44–46.90	0.21
<i>Set 2 only (n = 130)</i>				
IRS-2 staining pattern ^a				
Membrane	0.58	1.00	–	–
Diffuse		0.48	0.19–1.21	0.12
Punctate		0.70	0.26–1.90	0.48
IRS-2 membrane staining ^a				
Negative	0.29	1.00	–	–
Positive		1.94	0.80–4.71	0.14
Membrane staining (PR [–] only) ^{a,c}				
Negative	0.85	1.00	–	–
Positive		5.72	1.44–22.65	0.01
PR/membrane ^a				
PR [–] /membrane [–]	0.021	1.00	–	–
PR ⁺ /membrane [–]		0.37	0.18–0.79	0.01
PR [–] /membrane ⁺		3.06	0.96–9.78	0.06
PR ⁺ /membrane ⁺		0.42	0.09–1.88	0.26
<i>Pooled (n = 258)</i>				
IRS-2 staining pattern ^a				
Membrane	0.57	1.00	–	–
Diffuse		0.43	0.22–0.82	0.01
Punctate		0.52	0.26–1.05	0.07

	Univariate	Multivariate		
	<i>P</i> value	HR	95% CI	<i>P</i> value
IRS-2 membrane staining ^a				
Negative	0.31	1.00	–	–
Positive		2.20	1.20–4.06	0.01
Membrane staining (PR– only) ^{a,d}				
Negative	0.30	1.00	–	–
Positive		6.15	2.25–16.80	<0.001
PR/membrane ^a				
PR–/membrane–	0.010	1.00	–	–
PR+/membrane–		0.53	0.28–0.99	0.05
PR–/membrane+		3.36	1.58–7.16	0.002
PR+/membrane+		0.60	0.20–1.84	0.37

^a Multivariate results were obtained from a model that adjusted for the core covariates including age, study (for the combined analysis), tumor size, node status, grade, adjuvant therapy, ER, PR, and HER2. The results for IRS-2 staining pattern and IRS-2 membrane staining were obtained from separate models adjusting for the same set of the core covariates

^b *n* = 40, Set 1 contained only 29 PR– cases which had all covariates

^c *n* = 56

^d *n* = 96