Published in final edited form as:

Cancer Lett. 2013 September 28; 338(2): 239–248. doi:10.1016/j.canlet.2013.03.030.

IRS1 is highly expressed in localized breast tumors and regulates the sensitivity of breast cancer cells to chemotherapy, while IRS2 is highly expressed in invasive breast tumors

Holly A. Porter^a, Anthony Perry^d, Chris Kingsley^e, Nhan L. Tran^f, and Achsa D. Keegan^{a,b,c,*}

^aCenter for Vascular and Inflammatory Diseases, University of Maryland School of Medicine, Baltimore, MD 21201, USA

^bMarlene and Stewart Greenebaum Cancer Center, University of Maryland School of Medicine, Baltimore, MD 21201, USA

^cCentre for Vascular and Inflammatory Diseases, University of Maryland School of Medicine, Baltimore, MD 21201, USA

^dBrigham and Women's Hospital and Harvard Medical School, Boston, MA 02115, USA

^eDiabetes, Cardiovascular and Metabolic Diseases Division, Translational Genomics Research Institute, Phoenix, AZ 85004, USA

^fCancer and Cell Biology Division, Translational Genomics Research Institute, Phoenix, AZ 85004, USA

Abstract

Insulin receptor substrate (IRS) proteins have been shown to play an important role in breast cancer by differentially regulating cancer cell survival, proliferation, and motility. Furthermore, the IL-4-induced tyrosine phosphorylation of the transcription factor STAT6 was shown to protect breast cancer cells from apoptosis. Here, we analyzed human breast cancer tissues for the expression of IRS1, IRS2, STAT6, and tyrosine phosphorylated STAT6 (pSTAT6). We found that IRS1 and pSTAT6 were both highly expressed in ductal carcinoma *in situ* (DCIS). On the other hand, IRS2 expression was low in DCIS, but increased significantly in relation to tumor invasiveness. We utilized cell lines with disparate IRS1 expression, MDA-MB-231, MCF7, and MCF7 cells with depleted IRS1 due to shRNA lentiviral infection, to examine the role of IRS1 and IRS2 in the responsiveness of breast cancer cells to chemotherapy. We report that high IRS1 sensitized MCF7 cells to specific chemotherapeutic agents. These results suggest that high IRS1 with low IRS2 expression may predict the effectiveness of specific types of chemotherapy in breast cancer.

Keywords

Insulin receptor substrate; STAT6; Breast cancer; Chemotherapy; Cell death

1. Introduction

Breast cancer is the second most commonly diagnosed cancer and the most frequent cancer among women worldwide [1]. The two most common types of breast cancer are ductal and lobular; ductal and lobular cancers that are *in situ*, localized to a specific region, make up 85–90% and 8% of breast cancers, respectively. There are also invasive ductal and lobular cancers, as well as inflammatory breast cancers which are the most aggressive [2,3]. Although chemotherapy has proven to increase the survival of breast cancer patients, there are a significant number of patients who only transiently respond to the treatment and ultimately succumb to metastatic disease [4].

IL-4 is a cytokine found in the microenvironment of many breast tumors [5–8]. IL-4 activates two major signaling pathways, the signal transducer and activator of transcription (STAT) 6 pathway and the insulin receptor substrate (IRS) pathway, which have both been implicated in mammary gland development and breast cancer. IL-4 was shown to protect breast cancer cells from chemotherapy-induced death *in vitro*. IL-4 can be produced by the cancer cells themselves and act in an autocrine fashion or by CD4⁺ tumorinfiltrating lymphocytes (TILs) [5–8]. STAT6 was important for the protection of human breast cancer cells from spontaneous cell death in the presence or absence of IL-4 [9].

Studies on the role of IRS in breast cancer were predominantly performed independently of IL-4. Although IRS1 and IRS2 have similar structure, they have unique functions in cancer progression. They differentially regulate cellular processes such as survival, proliferation, and motility [10–17]. IRS2 has been proposed to regulate mammary tumor metastasis in a mouse model of breast cancer and in breast cancer cell lines since the lack of IRS2 rendered tumor cells less invasive [16,18–23]. IRS1 was not required for metastasis and was actually suggested to be a suppressor of metastasis in breast cancer; instead IRS1 enhanced breast cancer cell proliferation. Knocking out IRS2 rendered mammary tumor cells more apoptotic in basal conditions and also after serum deprivation; conversely, knocking out IRS1 rendered the cells resistant to apoptotic stimuli under similar conditions [16,18]. Furthermore, IRS2 suppressed the function of IRS1 by strongly activating the Akt/ mTOR signaling pathway and inducing the serine phosphorylation of IRS1 [16,18–20]. Studies by Byron *et al.* using the T47D breast cancer cell line similarly showed roles for IRS1 in proliferation and IRS2 in motility [15].

While these studies suggest that IRS1 and IRS2 regulate many cancer cell processes, the roles of IRS1 and IRS2 in protecting tumor cells from chemotherapy-induced apoptosis has not been extensively studied. We recently showed that expression of IRS1, in contrast to IRS2, led to enhanced sensitivity to chemotherapy-induced apoptosis in 32D myeloid progenitor cells [24]. We hypothesized that IRS1 and IRS2 would be differentially expressed in breast tumors and that they would differentially control the sensitivity of breast cancer cell lines to chemotherapy-induced cell death. To test this, we stained human breast cancer tissue microarrays with antibodies that detect IRS1, IRS2, STAT6, and tyrosine phosphorylated STAT6 (pSTAT6) to investigate whether expression of these proteins correlate with tumor type or grade. We also utilized MCF7 cells and MDA-MB-231 cells to determine the role of IRS1 and IRS2 in the protection of breast cancer cells from chemotherapy-induced apoptosis. We found that human breast tumors and cell lines had varied IRS1, IRS2, STAT6 and pSTAT6 expression depending on the tumor type and grade. Elevated IRS2 expression correlated with increased tumor grade. IRS1 and pSTAT6 showed a highly significant positive correlation with each other and were both elevated in ductal carcinoma *in situ*. We also found that IRS1 sensitized MCF7 cells to specific chemotherapeutic agents and that decreasing IRS1 resulted in a decrease in ER α expression levels.

2. Materials and methods

2.1. Materials

MCF7 and MDA-MB-231 cells were obtained from Dr. T. Antalis (University of Maryland School of Medicine, Baltimore). U937 cells were obtained from ATCC (Rockville, MD). IL-4 was purchased from R & D and used at a final concentration of 10 ng/ml. Puromycin, taxol, and doxorubicin were purchased from Sigma–Aldrich. Taxol was also purchased from LC Laboratories. Antibodies for STAT6 (S-20), ER α , RAD51, BCL-2, BCL-xL, BAK, and Annexin A2 were purchased from Santa Cruz Biotechnology. Antibody for BAX was purchased from Cell Signaling Technology. Antibody for IRS1 was purchased from Upstate Biotechnology. Antibody specific for human IRS2 was generated and tested as previously described [24]. Antibodies for phosphotyrosines (PY 20) and tyrosine phosphorylated (pY 641) STAT6 were purchased from BD Biosciences. Antibody for alpha-tubulin was purchased from ebioscience.

2.2. Cell culture

MDA-MB-231 and MCF7 cells were maintained in complete DMEM (Lonza) containing 4.5 g/L glucose and supplemented with 10% fetal bovine serum (FBS), 1% glutamine, 1% pen-strep and 1% sodium pyruvate. MCF7 cells infected with either IRS1 shRNA lentivirus or non-target shRNA lentivirus were maintained in complete DMEM and 700 ng/ml puromycin (Sigma).

2.3. Generation of IRS1-depleted MCF7 Cells

MCF7 cells were plated to 80% confluency in 2 mls of culture media. IRS1 shRNA lenti-viral supernatant (Sigma TRCN0000039908) or non-target control shRNA lenti-viral supernatant (Sigma SHC002V) was used to infect the cells. Two days after infection, cells were selected in 700 ng/ml puromycin. Puromycin-resistant cells were tested for the expression of IRS1 by immunoblotting using an IRS1-specific antibody. The phosphorylation of IRS1 was tested by immunoprecipitation with an IRS1-specific antibody and then immunoblotting using a phosphotyrosine-specific antibody.

2.4. Immunoblotting

Cells were washed and treated with 10 ng/ml IL-4 (R&D Systems) for 30 minutes. Cells were lysed with lysis buffer (50 mM HEPES at pH 7.5, 0.5% NP-40, 5 mM EDTA, 50 mM NaCl, 10 mM Na pyrophosphate, 50 mM NaF, fresh 1 mM NaOV, fresh 1 mM PMSF, fresh 1 $\times$ protease cocktail, fresh 100 nM calyculin A). In some experiments a protein subcellular fractionation kit (Pierce) was used to obtain lysates. Lysates were quantified with a BCA Kit (Pierce Biotechnology). Equal amounts of protein were loaded on the gel, separated by SDS-PAGE and transferred to a nitrocellulose membrane (Millipore). The blot was blocked with 3% BSA in TBST (25 mM Tris–HCl pH 7.4, 150 mM NaCl, 0.1% Tween-20) wash buffer for 1 h. The appropriate primary antibody was added for either 1 h or overnight. The blot was then washed and the secondary antibody conjugated with horseradish peroxidase was added for 1 h. The blot was washed again and chemiluminescence reagents (Denville Scientific) were used to visualize the signal on X-ray film. In some experiments the membranes were stripped with a 37 °C stripping solution (Millipore) for 30 min at room temperature. These membranes were then used for immunoblotting with another antibody. For immunoprecipitation experiments, specific antibodies were added to equal amounts of lysate. Agarose beads were used to pull down antibody-bound proteins prior to analysis by SDS-PAGE and western blotting.

2.5. Chemosensitivity assay

Cells were washed with culture medium, resuspended in culture medium, and plated in triplicate. One day later, the cells were treated in the presence or absence of various chemotherapeutic agents for 24 h. Cell viability was measured using the MTS assay (Promega) as described by the manufacturer.

2.6. Tumor tissue microarray (TMA)

Three tumor-specific tissue microarrays (TMAs) were constructed from paraffin-embedded blocks of breast tumors with varying grade and diagnosis by the Translational Genomics Research Institute TMA core facility (Phoenix, AZ) as previously described [25,26]. All tumor cases were histologically reviewed for tumor type, grade, and for expression of estrogen and progesterone receptors. There were three tissue microarrays with 216, 246, or 231 tissue samples that were spotted in triplicate or duplicate. Control tissues were included to represent normal tissue counterparts. Sections (5 μ m) were sequentially cut and stained with antibodies specific for IRS1, IRS2, tyrosine phosphorylated (pY 641) STAT6, and STAT6 using a BondMax TMA autostainer (Vision Biosystems, Inc.). Endogenous peroxidase activity was quenched by incubating slides in 3% hydrogen peroxide for 10 minutes at 25 °C. Slide blocking was done with background Sniper from Biocare and slides were counterstained with hematoxylin. The stained slides were evaluated by a pathologist and scored for staining intensity where 0 = negative, 1 = weak, 2 = moderate, and 3 = strong. Tests for pairwise correlations, using the Kendall's tau rank correlation test, were calculated using the cor.test function in the R statistical package as described by Whitsett et al. [27,28].

3. Results

3.1. Expression of IRS1, IRS2, STAT6, and tyrosine phosphorylated STAT6 in human breast tumors

Previously published studies showed that IRS1 was expressed at moderate to high levels in human normal breast tissue and well differentiated primary breast tumors [19,29]. Conversely, IRS1 expression was decreased in poorly differentiated, high grade tumors [19,29]. On the other hand, membrane localization of IRS2 was associated with decreased overall survival in progesterone receptor (PR) negative breast tumors [30]. In our studies we used immunohistochemistry to examine cytoplasmic expression of IRS1, IRS2, STAT6 and tyrosine phosphorylated STAT6 in human breast tumors. We found that normal breast epithelium expressed low levels of IRS1, IRS2, STAT6, and pSTAT6 (Fig. 1). Normal breast undergoing lactational changes was also positive for IRS1, STAT6, and pSTAT6, but did not express IRS2. In contrast, localized tumors such as ductal carcinoma *in situ* (DCIS) showed high levels of IRS1 and pSTAT6 (Fig. 1). The IRS2 levels in such samples were lower comparatively and showed specific localization within the tumor. Conversely, invasive ductal carcinomas (IDC) showed high expression of IRS2 and varied expression of IRS1 (Fig. 1 and Table 1). Varied expression of pSTAT6 was also detected, while STAT6 was expressed only at low levels in IDC (Fig. 1 and Table 2). Overall global analysis revealed a highly significant correlation between expression of IRS1 and either STAT6 or pSTAT6 (p values < 0.0001 in both cases). As reported previously, we also found that IRS1 expression positively correlated with estrogen receptor (ER) α (p value < 0.0001, data not shown) [31]. Surprisingly, we observed that expression of IRS1 and IRS2 were strongly correlated (p value < 0.0001). However, IRS2 expression was not found to be strongly associated with either STAT6 (p value = 0.943) or pSTAT6 (p value = 0.0044).

3.2. Localized breast ductal carcinomas express high levels of IRS1 and pSTAT6, while more invasive human breast tumors express high levels of IRS2

We further analyzed whether expression of IRS1, pSTAT6, or IRS2 correlated with tumor type and grade. The vast majority of nonneoplastic breast tissue expressed little to no IRS1 or pSTAT6 (83.4% and 100% with a score of 0 or 1, respectively) (Tables 1 and 2). Strikingly, we found that 100% of the DCIS tissues showed an IRS1 staining score of 3 (Table 1) and a pSTAT6 staining score of 3 (Table 2). Invasive lobular carcinoma tissues showed IRS1 staining score of 3 in only 35% of samples (Table 1) and pSTAT6 staining score of 3 in only 37.5% of samples (Table 2). IDC tissues showed IRS1 staining score of 3 in only 57.2% of samples (Table 1) and pSTAT6 staining score of 3 in 27.5% of samples (Table 2). Furthermore, the percentage of tumor samples with high staining scores for IRS1 and pSTAT6 remained constant as the grade of IDC increased. In contrast, IRS2 staining intensity was strongly associated with an invasive phenotype and high IDC grade (Table 3 and Fig. 2). Nonneoplastic human breast tissues and DCIS showed low IRS2 staining (100% and 89% with a score of 0 or 1, respectively), while the majority of invasive lobular and ductal carcinomas (70% and 72%, respectively) demonstrated strong staining for IRS2 (Fig. 2). Furthermore, IRS2 staining intensities increased in direct relation to increasing grades of IDC (Fig. 2 and Table 3). Less than half (~48%) of IDC-grade 1 showed a staining score 2, while 63% of IDC-grade 2 and 97% of IDC-grade 3 had a score 2. These analyses show that elevated expression of IRS1 and pSTAT6 strongly correlate with DCIS, while IRS2 expression strongly correlates with the invasive phenotype and with higher grade of IDC. This pattern of IRS2 expression in human breast tissue is consistent with the *in vitro* analyses of cell lines and with mouse models of breast cancer metastasis [20,22–27].

3.3. MCF7 and MDA-MB-231 cells have differential IRS and STAT6 expression and tyrosine phosphorylation

To determine whether human breast cancer cell lines also have varied IRS and STAT6 expression we utilized MCF7 and MDA-MB-231 cells. MCF7 cells are ER α positive and have low metastatic potential; whereas, MDA-MB-231 cells are ER α negative and have much higher metastatic potential [22,32]. Published studies show that IL-4 receptor expression is increased on numerous cancer cells, including breast cancer cells, as compared to normal cells [33,34]. Furthermore, the affinity of IL-4 binding to its receptors is also increased, especially in breast cancer, as compared to normal cells [33,34]. FACS analysis confirmed that both MCF7 and MDA-MB-231 cells express the type I IL-4 receptor (data not shown). While MCF7 cells and MDA-MB-231 cells expressed similar levels of IRS2, MCF7 cells expressed much higher levels of IRS1 than MDA-MB-231 cells. In the presence of IL-4, MCF7 cells showed higher tyrosine phosphorylation of both IRS1 and IRS2 than MDA-MB-231 cells (Fig. 3A). This supports published data that shows that in estrogen-receptor positive human breast cancer cell lines, IRS1, not IRS2, is the predominant signaling molecule that is activated by IGF1, insulin, and IL-4 [35].

We also found that MDA-MB-231 cells expressed higher levels of total STAT6 than MCF7 cells (Fig. 3B). Treatment of cells with IL-4 induced tyrosine phosphorylation of STAT6 in both cell types, although the signal was greater for MDA-MB-231 cells. It is important to note that although MDA-MB-231 cells express higher STAT6 and pSTAT6, results from our human breast tumor tissues showed a significant positive correlation between IRS1 and STAT6 and pSTAT6, but not IRS2 and STAT6 or pSTAT6.

3.4. MCF7 cells are more sensitive to taxol-induced death than MDA-MB-231 cells

Studies exploring the role of IRS1 and IRS2 in protecting tumor cells from chemotherapy-induced apoptosis are limited. De et al. reported that MCF7 and MDA-MB-231 cells are differentially sensitive to docetaxol [36]. In a previously published study, we showed that

IRS1, but not IRS2, expression sensitized 32D cells to chemotherapy-induced death [24]. Breast cancer is commonly treated with the chemotherapy drugs taxol or doxorubicin. Taxol inhibits microtubule disassembly and doxorubicin intercalates in DNA [37–40]. In order to determine if IRS1 would also sensitize breast cancer cells to chemotherapy-induced death, we first treated MCF7 and MDA-MB-231 cells with taxol or doxorubicin. As we hypothesized based on their high IRS1 expression, MCF7 cells were more sensitive to taxol-induced death than MDA-MB-231 cells (Fig. 4). On the other hand, doxorubicin-induced cell death was similar in both cell lines, with MCF7 cells being only modestly more sensitive than MDA-MB-231 cells. These results suggest that breast cancer cells with unique IRS expression patterns respond differently to chemotherapeutic agents. Moreover, our results revealed that treatment with IL-4 did not protect MCF7 cells from doxorubicin-induced death (data not shown).

3.5. Decreasing IRS1 expression in MCF7 cells de-sensitizes these cells to some but not all chemotherapeutic agents

To further examine the role of IRS1 in sensitizing cells to chemotherapy, we infected MCF7 cells with an IRS1-shRNA lentivirus and generated two IRS1-deficient cell lines (99-2W1 and 99-2W3). We quantified the expression of IRS1, IRS2, and STAT6 by western blotting and densitometry. IRS1 levels were decreased by 50–70% in the transduced cells, with the greatest decrease observed in 99-2W3 (Fig. 5A). The IRS2 and STAT6 levels were either increased (99-2W1) or not affected (99-2W3).

To determine if MCF7 cells with depleted IRS1 levels would be less responsive to chemotherapy, we treated MCF7, 99-2W1, and 99-2W3 with taxol or doxorubicin and measured cell death. MCF7 cells with decreased IRS1 levels were less sensitive to taxol-induced cell death, but not to doxorubicin-induced cell death compared to parental MCF7 cells (Fig. 5B and C). Given the differential responses to taxol and doxorubicin, we examined cell sensitivity to additional chemotherapeutic agents. We treated the cells with vincristine, an agent that inhibits microtubule assembly, etoposide, which binds DNA-topoisomerase II, daunorubicin, which intercalates in DNA, or camptothecin, which binds DNA-topoisomerase I [38,41–43]. We found that MCF7 cells with the greatest decrease in IRS1 levels (99-2W3) were significantly less sensitive to vincristine- and etoposide-induced cell death than the parental MCF7 cells (data not shown). On the other hand, decreasing IRS1 in MCF7 cells did not change the responses of these cells to daunorubicin or camptothecin (data not shown). Together these results suggest that IRS1 plays an important role in sensitizing MCF7 cells to death induced by some, but not all chemotherapeutic drugs.

3.6. Decreasing IRS1 expression alters Annexin A2 localization

To investigate the mechanism by which IRS1 expression sensitized MCF7 cells to chemotherapy, we analyzed the levels of various proteins that regulate survival of breast cancer cells including ER α , BCL-xL, BAK, BCL-2, BAX, Annexin A2 and the levels of RAD51, a DNA repair protein. We found that decreasing IRS1 in MCF7 cells resulted in a decrease in ER α levels (Fig. 6A). These results are consistent with previous published studies demonstrating cross-regulation between IRS1 and ER α [19,31]. High expression of IRS1 was partially due to ER α activity, as estrogens can enhance IRS1 expression and function. Furthermore, IRS1 interacted with ER α in the nucleus and modulated its transcriptional activity at estrogen response elements [19,31]. Since we observed a decrease in ER α expression, we examined the sensitivity of MCF7, 99-2W1, and 99-2W3 to acute tamoxifen treatment. Tamoxifen antagonizes the estrogen receptor [44,45]. We found that decreasing IRS1 in MCF7 cells did not change the responses of these cells to tamoxifen in the short term, even though they expressed reduced levels (~50%) of ER α (data not shown).

We found that decreasing IRS1 did not appreciably alter the levels of BCL-xL, BAK, BCL-2, BAX, or RAD51 (Fig. 6A). In our recently published study using the 32D model, we found that expressing IRS1 in 32D cells led to increased Annexin A2 levels and altered Annexin A2 localization compared to 32D parental cells [24]. Although we detected no change in Annexin A2 expression in MCF7 cells when decreasing IRS1 expression levels (Fig. 6A), we next examined Annexin A2 subcellular localization since Annexin A2 localization can influence its function. Soluble extracts of the fractions were separated by SDS-PAGE. Western blotting of MCF7 cells showed that IRS1 was predominantly located in the cytoplasmic and soluble nuclear fractions (Fig. 6B). Depleting IRS1 resulted in IRS1 being predominantly located in the cytoplasmic fraction and only very weakly located in the soluble nuclear fraction. In addition, we found that in MCF7 cells Annexin A2 was similarly predominantly located in the cytoplasmic and soluble nuclear fractions, and only weakly located in the membrane, chromatin-bound nuclear, and cytosolic fractions. Interestingly, when IRS1 was depleted, Annexin A2 was localized in all of the fractions at similar levels. Control blots demonstrated that HSP90 was predominantly located in the cytoplasmic fraction of all cells and only weakly located in some membrane fractions as expected, while Sp1 was limited to the soluble nuclear fraction.

4. Discussion and conclusions

IRS proteins mediate signaling downstream of hormones, such as growth hormone, prolactin, estrogen and progesterone, and growth factors and cytokines, such as IGF-1 and IL-4, respectively, that play a role in normal mammary gland development and breast cancer development and progression [13,18,19,22,46–48]. IRS1 and IRS2 gene transcription can be enhanced by steroids, such as estrogen and progesterone, respectively, in the mouse mammary gland and in breast cancer cells. In breast cancer cells, both IRS1 and IRS2 are expressed, but in response to insulin, IGF-1, and IL-4, estrogen receptor-positive cells mainly signal via IRS1, while estrogen receptor-negative cells mainly signal via IRS2 in response to IGF-1 [21,35,46].

Prior studies examining the role of IRS1 in human breast cancer have been contradictory. Several studies reported that IRS1 expression correlated with poorly differentiated breast cancer and lymph node involvement and that high levels of IRS1 predicted worse disease-free survival for breast cancer patients [49,50]. Conversely, other studies showed that normal breast tissue and well-differentiated primary tumors expressed moderate to high IRS1 levels and poorly differentiated, high grade tumors showed decreased IRS1 levels [29,31]. Clark et al. showed that in invasive ductal carcinomas, IRS1 is expressed in the nuclei and the cytoplasm, but IRS2 is expressed in the cytoplasm and membrane and not the nucleus [30]. Furthermore, membrane localization of IRS2 was associated with decreased overall survival in progesterone receptor (PR) negative breast cancer patients which places importance on hormone receptor expression and subcellular localization of the IRS proteins in understanding IRS functions in breast cancer [30]. We found that normal breast tissue expressed low levels of IRS1, IRS2, and pSTAT6. Ductal carcinoma *in situ* (DCIS) expressed high IRS1 and pSTAT6, but low-moderate levels of IRS2. Conversely, more invasive human breast tumors such as invasive ductal carcinomas (IDC), which are the most common types of breast tumors, were characterized by high expression of IRS2. IRS1 and pSTAT6 expression in these tumors was variable. These observations suggest that localized tumors may initially express high levels of IRS1 and pSTAT6, but that as tumors become more aggressive they decrease IRS1 and pSTAT6 and increase IRS2 expression. We propose that this differential expression could impact breast cancer patient prognosis.

Exogenous factors that are implicated in breast cancer progression such as elevated free fatty acids, obesity, and oxidative stress can induce the serine phosphorylation of IRS1 which will

negatively regulate IRS1 function [20,51]. Therefore, IRS expression may not be the sole predictor of breast cancer phenotype and response to chemotherapy. It will be informative to consider the tyrosine and serine phosphorylation status of IRS1 and IRS2 within the breast cancer tissue samples in order to more directly correlate the *in vitro* studies with the *in situ* studies.

Few studies have looked at the role of IRS proteins in responsiveness of breast cancer cells to therapeutic modalities. Our results showed that MCF7 cells, which expressed high IRS1 levels, are more sensitive to taxol-induced death than MDA-MB-231 cells, which expressed lower IRS1 levels. In support of this observation, De *et al.* previously showed that MCF7 cells were more sensitive to docetaxel-induced death compared to MDA-MB-231 cells [36]. MCF7 and MDA-MB-231 cells showed similar cell death when treated with doxorubicin. In addition, we found that decreasing IRS1 made MCF7 cells more resistant to taxol, etoposide, and vincristine, which can all arrest cells in the G2 phase of the cell cycle [37,38,41]. Decreasing IRS1 did not change the sensitivity of MCF7 cells to doxorubicin, camptothecin, and daunorubicin which all arrest cells in the S phase of the cell cycle [39,40,42,43]. These results suggest that IRS1 may only sensitize cells to death by drugs that arrest cells in the G2 phase of the cell cycle. It is also possible that IRS1 sensitizes cells to drugs that cause cell cycle arrest in multiple phases, but that additional pathways compensate for the loss of IRS1 in the presence of drugs that target cells in phases other than G2. We have reported that expression of IRS2 inhibited the ability of IRS1 to sensitize 32D cells to chemotherapy [24]. Future studies should determine if over-expressing IRS2 in MCF7 cells will result in less sensitivity to taxol, etoposide and vincristine. Since our results also showed that depletion of IRS1 altered the subcellular localization of Annexin A2, further studies should explore whether changes in Annexin A2 localization mediates the ability of IRS1 to sensitize MCF7 cells to chemotherapy.

IRS1 expression positively correlated with ER α (estrogen receptor α) levels in human ductal breast tumors [31]. It has been demonstrated that IRS1 and ER α regulate each other's expression and function. 17 β estradiol can upregulate IRS1 expression and function and ER α can interact with IRS1 and increase the stability of IRS1. On the other hand, IRS1 can interact with ER α in the nucleus and modulate its transcriptional activity at the estrogen DNA response element [19,29,31]. Our studies extend that data showing that decreasing IRS1 in MCF7 cells resulted in a 50% decrease in ER α expression levels. Studies pertaining to estrogen receptor expression and response to chemotherapy have been conflicting. Studies by Lippman *et al.* showed that patients with estrogen receptor negative breast tumors had an enhanced response to chemotherapy compared to those with estrogen receptor positive tumors [52]. On the other hand, Kiang *et al.* reported that ER positive tumors showed better responses to chemotherapy compared to ER negative tumors [53]. Our studies suggest that localized breast tumors that express high ER α and low IRS2 would be much more sensitive to death induced by specific chemotherapeutic agents due to their higher IRS1 levels. The human breast samples analyzed in this study were not supported by follow up data on responses to treatment. Thus, it will be beneficial to prospectively study the relationship between IRS1 expression and response to chemotherapy in human patients.

Our finding that IRS1 plays an important role in sensitizing MCF7 cells to taxol, etoposide, and vincristine suggests that localized ER α positive breast tumors that express high cytoplasmic IRS1 may be highly sensitive to specific chemotherapeutic agents in combination with hormone therapies like tamoxifen and aromatase inhibitors. While ER α + tumors are treated with these anti-estrogen agents [44,45], it was shown that over-expression of IRS1 in MCF7 cells, resulted in the development of estrogen independence, thereby mediating resistance to anti-estrogens [31,54– 56]. Moreover, ER positive tumors have been shown to convert to ER negative tumors in mouse mammary tumor models and in human

breast cancer during disease progression or upon therapy [53]. Previously published studies showed that tamoxifen treatment of MCF7 cells that over-express IRS1 resulted in dissociation of IRS1 from the p85 subunit of PI-3 kinase [55]; ICI 182,780, another anti-estrogen, induced a similar response [56]. Furthermore, both tamoxifen and ICI 182,780 decreased total IRS1 levels and reduced both basal and IGF-1-induced tyrosine phosphorylation of IRS1 [32,55,56]. Taking into consideration our results and the published studies mentioned above, it is possible that chemotherapy treatment of tumors recurring after initial hormone therapy will be ineffective since long-term hormone therapy can decrease IRS1 expression, thereby de-sensitizing the cells to chemotherapy.

In conclusion, these results showed that human breast tumors and cell lines had varied IRS1, IRS2, STAT6 and pSTAT6 expression depending on the tumor type and grade. In human tumors, IRS1 and tyrosine phosphorylated STAT6 showed a highly significant positive correlation and both were more highly expressed in the less aggressive DCIS tumors, while elevated IRS2 expression was associated with high grade IDC. Studies in cancer cell lines reflect the findings in tumor samples and suggest that high IRS1 could be a good indicator of the effectiveness of specific types of chemotherapy in breast cancer. We found that decreasing IRS1 resulted in a decrease in ER α expression levels. We predict that more differentiated, ER α positive breast tumors that express high IRS1 would respond favorably to chemotherapy. Understanding the molecular programming of a cancer cell may allow for the better prediction of a patient's response to treatment.

Acknowledgments

We acknowledge the excellent technical assistance of Ms. Xiulan Qi and the advice of Dr. Jeff Winkles during the course of this work. This work was supported by the United States Public Health Service grants from the National Institutes of Health: Transfusion Medicine Research Training Program T32 HL007698, NRSA Predoctoral Diversity Fellowship Grant F31CA142165, and NIGMS Initiative for Maximizing Student Development Grant R25-GM55036, Procter and Gamble (to H.A.P), and R01AI038985 (to A.D.K) and R01CA130940 (to N.L.T).

References

1. Ferlay J, Shin HR, Bray F, Forman D, Mathers C, Parkin DM. Estimates of worldwide burden of cancer in 2008: GLOBOCAN. *Int J Cancer*. (2010) (2008); 127:2893–2917. [PubMed: 21351269]
2. Munagala R, Aqil F, Gupta RC. Promising molecular targeted therapies in breast cancer. *Indian J Pharmacol*. 2011; 43:236–245. [PubMed: 21713084]
3. A.D.A.M. [Accessed 23.02.12] Breast cancer. 1993. <http://www.nlm.nih.gov/medlineplus/ency/article/000913.htm#Definition>
4. Wang MY, Chen PS, Prakash E, Hsu HC, Huang HY, Lin MT, et al. Connective tissue growth factor confers drug resistance in breast cancer through concomitant up-regulation of Bcl-xL and cIAP1. *Cancer Res*. 2009; 69:3482–3491. [PubMed: 19351859]
5. Romagnani S. The Th1/Th2 paradigm. *Immunol Today*. 1997; 18:263–266. [PubMed: 9190109]
6. Conticello C, Pedini F, Zeuner A, Patti M, Zerilli M, Stassi G, et al. IL-4 protects tumor cells from anti-CD95 and chemotherapeutic agents via up-regulation of antiapoptotic proteins. *J Immunol*. 2004; 172:5467–5477. [PubMed: 15100288]
7. Todaro M, Lombardo Y, Francipane MG, Alea MP, Cammareri P, Iovino F, et al. Apoptosis resistance in epithelial tumors is mediated by tumor-cell-derived interleukin-4. *Cell Death Differ*. 2008; 15:762–772. [PubMed: 18202702]
8. Li Z, Chen L, Qin Z. Paradoxical roles of IL-4 in tumor immunity. *Cell Mol Immunol*. 2009; 6:415–422. [PubMed: 20003817]
9. Zhang WJ, Li BH, Yang XZ, Li PD, Yuan Q, Liu XH, et al. IL-4-induced Stat6 activities affect apoptosis and gene expression in breast cancer cells. *Cytokine*. 2008; 42:39–47. [PubMed: 18342537]

10. White MF. The IRS-signalling system: a network of docking proteins that mediate insulin action. *Mol Cell Biochem.* 1998; 182:3–11. [PubMed: 9609109]
11. Bruning JC, Winnay J, Cheatham B, Kahn CR. Differential signaling by insulin receptor substrate 1 (IRS-1) and IRS-2 in IRS-1-deficient cells. *Mol Cell Biol.* 1997; 17:1513–1521. [PubMed: 9032279]
12. Withers DJ, White M. Perspective: the insulin signaling system—a common link in the pathogenesis of type 2 diabetes. *Endocrinology.* 2000; 141:1917–1921. [PubMed: 10830270]
13. Keegan AD, Zamorano J. Regulation of gene expression, growth, and cell survival by IL-4: contribution of multiple signaling pathways. *Cell Res.* 1998; 8:1–13. [PubMed: 9570012]
14. Saltiel AR, Kahn CR. Insulin signalling and the regulation of glucose and lipid metabolism. *Nature.* 2001; 414:799–806. [PubMed: 11742412]
15. Byron SA, Horwitz KB, Richer JK, Lange CA, Zhang X, Yee D. Insulin receptor substrates mediate distinct biological responses to insulin-like growth factor receptor activation in breast cancer cells. *Br J Cancer.* 2006; 95:1220–1228. [PubMed: 17043687]
16. Ma Z, Gibson SL, Byrne MA, Zhang J, White MF, Shaw LM. Suppression of insulin receptor substrate 1 (IRS-1) promotes mammary tumor metastasis. *Mol Cell Biol.* 2006; 26:9338–9351. [PubMed: 17030605]
17. Shaw LM. Identification of insulin receptor substrate 1 (IRS-1) and IRS-2 as signaling intermediates in the $\alpha 6 \beta 4$ integrin-dependent activation of phosphoinositide 3-OH kinase and promotion of invasion. *Mol Cell Biol.* 2001; 21:5082–5093. [PubMed: 11438664]
18. Nagle JA, Ma Z, Byrne MA, White MF, Shaw LM. Involvement of insulin receptor substrate 2 in mammary tumor metastasis. *Mol Cell Biol.* 2004; 24:9726–9735. [PubMed: 15509777]
19. Mardilovich K, Pankratz SL, Shaw LM. Expression and function of the insulin receptor substrate proteins in cancer. *Cell Commun Signal.* 2009; 7:14. [PubMed: 19534786]
20. Gibson SL, Ma Z, Shaw LM. Divergent roles for IRS-1 and IRS-2 in breast cancer metastasis. *Cell Cycle.* 2007; 6:631–637. [PubMed: 17361103]
21. Jackson JG, Zhang X, Yoneda T, Yee D. Regulation of breast cancer cell motility by insulin receptor substrate-2 (IRS-2) in metastatic variants of human breast cancer cell lines. *Oncogene.* 2001; 20:7318–7325. [PubMed: 11704861]
22. Cui X, Kim HJ, Kuitse I, Kim H, Brown PH, Lee AV. Epidermal growth factor induces insulin receptor substrate-2 in breast cancer cells via c-Jun NH(2)-terminal kinase/activator protein-1 signaling to regulate cell migration. *Cancer Res.* 2006; 66:5304–5313. [PubMed: 16707456]
23. Zhang X, Kamaraju S, Hakuno F, Kabuta T, Takahashi S, Sachdev D, et al. Motility response to insulin-like growth factor-I (IGF-I) in MCF-7 cells is associated with IRS-2 activation and integrin expression. *Breast Cancer Res Treat.* 2004; 83:161–170. [PubMed: 14997047]
24. Porter HA, Carey GB, Keegan AD. Insulin receptor substrate 1 expression enhances the sensitivity of 32D cells to chemotherapy-induced cell death, *Exp. Cell Res.* 2012
25. Willis AL, Tran NL, Chatigny JM, Charlton N, Vu H, Brown SA, et al. The fibroblast growth factor-inducible 14 receptor is highly expressed in HER2-positive breast tumors and regulates breast cancer cell invasive capacity. *Mol Cancer Res.* 2008; 6:725–734. [PubMed: 18505918]
26. Teo H, Ghosh S, Luesch H, Ghosh A, Wong ET, Malik N, et al. Telomere-independent Rap1 is an IKK adaptor and regulates NF-kappaB-dependent gene expression. *Nat Cell Biol.* 2010; 12:758–767. [PubMed: 20622870]
27. Whitsett TG, Cheng E, Inge L, Asrani K, Jameson NM, Hostetter G, et al. Elevated expression of Fn14 in non-small cell lung cancer correlates with activated EGFR and promotes tumor cell migration and invasion. *Am J Pathol.* 2012
28. Fortin SP, Ennis MJ, Savitch BA, Carpentieri D, McDonough WS, Winkles JA, et al. Tumor necrosis factor-like weak inducer of apoptosis stimulation of glioma cell survival is dependent on Akt2 function. *Mol Cancer Res.* 2009; 7:1871–1881. [PubMed: 19861406]
29. Schnarr B, Strunz K, Ohsam J, Benner A, Wacker J, Mayer D. Down-regulation of insulin-like growth factor-I receptor and insulin receptor substrate-1 expression in advanced human breast cancer. *Int J Cancer.* 2000; 89:506–513. [PubMed: 11102895]

30. Clark JL, Dresser K, Hsieh CC, Sabel M, Kleer CG, Khan A, et al. Membrane localization of insulin receptor substrate-2 (IRS-2) is associated with decreased overall survival in breast cancer. *Breast Cancer Res Treat.* 2011
31. Sisci D, Morelli C, Garofalo C, Romeo F, Morabito L, Casaburi F, et al. Expression of nuclear insulin receptor substrate 1 in breast cancer. *J Clin Pathol.* 2007; 60:633–641. [PubMed: 16882697]
32. Molloy CA, May FE, Westley BR. Insulin receptor substrate-1 expression is regulated by estrogen in the MCF-7 human breast cancer cell line. *J Biol Chem.* 2000; 275:12565–12571. [PubMed: 10777546]
33. Obiri NI, Siegel JP, Varricchio F, Puri RK. Expression of high-affinity IL-4 receptors on human melanoma, ovarian and breast carcinoma cells. *Clin Exp Immunol.* 1994; 95:148–155. [PubMed: 8287600]
34. Burt BM, Bader A, Winter D, Rodig SJ, Bueno R, Sugarbaker DJ. Expression of interleukin-4 receptor alpha in human pleural mesothelioma is associated with poor survival and promotion of tumor inflammation. *Clin Cancer Res.* 2012; 18:1568–1577. [PubMed: 22261806]
35. Jackson JG, White MF, Yee D. Insulin receptor substrate-1 is the predominant signaling molecule activated by insulin-like growth factor-I, insulin, and interleukin-4 in estrogen receptor-positive human breast cancer cells. *J Biol Chem.* 1998; 273:9994–10003. [PubMed: 9545345]
36. De S, Cipriano R, Jackson MW, Stark GR. Overexpression of kinesins mediates docetaxel resistance in breast cancer cells. *Cancer Res.* 2009; 69:8035–8042. [PubMed: 19789344]
37. Wang TH, Popp DM, Wang HS, Saitoh M, Mural JG, Henley DC, et al. Microtubule dysfunction induced by paclitaxel initiates apoptosis through both c-Jun N-terminal kinase (JNK)-dependent and -independent pathways in ovarian cancer cells. *J Biol Chem.* 1999; 274:8208–8216. [PubMed: 10075725]
38. Zhou J, Giannakakou P. Targeting microtubules for cancer chemotherapy. *Curr Med Chem Anticancer Agents.* 2005; 5:65–71. [PubMed: 15720262]
39. Momparler RL, Karon M, Siegel SE, Avila F. Effect of adriamycin on DNA, RNA, and protein synthesis in cell-free systems and intact cells. *Cancer Res.* 1976; 36:2891–2895. [PubMed: 1277199]
40. Koehn H, Magan N, Isaacs RJ, Stowell KM. Differential regulation of DNA repair protein Rad51 in human tumour cell lines exposed to doxorubicin. *Anticancer Drugs.* 2007; 18:419–425. [PubMed: 17351394]
41. Beere HM, Chresta CM, Alejo-Herberg A, Skladanowski A, Dive C, Larsen AK, et al. Investigation of the mechanism of higher order chromatin fragmentation observed in drug-induced apoptosis. *Mol Pharmacol.* 1995; 47:986–996. [PubMed: 7746285]
42. Zhao H, Rybak P, Dobrucki J, Traganos F, Darzynkiewicz Z. Relationship of DNA damage signaling to DNA replication following treatment with DNA topoisomerase inhibitors camptothecin/topotecan, mitoxantrone, or etoposide. *Cytometry A.* 2012; 81:45–51. [PubMed: 22140093]
43. Aubel-Sadron G, Londos-Gagliardi D. Daunorubicin and doxorubicin, anthracycline antibiotics, a physicochemical and biological review. *Biochimie.* 1984; 66:333–352. [PubMed: 6380596]
44. Chumsri S, Howes T, Bao T, Sabnis G, Brodie A. Aromatase, aromatase inhibitors, and breast cancer. *J Steroid Biochem Mol Biol.* 2011; 125:13–22. [PubMed: 21335088]
45. Glick JH, Creech RH, Torri S, Holroyde C, Brodovsky H, Catalano RB, et al. Tamoxifen plus sequential CMF chemotherapy versus tamoxifen alone in postmenopausal patients with advanced breast cancer: a randomized trial. *Cancer.* 1980; 45:735–741. [PubMed: 6986969]
46. Dearth RK, Cui X, Kim HJ, Hadsell DL, Lee AV. Oncogenic transformation by the signaling adaptor proteins insulin receptor substrate (IRS)-1 and IRS-2. *Cell Cycle.* 2007; 6:705–713. [PubMed: 17374994]
47. Cui X, Lazard Z, Zhang P, Hopp TA, Lee AV. Progesterone crosstalks with insulin-like growth factor signaling in breast cancer cells via induction of insulin receptor substrate-2. *Oncogene.* 2003; 22:6937–6941. [PubMed: 14534541]
48. Vassen L, Wegrzyn W, Klein-Hitpass L. Human insulin receptor substrate-2 (IRS-2) is a primary progesterone response gene. *Mol Endocrinol.* 1999; 13:485–494. [PubMed: 10077005]

49. Rocha RL, Hilsenbeck SG, Jackson JG, VanDenBerg CL, Weng C, Lee AV, et al. Insulin-like growth factor binding protein-3 and insulin receptor substrate-1 in breast cancer: correlation with clinical parameters and disease-free survival. *Clin Cancer Res.* 1997; 3:103–109. [PubMed: 9815544]
50. Koda M, Sulkowska M, Kanczuga-Koda L, Sulkowski S. Expression of insulin receptor substrate 1 in primary breast cancer and lymph node metastases. *J Clin Pathol.* 2005; 58:645–649. [PubMed: 15917419]
51. Gual P, Le Marchand-Brustel Y, Tanti JF. Positive and negative regulation of insulin signaling through IRS-1 phosphorylation. *Biochimie.* 2005; 87:99–109. [PubMed: 15733744]
52. Lippman ME, Allegra JC, Thompson EB, Simon R, Barlock A, Green L, et al. The relation between estrogen receptors and response rate to cytotoxic chemotherapy in metastatic breast cancer. *N Engl J Med.* 1978; 298:1223–1228. [PubMed: 651963]
53. Kiang DT, Frenning DH, Goldman AI, Ascensao VF, Kennedy BJ. Estrogen receptors and responses to chemotherapy and hormonal therapy in advanced breast cancer. *N Engl J Med.* 1978; 299:1330–1334. [PubMed: 714108]
54. Surmacz E, Burgaud JL. Overexpression of insulin receptor substrate 1 (IRS-1) in the human breast cancer cell line MCF-7 induces loss of estrogen requirements for growth and transformation. *Clin Cancer Res.* 1995; 1:1429–1436. [PubMed: 9815941]
55. Guvakova MA, Surmacz E. Tamoxifen interferes with the insulin-like growth factor I receptor (IGF-IR) signaling pathway in breast cancer cells. *Cancer Res.* 1997; 57:2606–2610. [PubMed: 9205064]
56. Salerno M, Sisci D, Mauro L, Guvakova MA, Ando S, Surmacz E. Insulin receptor substrate 1 is a target for the pure antiestrogen ICI 182,780 in breast cancer cells. *Int J Cancer.* 1999; 81:299–304. [PubMed: 10188734]

Fig. 1.

Expression of IRS1, IRS2, STAT6 and tyrosine phosphorylated STAT6 in human breast tumors. Three human breast cancer tissue microarrays were analyzed by immunohistochemistry using antibodies specific for IRS1, IRS2, STAT6 or pSTAT6. Representative samples from the microarray including normal breast tissue from reduction mammoplasty, normal lactating breast, ER α positive ductal carcinoma *in situ* (DCIS), and ER α negative invasive ductal carcinoma (IDC) are shown. The microarrays were analyzed by a pathologist and scored for intensity of cytoplasmic staining on a scale 0–3: 0, negative; 1, weak; 2, moderate; 3, strong. Scores are shown in the bottom right corner of each sample.

Fig. 2.

Higher grade, invasive ductal carcinomas express more IRS2. Representative samples from the microarray showing specific IRS2 staining (Table 3) are shown with a high power inset. (A) Normal breast tissue, (B) invasive ductal carcinoma (IDC) grade 1, and (C) IDC grade 3. (D) The IRS2-stained microarrays were analyzed by a pathologist and scored for intensity of cytoplasmic staining on a scale 0–3. The percentage of samples scoring in each level is shown graphically for DCIS, IDC grade 1, grade 2, and grade 3.

Fig. 3.

MCF7 and MDA-MB-231 have differential IRS and STAT6 expression and tyrosine phosphorylation. (A) MCF7 and MDA-MB-231 cells were treated in the presence or absence of IL-4 (10 ng/mL) for 30 min. Cell lysates were prepared and immunoprecipitated with anti-IRS1 or anti-IRS2 antibodies. Western blots were probed with anti-phosphotyrosine, anti-IRS1 or anti-IRS2 antibodies as appropriate. Several exposures of the anti-pY blot are shown. (B) MCF7 and MDA-MB-231 cells were treated in the presence or absence of IL-4 (10 ng/mL) for 30 min. Cell lysates were prepared and the western blot was probed with anti-pSTAT6 or anti-STAT6 antibodies. Lysates from U937 cells treated with IL-4 were used as a positive control.

Fig. 4.

MCF7 cells are more sensitive to taxol-induced death than MDA-MB-231 cells. MCF7 and MDA-MB-231 cells were cultured at 8×10^3 cells/100 μ l of complete media in the presence or absence of 10 μ M taxol or 10 μ M doxorubicin for 24 h. Cell survival was measured using the MTS assay shown as maximum absorbance relative to untreated control. Two-tailed, unpaired, *t*-test was used to determine statistical significance.

Fig. 5.

Decreasing IRS1 expression by shRNA-knock-down in MCF7 cells de-sensitizes these cells to some, but not all chemotherapeutic agents. (A) MCF7 cells were infected with IRS1 shRNA as described in Section 2 and two stable cell lines were generated (99-2W1 and 99-2W3). Cell lysates were prepared and the western blot was probed with anti-IRS1, anti-IRS2, anti-STAT6, or anti-Tubulin antibodies as indicated. MCF7 cells and MCF7 IRS1-depleted cells were cultured at 8×10^3 cells/100 μ l of complete media and treated with or without taxol (10 μ M), (B) or doxorubicin (10 μ M), (C) for 24 h. Cell survival was measured using the MTS assay and shown as maximum absorbance relative to untreated control. Two-tailed, unpaired, *t*-test was used to determine statistical significance. Comparisons with significant differences are indicated.

Fig. 6. Decreasing IRS1 expression alters Annexin A2 localization. (A) Cell lysates were prepared from MCF7 cells and MCF7 IRS1-depleted cells. The western blot was probed with anti-ER α , anti-BCL-xL, anti-BAK, anti-BCL-2, anti-BAX, anti-Annexin A2, anti-RAD51, or anti-Tubulin antibodies. (B) MCF7 cells and MCF7 IRS1-depleted cells were lysed using a subcellular fractionation kit. The subcellular fractions were prepared and analyzed by western blotting with anti-IRS1 and anti-Annexin A2 antibodies. A = cytoplasmic extract, B = membrane extract, C = soluble nuclear extract, D = chromatin-bound nuclear extract, E = cytoskeletal extract. Antibodies to HSP90 and Sp1 were used as positive controls for the cytoplasmic and soluble nuclear fractions, respectively.

Table 1

Cytoplasmic staining intensity of IRS1.

Breast tumor vs. control	Score 0	Score 1	Score 2	Score 3
Nonneoplastic breast tissue	1/6 (16.7%)	4/6 (66.7%)	1/6 (16.7%)	0/6 (0.0%)
Ductal carcinoma <i>in situ</i> (DCIS)	0/6 (0.0%)	0/6 (0.0%)	0/6 (0.0%)	6/6 (100%)
invasive lobular carcinoma	0/20 (0.0%)	5/20 (25.0%)	8/20 (40.0%)	7/20 (35.0%)
Invasive ductal carcinoma (IDC)	13/145 (9.0%)	22/145 (15.2%)	27/145 (18.6%)	83/145 (57.2%)
<i>Grades of IDC</i>				
Grade 1	4/30 (13.3%)	1/30 (3.3%)	5/30 (16.7%)	20/30 (66.7%)
Grade 2	6/53 (11.3%)	5/53 (9.4%)	8/53 (15.1%)	34/53 (64.2%)
Grade 3	0/41 (0.0%)	8/41 (19.5%)	8/41 (19.5%)	25/41 (61.0%)

Breast cancer tissue microarrays were stained with an anti-IRS1 antibody. They were analyzed by a pathologist and scored for tumor type, grade, and intensity of IRS1 cytoplasmic staining on a scale of 0–3 as described in Section 2. The numbers of samples scored in each category are shown and the percentages were calculated.

Table 2

Cytoplasmic staining intensity of pSTAT6.

Breast tumor vs. control	Score 0	Score 1	Score 2	Score 3
Nonneoplastic breast tissue	3/6 (50%)	3/6 (50%)	0/6 (0.0%)	0/6 (0.0%)
Ductal carcinoma <i>in situ</i> (DCIS)	0/5 (0.0%)	0/5 (0.0%)	0/5 (0.0%)	5/5 (100%)
Invasive lobular carcinoma	2/16 (12.5%)	4/16 (25.0%)	4/16 (25.0%)	6/16 (37.5%)
Invasive ductal carcinoma (IDC)	34/149 (22.8%)	51/149 (34.2%)	23/149 (15.4%)	41/149 (27.5%)
<i>Grades of IDC</i>				
Grade 1	4/27 (14.8%)	11/27 (40.7%)	4/27 (14.8%)	8/27 (29.6%)
Grade 2	14/52 (26.9%)	19/52 (36.5%)	8/52 (15.4%)	11/52 (21.2%)
Grade 3	9/40 (22.5%)	9/40 (22.5%)	8/40 (20.0%)	14/40 (35.0%)

Breast cancer tissue microarrays were stained with an anti-phospho-STAT6 antibody. They were analyzed by a pathologist and scored for tumor type, grade, and intensity of pSTAT6 cytoplasmic staining on a scale of 0–3 as described in Section 2. The numbers of samples scored in each category are shown and the percentages were calculated.

Table 3

Cytoplasmic staining intensity of IRS2.

Breast tumor vs. control	Score 0	Score 1	Score 2	Score 3
Nonneoplastic breast tissue	1/6 (16.7%)	5/6 (83.3%)	0/6 (0.0%)	0/6 (0.0%)
Ductal carcinoma <i>in situ</i> (DCIS)	4/9 (44.4%)	4/9 (44.4%)	1/9 (11.1%)	0/9 (0.0%)
Invasive lobular carcinoma	2/20 (10.0%)	4/20 (20.0%)	10/20 (50.0%)	4/20 (20.0%)
Invasive ductal carcinoma (IDC)	7/149 (4.7%)	35/149 (23.5%)	48/149 (32.2%)	59/149 (39.6%)
<i>Grades of IDC</i>				
Grade 1	4/27 (14.8%)	10/27 (37.0%)	10/27 (37.0%)	3/27 (11.1%)
Grade 2	1/43 (2.3%)	15/43 (34.9%)	17/43 (39.5%)	10/43 (23.3%)
Grade 3	0/44 (0.0%)	1/44 (2.3%)	7/44 (15.9%)	36/44 (81.8%)

Breast cancer tissue microarrays were stained with an anti-IRS2 antibody. They were analyzed by a pathologist and scored for tumor type, grade, and intensity of IRS2 cytoplasmic staining on a scale of 0–3 as described in Section 2. The numbers of samples scored in each category are shown and the percentages were calculated.