Elevated Levels of p66 Shc Are Found in Breast Cancer Cell Lines and Primary Tumors with High Metastatic Potential

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ABSTRACT

The adapter molecule Shc has been implicated in specific steps of metastasis. In the current study, we show that the expression and activation of the p66 Shc isoform increased in a highly metastatic variant (F-11) of the human breast cancer cell line MDA-MB-231 compared to the parent cell line, whereas the p46 and p52 Shc isoforms were unchanged. Despite reports that p66 Shc can negatively regulate epidermal growth factor signaling to the mitogen-activated protein kinase pathway, we found no change in epidermal growth factor-stimulated activation of mitogen-activated protein kinase in the F-11 cell line. We determined the level of Shc expression by immunoblot in primary breast cancer specimens obtained from patients with or without axillary node involvement. p66 Shc expression increased in tumors obtained from node-positive patients (Spearman correlation coefficient = 0.43377; \( P = 0.0058 \)) compared to the node-negative specimens. Furthermore, increasing levels of p66 Shc correlated with an increasing number of positive nodes (\( P = 0.032 \)). This study shows that p66 Shc expression increased in cultured human breast cancer cells selected for metastasis and in primary human breast cancer specimens obtained from patients with lymph node involvement, suggesting a possible role for Shc in human breast cancer metastasis.

INTRODUCTION

Breast cancer is a lethal disease because of uncontrolled proliferation at metastatic sites. Metastasis is a complex, multi-step process involving recruitment of blood vessels, motility and invasion of transformed cells into surrounding stroma, and intravasation of tumor cells into the vasculature. After reaching the circulation or lymphatic channels, some metastatic cells must then arrest in the intraluminal space, adhere to the endothelial walls, extravasate into the organ, and interact with the new environment (1). Each of these steps is, at least in part, controlled by various growth factors, adhesion molecules, and their receptor tyrosine kinases. Growth factors that are known to be involved in cell motility, invasion, or migration of various cell types include nerve growth factor (2, 3), fibroblast growth factor (2, 4, 5), platelet-derived growth factor (2, 6), EGF (2, 7), keratin growth factor (3), hepatocyte growth factor (3, 8), interleukin 2 (9), and insulin and IGFs (10).

Many growth factors, cytokines, and adhesion molecules exert their effects by activating specific tyrosine kinases. The activated receptor allows the docking of additional SH2 and PTB domain containing adapter molecules that coordinate and integrate intracellular signaling events. Interestingly, the adapter molecule Shc is activated by each of the receptors for the ligands listed, suggesting a possible role for Shc in the metastatic process. Shc is a SH2 and PTB domain-containing protein encoded by one gene that produces three isoforms with molecular weights of 46,000, 52,000, and 66,000 respectively (11). Each isoform can serve as a substrate for activated cytoplasmic or receptor tyrosine kinases via either PTB or SH2 domains and can then associate with other SH2 domains containing signaling molecules, such as Grb2. It has previously been shown that Shc can amplify the motogenic signal delivered by the hepatocyte growth factor receptor Met in BN-14 mouse bile duct and A549 lung carcinoma cells (8). Furthermore, Shc binding has been shown to be essential in cell motility induced by the nerve growth factor receptor trkA (3). Even more compelling evidence for a role by Shc in metastasis was found in a study of transgenic mice strains expressing polyomavirus middle T antigen with a mutated Shc binding site (12). Focal mammary tumors arose in the female transgenic mice, but surprisingly, there was reversion of the mutated middle T Shc binding site to wild-type in a number of the metastatic tumors forming in these mice. This important study demonstrates a strong, positive selective pressure for normal Shc function in the establishment of breast cancer metastases.

In the current study, we used a highly metastatic variant (F-11) isolated from a xenograft bone metastasis of breast cancer cell line MDA-MB-231 to investigate Shc signaling. Using EGF to activate Shc in these cells, we found that phosphorylation of the p66 isoform was greatly increased in the...
metastatic variant compared to the parental cell line. We also found that levels of p66 Shc protein were increased in F-11. No significant difference in EGF-induced activation of MAP kinase was detected in F-11 compared to the parental MDA-MB-231 cell line. To further investigate the importance of Shc in breast cancer metastasis, we measured levels of Shc protein in primary breast cancer specimens. In breast cancer specimens associated with lymph node metastases, we found that p66 Shc expression was correlated with a greater number of positive nodes. In light of previous work, these data further implicate Shc in the metastatic process and suggest that the p66 Shc isoform may be involved.

MATERIALS AND METHODS

Reagents. All chemicals and reagents were purchased from Sigma (St. Louis, MO) unless otherwise noted. MAP kinase and Shc antibodies were purchased from Upstate Biotechnology (Lake Placid, NY). Phospho-specific (activated) MAP kinase was purchased from New England Biolabs (Beverly, MA); RC-20 antiphosphotyrosine was purchased from Transduction Laboratories (Lexington, KY); horseradish peroxidase-linked antirabbit antibody and rainbow molecular weight markers were purchased from Amersham Pharmacia Biotech. IGF-I was purchased from Gro Pep (Adelaide, Australia). Acrylamide/bis was purchased from Bio-Rad (Hercules, CA). 435A cells were a gift of Nils Brunner (Finneslab, Copenhagen, Denmark) and have been described previously (13); MDA-MB-231 cells were originally purchased from American Type Culture Collection (Manassas, VA). The F-11 metastatic variant was created in the laboratory of Dr. Toshiyuki Yoneda (University of Texas Health Science Center at San Antonio, San Antonio, TX) and will be described elsewhere.4 Briefly, an athymic mouse received an intracardiac injection of MDA-MB-231 cells, and bone metastases that formed were established in vitro after 11 serial passages in vivo.

Cell Stimulation and Lysis. For stimulation experiments, cells were treated as described previously (14). After cells were allowed to attach overnight in serum-containing medium, cells were washed and incubated with SFM for 24 h. To stimulate cells, medium was replaced with SFM plus indicated growth factors for 10 min at the following concentrations: 5 nM (40 ng/ml) IGF-I and 50 ng/ml EGF. Cells were washed twice in ice-cold PBS and lysed with 500 μl of TNESV buffer. Protein concentration of the cleared lysates was determined by the copper-bicinchoninic acid method with a Pierce Laboratory kit (Rockford, IL).

Immunoprecipitations. All steps were performed on a platform rocker at 4°C. Equal amounts of protein were precleared with 25 μl of protein A-agarose for 30 min and then incubated overnight with 2 μl of Shc antibody. Next, 25 μl of protein A-agarose (Pierce) was added for 4 h, followed by three washes with TNESV buffer. Beads were resuspended in 2× Laemmli loading buffer with 30 mg/ml DTT, boiled, and then separated by 9% SDS-PAGE.

Preparation of Breast Tumor Specimens. Twenty primary tumor specimens from patients with pathological evidence of disease in the lymph nodes and 19 node-negative breast tumor specimens obtained from the San Antonio Tumor Bank were separated into two blinded groups. These tumors were obtained previously for the measurement of ER. Frozen, pulverized tumor powder (50 mg) was lysed in 500 μl of TNESV buffer. After centrifugation, protein concentration of the supernatant was determined as described above. Tumor protein (150 μg; 13 specimens were run on one gel, seven from one blinded group and six from the other), 50 μg of 435A control protein, and rainbow marker (15 total lanes) were separated by SDS-PAGE on three gels.

Immunoblotting. After SDS-PAGE, proteins were transferred overnight to nitrocellulose membranes (Bio-Rad). All blotting steps were carried out at room temperature with gentle rocking as described previously (14). The membranes were blocked in 5% nonfat dry milk in TBST [0.15 M NaCl, 0.01 M Tris-HCl (pH 7.4), and 0.05% Tween 20]. For antiphosphotyrosine blotting with RC-20, membranes were washed five times for more than 30 min with TBST after blocking, incubated with a 1000:1 dilution in TBST for 2 h, and washed. Chemiluminescence was then performed as described below. Shc blots were incubated with a 2000:1 dilution of the indicated antibody in blocking buffer for 1 h. After washing, blots were incubated with a 2000:1 dilution of horseradish peroxidase-linked antirabbit secondary antibody in blocking buffer for 1 h, followed by further washing. Enhanced chemiluminescence was performed according to the manufacturer’s instructions (Pierce).

Densitometry and Statistics. Densitometry was performed by scanning the radiographs (ScanJet IICx; Hewlett-Packard) and then analyzing the bands with ImageTool version 2.0 software (University of Texas Health Science Center at San Antonio). Radiographs with shorter exposure times than those shown in Fig. 3 were used for quantitating the p52 and p46 Shc bands. Background-subtracted densitometry units were normalized among the different gels by dividing by the values for the 435A control cell line.

Statistical Analysis. Levels of p46, p52, and p66 isoforms of Shc were compared using t tests. Spearman correlation coefficients were used to correlate the levels of p46, p52, and p66 isoforms of Shc with the number of positive lymph nodes, tumor size, ER and PgR content, S-phase fraction, and age of patients with primary breast cancer. t tests and Wilcoxon’s rank-sum test were used to compare the levels of p46, p52, and p66 across categorical representations of nodal status (0, 1–3, 4+), tumor size (≤2 cm, 2–5 cm, >5 cm), ER (ER+, ER−), PgR (PgR+, PgR−), S-phase fraction (low, high), and age (<50 years, ≥50 years). All calculations were performed using SAS software version 6.11 (SAS Institute, Cary, NC).

RESULTS
Shc Activation and Expression in MDA-MB-231 and F-11. We first compared Shc activation in MDA-MB-231 and a metastatic variant of this cell line selected from a bone metastasis, F-11. After EGF treatment and Shc immunoprecipi-

tation, antiphosphotyrosine immunoblotting shows MDA-MB-231 cells strongly phosphorylated the p46 and p52 isoforms of Shc, but only a weak activation of p66 Shc was observed (Fig. 1, top). In contrast, F-11 cells showed a much stronger activation of p66 Shc in response to EGF. Activation of the p46 and p52 isoforms was not significantly different in F-11 compared to parental MDA-MB-231 cells (Fig. 1, top). IGF-I treatment did not result in activation of any Shc isoform in either cell line (Fig. 1, top). After immunoblotting MDA-MB-231 and F-11 cell lysates for Shc, we found that the levels of p46 and p52 isoforms were unchanged between the two cell lines, but that F-11 cells had a 3-fold increased expression of the p66 isoform (Fig. 1, bottom).

Because transfected p66 Shc has been reported to inhibit MAP kinase activation in Chinese hamster ovary cells (15), we next examined by immunoblot the activation of MAP kinase by EGF in these cell lines. We found no significant difference in the level of phospho-MAP kinase between the MDA-MB-231 and F-11 cell lines (Fig. 2) in cells treated with EGF for 10 min. Thus, the elevated levels of p66 Shc seen in F-11 cells did not inhibit EGF-mediated MAP kinase activation. In fact, the F-11 cell line consistently displayed a slightly greater basal level of MAP kinase activation than did the parental cell line (Fig. 2).

**Shc Expression in Primary Breast Cancer Tissues.** After the observation that p66 Shc was up-regulated in the F-11 variant, we reasoned that this isoform might be a necessary or preferred pathway in breast cancer metastasis. To further explore this possibility, we examined Shc protein expression in 20 primary tumor specimens from patients with pathological evidence of disease in the axillary lymph nodes and in 19 node-negative breast tumor specimens. After Shc immunoblotting, bands were quantitated according to densitometry and normalized according to Shc levels in 435A cell lysates. Fig. 3 shows a representative gel of seven node-positive and six node-negative tumors in breast cancer cell line 435A. Spearman rank correlation analysis was performed and showed that p66 Shc was significantly increased in the node-positive specimens (Spearman correlation coefficient = 0.43; P = 0.0058), whereas the p46 (Spearman correlation coefficient = 0.14; P = 0.39)

**Fig. 1** Activation and expression of p66 Shc in the MDA-MB-231 cell line versus the F-11 metastatic variant. Top, serum-starved 70% confluent cell monolayers were treated for 10 min with SFM, EGF, or IGF-I as indicated above the lanes. Cell lysates were immunoprecipitated with Shc antibody, separated by SDS-PAGE, and antiphosphotyrosine immunoblotted. Bottom, SFM-treated cell lysates were separated by SDS-PAGE and Shc immunoblotted. Migration of various Shc isoforms is indicated at the left; molecular weight markers are indicated at the right. Data shown are representative of at least three independent experiments. Levels of p66 Shc were significantly higher in F-11 cells compared to MDA-MB-231 cells (P = 0.0019), whereas levels of p52 (P = 0.84) and p46 (P = 0.22) did not differ between the cell lines.

**Fig. 2** Expression and activation of MAP kinase in the MDA-MB-231 cell line versus the F-11 metastatic variant. Serum-starved 70% confluent cell monolayers were treated for 10 min with SFM, EGF, or IGF-I as indicated above the lanes. Cell lysates were separated by SDS-PAGE and immunoblotted for phospho-MAP kinase (top) or total MAP kinase (bottom). Molecular weight markers are indicated at the right. Data shown are representative of two separate experiments.
and p52 (Spearman correlation coefficient = 0.17; \( P = 0.29 \)) isoforms were not associated with nodal status. Furthermore, as shown in Table 1, the levels of p66 Shc were higher in the group of patients with \( \geq 4 \) positive nodes than in the group of patients with 1–3 positive nodes, and levels of p66 Shc were higher in the group of patients with 1–3 positive nodes than in the group of patients with 0 positive nodes (\( P = 0.032 \)). p66 Shc levels were not correlated with tumor size, ER status, PgR status, S-phase fraction, or age. p46 and p52 Shc levels were not correlated with tumor size, ER status, PgR status, S-phase fraction, or age. p46 and p52 Shc levels were not correlated with any of the parameters evaluated.

**DISCUSSION**

Many studies have shown a role for Shc in cellular migration, motility, invasion, and, ultimately, metastasis. The strongest piece of evidence thus far for a direct involvement of Shc in metastasis comes from mice transgenic for polyoma virus middle T antigen with a mutated Shc binding site (12). The oncogenic capacity of polyoma virus middle T antigen results from its ability to couple with and activate signaling molecules like Src, Shc, and phosphatidylinositol 3’-kinase. In some of the metastatic tumors that had formed in these mice, the mutated middle T Shc binding site had reverted to wild type and was again functional. This demonstrates a very strong selective pressure for functional Shc in the process of metastasis *in vivo*. In our study, MDA-MB-231 cells expressed and activated Shc. However, we found that cells selected for bone metastases (F-11) had increased p66 Shc expression by 3-fold. *In vitro*, the F-11 cells also activated the increased p66 Shc in response to EGF. EGF was chosen because of its well-known ability to activate Shc; however, at least 15 other tyrosine kinases also activate Shc, and many of these influence migration, invasion, motility, and metastasis. It would be difficult to determine which ligand(s) is responsible for activating Shc *in an in vivo* environment or in the human tumor specimens. Also, the exact step(s) of the metastatic process that Shc influences has yet to be determined. However, given the presence of Shc in primary breast tumors that had already metastasized to axillary lymph nodes, it is possible that Shc is involved at an early step, such as during invasion through the basement membrane or during motility.

The observation that the p66 Shc isoform was increased in the F-11 cells and in the node-positive breast tumor specimens was unexpected. No transforming or growth-promoting properties of this isoform have been reported: (a) it does not transform mouse fibroblasts *in vitro* (11); (b) it decreases (15) or does not influence activation of MAP kinases; and (c) it inhibits fos promoter activation. However, these studies represent data from only three cell lines, Cos-1, HeLa, and Chinese hamster ovary cells, that overexpress p66 Shc by transfection. Furthermore, experiments on phenotypic changes in p66 Shc-expressing cells were limited to transformation. Few other data exist for a functional role for p66 Shc, particularly endogenous p66 Shc.

If p66 Shc participates in the metastatic pathway, then clearly additional downstream molecules must be involved. In human tissues, it has been suggested that activation of MAP kinase results in more aggressive clinical behavior (16). The majority of data show that p52 Shc mediates signaling by activating the MAP kinase pathway via its association with the Grb2/sos complex, which activates ras. In the F-11 cell line, we found no difference in MAP kinase activation after EGF treatment compared to the MDA-MB-231 parental cells, but we consistently found that F-11 cells had a slightly higher level of basal activation. It is not known whether this slight increase was due to p66 Shc up-regulation or whether it was even physiolog-

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**Table 1** Levels of p66 Shc were compared to number of positive lymph nodes

<table>
<thead>
<tr>
<th>Lymph node status</th>
<th>N</th>
<th>Mean</th>
<th>SD</th>
<th>SE</th>
<th>Median</th>
<th>Minimum/Maximum</th>
<th>( P )</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 nodes</td>
<td>19</td>
<td>0.201</td>
<td>0.306</td>
<td>0.070</td>
<td>0.043</td>
<td>0.000/1.012</td>
<td>0.032</td>
</tr>
<tr>
<td>1–3 nodes</td>
<td>8</td>
<td>0.253</td>
<td>0.207</td>
<td>0.073</td>
<td>0.202</td>
<td>0.017/0.531</td>
<td></td>
</tr>
<tr>
<td>4+ nodes</td>
<td>12</td>
<td>0.533</td>
<td>0.478</td>
<td>0.138</td>
<td>0.361</td>
<td>0.036/1.382</td>
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</tr>
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p66 Shc levels in the primary breast specimen increased as the number of positive lymph nodes increased. In primary tumors obtained from patients with \( >4 \) positive lymph nodes involved with cancer (4+), an increased mean was found.

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**Fig. 3** Shc expression in node-positive versus node-negative primary breast tumors. Total protein (150 \( \mu \)g) from lysates of blinded breast tumor specimens (seven node-positive specimens and six node-negative specimens) and 50 \( \mu \)g of protein from control 435A cells were separated by SDS-PAGE and immunoblotted for Shc blots. A representative immunoblot is shown (1 of 3 blots). A total of 39 specimens was studied for Shc expression. Molecular weight markers are indicated at the right. Data shown are representative of two separate experiments.
ically significant. The high levels of basal MAP kinase activation in both MDA-MB-231 and F-11 cell lines make MAP kinase stimulation experiments more difficult to interpret, although our data suggest that increased signaling through MAP kinase alone does not account for the enhanced metastatic behavior of the variant cell lines. Our data show that the elevated levels of p66 Shc expression did not influence MAP kinase signalling in the F-11 cell line. In addition, the cell lines and tumor tissues we examined were obtained from fully transformed malignant breast epithelial cells, suggesting that p66 Shc does not play a role in the transformation of these cells. However, higher levels of p66 Shc were observed in specimens with higher metastatic potential, and it is possible that p66 Shc functions in motility and invasion pathways distinct from MAP kinase.

It is also possible that Shc may deliver its signal in the metastatic process by a Grb2-independent pathway. It has been shown that Shc can exert mitogenic (17) and antiapoptotic (18) effects independent of ras signalling. Shc also binds other unidentified tyrosine-phosphorylated proteins other than Grb2 (19). Even phosphatidylinositol 3’-kinase has been shown to bind Shc in leukemia cells (20). Thus, one may speculate that although Shc-mediated MAP kinase activation was unchanged in vitro in the F-11 cells, a Shc-mediated change in the activation of additional downstream signalling pathways may be responsible for the increased metastatic phenotype of F-11 cells.

Metastasis of primary tumors to distant sites is a multistep process controlled by numerous growth factors. Many of these growth factors activate Shc. In the current study, we found that p66 Shc expression increased in a highly metastatic variant of the human breast cancer cell line MDA-MB-231. This xenograft observation was validated by the finding that p66 Shc increased in lymph node-positive breast tumors and that increased levels of p66 Shc correlated with an increased number of positive nodes. These data suggest that Shc may function in the metastatic pathway. Interestingly, only one of the three Shc isoforms, p66, increased in the more metastatic cell line and in the node-positive primary breast cancer specimens. Determining the upstream effectors and downstream regulators of p66 Shc function may help identify the mechanisms responsible for breast cancer metastasis. Moreover, because levels of p66 Shc found in primary breast cancer specimens correlated with lymph node involvement, measurement of p66 Shc could have prognostic implications in breast cancer.

REFERENCES


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