Loss of HSulf-1 Expression Enhances Autocrine Signaling Mediated by Amphiregulin in Breast Cancer*

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Heparan sulfate (HS) glycosaminoglycans are the oligosaccharide chains of heparan sulfate proteoglycans. The sulfation of HS glycosaminoglycan residues is required for its interaction with various heparin-binding growth factors to promote their biological activities to activate their high affinity receptor tyrosine kinases. We have identified HS glycosaminoglycan-6-O-endosulfatase HSulf-1 as a down-regulated gene in ovarian, breast, and several other cancer cell lines. Here we have shown that HSulf-1 inhibits autocrine activation of the EGFR-ERK (epidermal growth factor receptor-extracellular signal-regulated kinase) pathway induced by serum withdrawal in MDA-MB-468 breast cancer cells. Short hairpin RNA-mediated down-regulation of HSulf-1 in HSulf-1 clonal lines of MDA-MB-468 led to a significant increase in autocrine activation of ERK compared with vector only control. The autocrine signaling was also inhibited with neutralization antibodies against amphiregulin and HB-EGF, the heparin-binding growth factor family of the EGF superfamily. Furthermore, HSulf-1-mediated inhibition of autocrine signaling was associated with reduced cyclin D1 levels, leading to decreased S phase fraction and increased G2-M fraction, as well as increased cell death. Finally, evaluation of HSulf-1 expression levels in primary invasive breast tumors by RNA in situ hybridization indicated that HSulf-1 is down-regulated in the majority (60%) of tumors, with a predominant association with lobular histology. These data suggest a potential role of HSulf-1 down-regulation in mammary carcinogenesis.

Breast cancer is the most common type of cancer among women in the United States. Despite the advances in early detection and therapeutic treatment options, an estimated 215,000 new cases and 41,000 deaths were reported in 2006 (1). One of the molecular signatures known to be associated with poor prognosis is the epidermal growth factor receptor (EGFR).2 The EGFR expression in breast cancer is often associated with resistance to endocrine hormone therapy (2, 3). Recent studies also indicate that hormone-induced estrogen receptor (ER) activation leads to activation of EGFR, suggesting that cross-talk between the two signaling pathways may be a crucial drug-resistant mechanism in ER-positive breast tumors (4). In addition, gene expression profiling studies identified the basal-like tumor as one of the breast tumor subtypes characterized with EGFR expression and poor prognosis (5, 6).

EGFR signaling is induced by various EGF-like growth factor family ligands, such as EGF, amphiregulin, heparin-binding EGF-like growth factor (HB-EGF), betacellulin, epieregulin, and transforming growth factor-α (7). Amphiregulin is one of the most investigated EGFR ligands in relation to breast cancer. Amphiregulin is a heparin-binding growth factor that requires heparan sulfate (HS) glycosaminoglycans as a low affinity co-receptor (8) and is overexpressed in the majority of primary breast tumors (9). Expression of amphiregulin and EGFR are seen both in breast tumors and non-malignant breast tissues adjacent to tumors, suggesting the presence of autocrine and paracrine signaling (10). The correlation between amphiregulin expression levels and aggressive cancer phenotypes has also been observed in breast cancer cell lines (11, 12).

We have previously shown that HSulf-1, an extracellular sulfatase catalyzing the 6-O desulfation of HS glycosaminoglycans, is down-regulated in ovarian, breast, and several other types of cancer cell lines (13). Studies by us and other groups (13–17) demonstrate that the loss of HSulf-1 expression results in increased sulfation of HS glycosaminoglycans, leading to the increased affinity of various heparin-binding growth factors to their cognate receptor tyrosine kinases to enhance downstream signaling, culminating in higher cell proliferation, angiogenesis, and chemoresistance. However, most of the studies elucidating the regulatory role of HSulf-1 in heparin binding growth factor signaling have utilized exogenously added heparin-binding growth factors. What is not known is whether HSulf-1 is also involved in autocrine signaling. Here we have shown for the first time that the loss of HSulf-1 in MDA-MB-468 breast cancer cells augments both autocrine and paracrine proliferation signaling through the heparin-binding growth factors of the EGF superfamily, amphiregulin, and HB-EGF. In addition, determination of HSulf-1 expression levels in primary invasive breast tumors on tissue microarray by RNA in situ hybridization indicated that HSulf-1 is down-regulated in the majority of tumors, especially those with lobular histology. These results suggest a potential mechanism by which HSulf-1 down-regulation may contribute to breast cancer development.
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**EXPERIMENTAL PROCEDURES**

*Materials*—Recombinant human amphiregulin, HB-EGF, and EGF, neutralization antibodies against human amphiregulin, HB-EGF, and EGF (polyclonal goat IgG), and biotinylated secondary antibodies against goat IgG and rabbit IgG (polyclonal donkey IgG) were purchased from R & D Systems (Minneapolis, MN). Rabbit polyclonal antibodies against total ERK1/2, phospho-ERK1/2, (Thr-202/Tyr-204), total EGFR, and phospho-EGFR (Tyr-992 and Tyr-1068) were from Cell Signaling Technology (Beverly, MA). monoclonal anti-cyclin D1/D2 antibody was from Upstate Biotechnology (Lake Placid, NY). Monoclonal anti-tubulin antibody was from Sigma. Horseradish peroxidase-conjugated rabbit IgG and ECL Western blotting detection reagent were from Amersham Biosciences. Poly-horseradish peroxidase streptavidin was from Endogen (Rockford, IL). The Rabbit polyclonal anti-v5 antibody was from Novus Biologicals (Littleton, CO). Other chemicals were the highest quality commercially available.

**Cell Culture and Induction of Autocrine Signaling**—Stable clones of the MDA468 breast cancer cell line expressing HSulf-1 or mock vector were cultured as described previously (17). To induce autocrine signaling, cells were incubated in plain Dulbecco’s modified Eagle’s medium at 37 °C for various time points. In some experiments, neutralization antibodies were added in Dulbecco’s modified Eagle’s medium with the following concentrations: anti-amphiregulin (10 µg/ml), anti-HB-EGF (10 µg/ml), and anti-EGF (1 µg/ml).

**Protein Extracts and Western Blot**—Cells were rinsed quickly with ice-cold phosphate-buffered saline and lysed at 4 °C in SDS sample loading buffer and analyzed by Western blot as described previously (13). The antibodies used for immunoblotting and their dilutions were as follows: phospho-ERK (dilution ratio 1:500), total ERK (1:500), phospho-EGFR (1:500), total EGFR (1:1000), cyclin D1/D2 (1:500), and tubulin (1:1000).

**Adeno-associated Viral Transduction to Express HSulf-1**—For construction of adeno-associated viral vector to express HSulf-1, a 2817-bp MfeI-SacI fragment containing full-length HSulf-1 cDNA was ligated into the EcoRI site of the pAAV-MCS vector. The plasmid DNA was purified with the EndFree Plasmid Maxi kit and packaged into infectious adeno-associated viral particles following the manufacturer’s protocol. For viral transduction, breast cancer cells grown to confluency were incubated for 48 h with a 4:1 mixture of the viral supernatants and fresh culture medium followed by a 24-h recovery in fresh culture medium. The expression of HSulf-1 was evaluated by reverse transcription (RT)-PCR (13) and immunoblotted for the V5 epitope tag.

**Retroviral Transduction for Short Hairpin RNA (shRNA)-mediated Knockdown of HSulf-1**—For down-regulation of HSulf-1, cells were incubated for 48 h with infectious retrovirus containing pSUPER.retro plasmid expressing HSulf-1 shRNAs as described previously (17). The empty vector served as the control. The efficacy of shRNA was evaluated by RT-PCR (13).

**Enzyme-linked Immunosorbent Assay**—For detection of soluble amphiregulin, EGF, and HB-EGF in conditioned medium, 100 µl of sample medium was incubated in 96-well Immulon HBX microtiter plates (Thermo Electron, Waltham, MA) for 1 h at room temperature. After blocking with 300 µl of Tris-buffered saline with Tween 20 (20 mM Tris, pH 8.0, 150 mM NaCl, 0.1% Tween 20) containing 3% bovine serum albumin for 5 min, the samples bound on the wells were incubated with 1.0 µg/ml primary antibody in the blocking solution for 1 h at room temperature and washed with TBST followed by incubations with 50 ng/ml biotin-conjugated secondary anti-goat antibody and tertiary poly-horseradish peroxidase streptavidin diluted at 1:5000. The peroxidase activity was detected using the 3,5′,5′,5′-tetramethyl benzidine substrate kit (Pierce). A standard curve was drawn for each plate using recombinant amphiregulin, EGF, or HB-EGF proteins for reference. Minimum detection limits of the assays for serum amphiregulin, EGF, and HB-EGF were 0.02, 0.05, and 0.01 ng/ml, respectively.

**Cell Proliferation Assay and Cell Cycle Analysis**—For measuring cell proliferation induced by amphiregulin, HB-EGF, or EGF, MDA468 clones seeded in 96-well plates (1000 cells/well) were serum-starved for 48 h in culture medium and then treated with 0.5 ng/ml amphiregulin, HB-EGF, or EGF in the presence of Alamar Blue dye (Invitrogen). The metabolically reduced Alamar Blue dye was quantified according to the protocol of the supplier. The cell cycle analysis by flow cytometry was performed as described previously (18).

**Breast Cancer Tissue Microarray**—Tissue microarrays (TMAs) containing 3 cores from 113 non-selected primary invasive breast cancers (stage I–IIIC) were constructed. An experienced surgical pathologist evaluated hematoxylin- and eosin-stained slides of all of the specimens before construction of the tissue microarray to identify representative tumor areas. Clinicopathologic data are summarized in Table 2.

**RNA in Situ Hybridization**—For preparation of single-stranded RNA probes for HSulf-1, a 510-bp EcoRI fragment containing the position 1–492 of HSulf-1 cDNA was ligated into the EcoRI site of the pGEM-T vector. For production of the sense probe labeled with digoxigenin-11-UTP (Roche Applied Science), the resulting plasmid construct was linearized with SacII and translated by Sp6 RNA polymerase using Riboprobe in vitro transcription systems (Promega, Madison, WI). Likewise, the antisense probe was produced using the plasmid linearized with SalI and T7 RNA polymerase. The RNA in situ hybridization on breast cancer TMA was performed as described previously (19).

**Statistics**—All data represent at least three independent experiments using cells from separate cultures and are expressed as the mean ± S.E. Differences between groups were compared using an unpaired two-tailed t test.

**RESULTS**

*HSulf-1 Inhibits Autocrine-mediated Phosphorylation of ERK1/2 and EGFR in the MDA-MB-468 Breast Cancer Cell Line*—Our recent study (17) demonstrates that HSulf-1 expression in HSulf-1-null, EGFR-overexpressing MDA-MB-468...
To eliminate the possibility that the above observations are due to clonal variations and/or are cell line-specific, we transiently expressed HSulf-1 in MDA468 and BT474 breast cancer cell lines using a high efficiency adeno-associated viral transduction system and then determined the levels of autocrine ERK phosphorylation. The expression of HSulf-1 in these cell lines following viral transduction was confirmed by RT-PCR and immunoblot for the V5 epitope tag fused at the C terminus (Fig. 2A). When the transduced cells were serum-starved for 2 h and the ERK1/2 phosphorylation levels compared with the vector-transduced control, significant reduction in ERK1/2 phosphorylation was observed in both cell lines (Fig. 2, B and C).

Consistent with the observation that forced expression of HSulf-1 leads to reduced autocrine signaling, HSulf-1 knockdown by RNA interference in MDA468 clone number 4 with stable HSulf-1 expression led to a significant increase in autocrine ERK phosphorylation compared with vector-transduced or untreated control samples (Fig. 3). The same trend was also observed in MDA468 clone number 5 (data not shown). Taken together, these data suggest that HSulf-1 inhibits autocrine activation of the EGF-ERK1/2 signaling cascade in breast cancer cells, potentially involving heparin-binding growth factors.

**Autocrine Phosphorylation of ERK1/2 in MDA468 Is Mediated by Amphiregulin and Heparin-binding EGF-like Growth Factor**—To identify the specific growth factors involved in this autocrine signaling, neutralization antibodies against three growth factors of EGF superfamily (amphiregulin, HB-EGF, or EGF) were added to serum-free medium, and their ability to inhibit autocrine ERK1/2 phosphorylation was investigated in the vector-transfected clone. When the cells were treated with neutralization antibodies against amphiregulin or HB-EGF, a significant inhibition (~50%) of autocrine activation of ERK1/2 was observed (Fig. 4, A and B). These data suggest that both amphiregulin and HB-EGF, the heparin-binding growth factors
of the EGF superfamily, stimulate autocrine signaling induced by serum withdrawal in MDA468. By contrast, the neutralization antibody against EGF enhanced the autocrine ERK phosphorylation (Fig. 4, A and B). Deprivation of EGF might be a key event for the initiation of autocrine signaling mediated by amphiregulin and HB-EGF. Quantification of amphiregulin, HB-EGF, and EGF in the conditioned medium from the vector-transfected and HSulf-1-expressing clones by enzyme-linked immunosorbent assay indicated no differences in the ligand levels between these clones (Fig. 4 C). Collectively, these data suggest that HSulf-1 inhibits the autocrine EGFR-ERK1/2 signaling triggered by amphiregulin and HB-EGF in MDA468 cells without affecting their expression level.

**HSulf-1 Inhibits Autocrine Cyclin D1 Expression, S Phase Entry, and the Cell Survival under Serum-free Conditions**—Because activation of the EGFR-ERK1/2 signaling cascade leads to mitogenic response, we next investigated the consequences of HSulf-1-mediated inhibition of autocrine activation on cell proliferation and survival under serum-free conditions. In the vector-transfected clone, cyclin D1 expression was down-regulated to a very low level in the first 6 h and remained so up to 12 h (Fig. 5). By contrast, in the HSulf-1 stable clone numbers 4 and 5, cyclin D1 protein was down-regulated to an undetectable level in the first 6 h and remained undetectable up to 12 h (Fig. 5). Cell cycle analysis at 72 h after serum withdrawal revealed a reduction in the S phase fraction in HSulf-1 clone numbers 4 and 5 (33.1 ± 0.6 and 36.8 ± 0.5%, respectively) compared with the vector-transfected clone (40.7 ± 0.5%) (Fig. 6 A and Table 1). HSulf-1 clone numbers 4 and 5 also showed an increase in G2–M phase (29.5 ± 0.9% and 21.8 ± 1.1%, respectively) compared with the vector-transfected clone (14.1 ± 0.4%). In addition, quantification of cell death by trypan blue exclusion following a 4-day serum withdrawal showed a significantly higher rate of cell death in HSulf-1 clones (~30%) compared with the vector-transfected clone (15%) (Fig. 6B). The mode of cell death

**FIGURE 3.** Knockdown of HSulf-1 by shRNA promoted autocrine phosphorylation of ERK in MDA468 stable clone number 4. A, MDA468 stable clone number 4 (CL4) was untreated (UT) or transduced virally with pSuperRetro empty vector (Vec) or HSulf-1 shRNA constructs (HS1si) for 48 h as described under “Experimental Procedures.” The knockdown of HSulf-1 was confirmed by RT-PCR. The lower panel shows GAPDH control. B, following HSulf-1 knockdown, the transduced cells were incubated in serum-free medium for 2 h and phosphorylation of ERK1/2 detected by Western blot. The immunoblot for total ERK1/2 shows the equal loading. C, quantification of the phospho-ERK1/2 levels by image analysis. *, p < 0.05 versus vector-transduced control.

**FIGURE 4.** Identification of amphiregulin and HB-EGF as the heparin-binding growth factors involved in autocrine activation of ERK in MDA468 cells. A, neutralization antibodies against amphiregulin (AR) and HB-EGF (but not EGF) inhibit autocrine ERK1/2 phosphorylation in the vector-transfected (Vec) clone. The cells were incubated in serum-free medium containing the indicated neutralization antibody for 2 h and the cell lysates used for immunoblotting. B, a quantification of the phospho-ERK1/2 levels by image analysis. *, p < 0.05 versus untreated control. C, the levels of amphiregulin, HB-EGF, and EGF secreted in the medium 2 h after serum withdrawal was determined by enzyme-linked immunosorbent assay.

**FIGURE 5.** HSulf-1 inhibits autocrine cyclin D1 expression under serum-free conditions. A, HSulf-1 inhibits autocrine cyclin D1 expression. The cells were serum-starved for the indicated time and the levels of cyclin D1 determined by immunoblotting. Note that MDA468 cells do not express cyclin D2 (28). B, a quantification of the cyclin D1 levels by image analysis. *, p < 0.05 versus HSulf-1 clones. Vec, vector.
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A.

FIGURE 6. HSulf-1 inhibits autocrine S phase entry and the cell survival under serum-free conditions. A, cell cycle analysis of the MDA468 clones after 72 h of serum withdrawal. B, quantification of cell death by trypan blue exclusion assay after 96 h of serum withdrawal. * p < 0.05 versus vector-transfected (Vec) control.

TABLE 1

Cell cycle analysis of MDA468 stable clones after 72 h of serum withdrawal

| Cell cycle | Vector | Clone 4 | Clone 5 |
|------------|--------|---------|---------|
| G0–G1      | 45.2 ± 0.9 | 37.4 ± 0.3 | 41.3 ± 0.7 |
| S          | 40.7 ± 0.5 | 33.1 ± 0.6 | 36.8 ± 0.5 |
| G2–M       | 14.1 ± 0.4 | 29.5 ± 0.9 | 21.8 ± 1.1 |

primary breast tumor samples (96 invasive ductal and 17 lobular carcinomas with varying grades and stages) of the patients treated at the Mayo Clinic were used in this study. The overall median age of the patients analyzed in this study was 60.4 years. HSulf-1 expression level was determined in 111 samples by RNA in situ hybridization (Fig. 8), and the intensity was scored from 1–2 (low) to 3–4 (high) by three independent scientists blinded to the clinical data. Our analysis indicated that 59.5% (66/111) of breast tumors had loss and/or lower levels of HSulf-1 expression (Table 2). There was a significant association between low HSulf-1 mRNA levels and low grade breast tumors (p = 0.0001, Table 2). Lower levels of HSulf-1 expression were detected in 87.5% (28/32) of low grade (grades 1 and 2) breast tumors compared with 48.1% (38/79) high grade (grades 3 and 4) breast tumors. Among the two histological subtypes on the TMA, 88.2% (15/17) of the invasive lobular carcinoma compared with 53.8% (50/93) of the invasive ductal carcinoma showed loss of HSulf-1 expression (p = 0.0076, Table 2). There was also a trend toward lower HSulf-1 expression levels in ER-positive (65.8%, 52/79) compared with ER-negative (44.4%, 12/27) tumors (p = 0.0682, Table 2). HSulf-1 expression level showed no significant association between the lymph node status and length to recurrence (Table 2). Statistical analysis of association between tumor histology and clinical data indicated that 64.7% (11/17) of invasive lobular breast carcinoma were low grade compared with 21.9% (21/96) of invasive ductal carcinoma (p = 0.0008, data not shown). In addition, 100% (0/17) of invasive lobular breast carcinomas were ER-positive compared with 70.3% (27/91) of invasive ductal carcinoma (p = 0.0059, data not shown). These data indicate that HSulf-1 expression is down-regulated in the majority of tumors with a predominant association with lobular histology.

DISCUSSION

We have shown previously that the loss of HSulf-1 in cancer cells results in an increase in HS glycosaminoglycan sulfation, promoting various heparin-binding growth factor activities to induce the MAPK signaling pathway (13–15). Our recent study using the MDA468 breast cancer cell line also demonstrates that HSulf-1 inhibits tumorigenesis and angiogenesis in vivo (17). During analysis of growth factor signaling in the MDA468 cell proliferation and ERK1/2 phosphorylation stimulated by amphiregulin and HB-EGF.

Loss of HSulf-1 Expression Is Associated with Low Grade Invasive Lobular Breast Cancers—To investigate the clinical significance of HSulf-1 down-regulation in breast cancer, the potential association between the HSulf-1 expression level and clinicopathological parameters (such as tumor histology, grade, and hormone receptor status) and the presence of lymph node and recurrent disease was analyzed in breast cancer TMA. A TMA containing a total of 113
stable clones expressing HSulf-1, we found that the HSulf-1 clones had diminished autocrine activation of EGFR-ERK1/2 signaling induced by serum withdrawal (Fig. 1). The inhibition of autocrine ERK phosphorylation was also observed in MDA468 and BT474 cells transiently transduced with the adeno-associated viral construct of HSulf-1 (Fig. 2), indicating that the above observations are not cell line-specific or due to clonal variations. In addition, knockdown of HSulf-1 expression by RNA interference promoted autocrine phosphorylation of ERK1/2 (Fig. 3), further lending support to the role of HSulf-1 in autocrine signaling. A more detailed analysis of this autocrine signaling indicated that the heparin-binding growth factors of the EGF superfamily, amphiregulin and HB-EGF, are involved in this process (Fig. 4). The autocrine activation of EGFR-ERK1/2 signaling by amphiregulin upon serum withdrawal has also been described in normal keratinocytes (20). Numerous reports have provided evidence that EGFR and amphiregulin play important roles in breast cancer pathology. The levels of amphiregulin in invasive breast tumors are increased following the tumor stage and grade (10, 21). Ma et al. (11) also demonstrated that an autocrine loop by overexpressed amphiregulin and EGFR is involved in the tumorigenic potential of transformed breast epithelial cell xenografts and that disrupting this autocrine loop with antisense-mediated down-regulation of either amphiregulin or EGFR is effective in inhibiting tumor growth (12). Our present study demonstrating that down-regulation of HSulf-1 augments amphiregulin-EGFR autocrine signaling without affecting the ligand levels provides a novel mechanism of acquired autocrine signaling in breast cancer. Furthermore, the inhibition of autocrine EGFR-ERK1/2 signaling by HSulf-1 was associated with diminished expression of cyclin D1 (Fig. 5), reduced S phase, and increased G2–M fractions (Fig. 6 and Table 1). This is consistent with a previous report by Yin et al. (22) demonstrating that inhibition of EGFR by apigenin reduced ERK phosphorylation and induced G2–M arrest in MCF-7 and MDA468 cells. A delayed G2–M transition by dominant negative MAPKK1 was also reported in fibroblasts (23), further supporting our observation. HSulf-1 clones incubated in serum-free medium showed more necrotic cell death compared with the vector-transfected control (Fig. 6), consistent with our previous observation that tumor xenografts of the HSulf-1 clones showed reduced tumorigenesis associated with enhanced necrosis (17). In addition, the mitogenic activity of exogenous amphiregulin and HB-EGF was also diminished in the HSulf-1 clones (Fig. 7), suggesting that

### FIGURE 7. Mitogenic activity of exogenous amphiregulin and HB-EGF but not EGF was inhibited by HSulf-1 in MDA468. A, MDA468 stable clones were serum-starved overnight and stimulated with 0.5 ng/ml amphiregulin or EGF for the indicated times. Whole cell lysates were subjected to immunoblot for total and phospho-ERK1/2. Vec, vector. B, a quantification of amphiregulin-induced phospho-ERK1/2 levels by image analysis. *, \( p < 0.05 \) versus HSulf-1 clones; **, \( p < 0.001 \) versus HSulf-1 clones. C, a quantification of EGF-induced phospho-ERK1/2 levels by image analysis. D, cell proliferation induced by exogenous amphiregulin, HB-EGF, or EGF was monitored by metabolic reduction of Alamar Blue. *, \( p < 0.05 \) versus HSulf-1 clones. AR, amphiregulin; FU, fluorescence units.

### FIGURE 8. Detection of HSulf-1 mRNA expression in breast cancer tissue microarray by RNA in situ hybridization. A, representative images of breast cancer tissue specimen expressing different levels of HSulf-1 mRNA. B, a representative tumor on the tissue microarray stained with the sense and antisense probes. The absence of staining in the sense-probed section indicates the specificity of the hybridization.
HSulf-1 down-regulation augments both the autocrine and paracrine signaling of heparin-binding growth factors. Finally, an analysis of association between HSulf-1 expression and clinicopathological parameters in breast tumor samples demonstrated that HSulf-1 expression is down-regulated in the majority of tumors with a predominant association with lobular histology (Table 2), which is often associated with better clinical parameters such as tumor grades and positive ER expression (24).

The association between HSulf-1 down-regulation and lobular histology may explain why this histological subtype does not show a better clinical outcome than the invasive ductal subtype, despite the various favorable biologic phenotypes. Arpino et al. (24) have demonstrated that invasive lobular carcinoma is more likely to be estrogen and progesterone receptor-positive, lower in S phase fraction, and to be more diploid, HER-2-, p53-, and EGFR-negative than invasive ductal carcinoma, yet show no advantage in clinical outcome. Our data suggest that this histological subtype might have an enhanced autocrine amphiregulin and/or HB-EGF signaling because of HSulf-1 down-regulation, providing a potential mechanism that makes lobular carcinomas more aggressive than predicted based on classical biomarkers. In addition, Bottini et al. (25) have demonstrated that low dose metronomic cyclophosphamide therapy, which inhibits tumor angiogenesis and therefore limits nutrition supply, is beneficial for ductal but not lobular carcinoma when used in combination with systemic letrozole therapy. This seems to be consistent with our data implicating that HSulf-1 down-regulation in invasive lobular carcinomas might confer resistance to cell death under low nutrition conditions similar to the in vitro setup of serum withdrawal. This might also suggest that the loss of HSulf-1 could serve as a biomarker to predict the response to anti-angiogenic therapy.

In addition, elevated EGFR signaling has also been implicated in acquired drug resistance to systemic hormone therapy in breast cancer, emerging as a rationale for the effective combination of endocrine hormone therapy and EGFR-specific tyrosine kinase inhibitors in breast tumors (26, 27). Our present data suggest that HSulf-1 could serve as a potential molecular target to inhibit EGFR signaling in low grade breast cancer. Our previous studies (13–15) demonstrate that HSulf-1 sensitizes cancer cells to chemotherapeutic agents. Introduction of HSulf-1 activity by enzyme replacement- or gene therapy–based approaches coupled to conventional chemotherapy may have added benefit in the treatment of breast cancer.

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TABLE 2
Association between breast characteristics and HSulf-1 expression

| Clinical parameters | HSulf-1 expression | $p$ value |
|---------------------|--------------------|----------|
|                     | Absent-low (1–2)   | Moderate-high (3–4) |
| Patients (n)        | 66                  | 45       | 0.1199 |
| Age (years; means ± SD) | 61.9 ± 13.2          | 58.0 ± 12.6          |
| Grade               |                     |           |
| Number with data    | 66                  | 45       | 0.0001 |
| Low (1–2)           | 28 (87.5%)          | 4 (12.5%) |
| High (3–4)          | 38 (48.1%)          | 41 (51.9%) |
| Histology           |                     |           |
| Number with data    | 65                  | 45       | 0.0076 |
| Ductal              | 50 (53.8%)          | 43 (46.2%) |
| Lobular             | 15 (88.2%)          | 2 (11.8%) |
| Estrogen receptor   |                     |           |
| Number with data    | 64                  | 42       | 0.0682 |
| Negative            | 12 (44.4%)          | 15 (55.6%) |
| Positive            | 52 (65.6%)          | 27 (34.2%) |
| Lymph node status   |                     |           |
| Number with data    | 66                  | 44       | 0.6821 |
| Negative            | 46 (61.3%)          | 29 (38.7%) |
| Positive            | 20 (57.1%)          | 15 (42.9%) |
| Recurrent disease   |                     |           |
| Number with data    | 65                  | 45       | 0.1509 |
| Negative            | 47 (64.4%)          | 26 (35.6%) |
| Positive            | 18 (48.6%)          | 19 (51.4%) |
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