SHON Is a Novel Estrogen-Regulated Oncogene in Mammary Carcinoma That Predicts Patient Response to Endocrine Therapy

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Abstract

Endocrine therapies are the primary systemic intervention for patients with estrogen receptor–positive (ER⁺) breast cancer. However, a significant proportion of initially responsive ER⁺ tumors develop resistance, with relapses occurring in up to 50% of patients. Lack of reliable predictive biomarkers remains an unmet need for enhanced clinical management of this disease. In this study, we address this need in identifying a novel estrogen-regulated gene called SHON (secreted hominoid-specific oncogene). Enforced expression of SHON in breast cancer cells increased their proliferation, survival, migration, and invasion in vitro. Furthermore, SHON enhanced the oncogenicity of these cells in xenograft models of human breast cancer and was also sufficient to oncogenically transform MCF10A human mammary epithelial cells. Conversely, SHON attenuation mediated by RNA interference- or antibody-based methods reduced the oncogenicity of breast cancer cells. Mechanistic investigations indicated that the oncogenic transforming properties of SHON were mediated by BCL-2 and NF-kB. In primary clinical specimens, SHON was immunohistochemically detected in 62% of breast cancers, in which its expression was positively correlated with ER expression. In this setting, SHON expression predicted a favorable response to endocrine therapy in high-risk patients with ER⁺ breast cancer. Taken together, our findings identify SHON as a novel human oncogene with predictive utility in ER⁺ breast cancer, perhaps offering a simple biomarker to predict the therapeutic efficacy of antiestrogen therapy in patients with breast cancer. Cancer Res; 73(23); 6951–62. ©2013 AACR.

Introduction

Breast cancer is the most prevalent cancer in females worldwide. Estrogen signaling and estrogen receptors (ER) play a pivotal role in the initiation and progression of breast cancer, with the majority of human breast cancer being initially estrogen dependent. In accepted models of ER function, estrogen binding to either ERα or ERβ results in dimerization and conformational change (1). The resulting estrogen–ER complex modulates gene transcription by binding to estrogen response elements in DNA, or by interaction with other transcription factors such as specificity protein 1 (SP1), activating protein 1 (AP-1), or NF-κB (2). ER signaling can also be modulated by other coregulatory proteins or through genomic and extranuclear actions (3, 4). Expression arrays have identified early and late estrogen responsive genes (5), including genes involved in cancer cell proliferation and survival, enhancing our understanding of the molecular events involved in estrogen action.

Antiestrogens remain the most effective form of systemic intervention for patients with ER-positive (ER⁺) tumors, which account for approximately 75% of breast cancers. Current approaches include the use of selective ER modulators (SERM; e.g., tamoxifen) that compete with estrogen for ER binding or aromatase inhibitors (e.g., letrozole, anastrozole, and exemestane), which inhibit the synthesis of estrogen. ERα, the currently used predictive biomarker for antiestrogen responsiveness, is not consistently accurate in predicting response due to de novo or acquired tumor resistance. Although a significant high proportion of ER⁺ breast tumors initially respond to antiestrogens, resistance...
eventually develops, and up to 40% to 50% of patients with ER\(^+\) tumors relapse (6–9), through molecular mechanisms that are yet to be fully understood. These tumors typically continue to express ER\(_x\) (10) and demonstrate earlier metastatic recurrence (11). The expression of ER\(_x\)-regulated progesterone receptor (PR) and BCL-2 genes is associated with improved survival of patients with breast cancer (12, 13) and serves as a favorable prognostic marker for endocrine therapy (12, 14). However, there is still no definitive methodology to distinguish ER\(_x\) tumors that will or will not respond to endocrine therapy.

There appears to be as few as 168 human-specific genes among the approximately 23,000 or so genes identified from the completion of the human genome project (15). When expanded to include primate lineages, there may be only several hundred unique hominoid genes. The identification of such human/hominoid-specific genes and analysis of their molecular functions might increase the understanding of the contributions these genes likely make to human disease processes. Studies have demonstrated that some human/hominoid-specific genes and their regulated signaling pathways may serve as biomarkers in human disease (16) and a number of human/hominoid-specific genes, such as Tre2 (17) and TBC1D3 (18–20), have been linked to human cancer. However, only a few proteins translated from human/hominoid-specific genes have been studied and the functions of many others remain as yet uncharacterized.

Here, we report the identification of a novel estrogen regulated oncogene, secreted hominoid-specific oncogene (SHON) in mammary carcinoma and determine the predictive significance of SHON expression for response to endocrine therapy in high-risk patients with ER\(^+\) breast cancer.

Materials and Methods

Cell lines

All cell lines used in this study were obtained from the American Type Culture Collection and were cultured in conditions as recommended except the MCF-7 cell line that was cultured in RPMI 1640 supplemented with 10% heat-inactivated FBS, 100 U/mL penicillin, 100 \(\mu\)g/mL streptomycin, and 2 mmol/L L-glutamine in a 37°C humidified incubator with 5% CO\(_2\).

Plasmid constructs

PIKR2786 (Genentech UNQ ID: UNQ2786) represented by an expressed sequence tag (EST; GenBank AY358103) was originally identified, through bioinformatic analysis, as a potential secreted or transmembrane protein (21). We have demonstrated that UNQ2786 is a secreted protein and is a human mammary epithelial oncogene and have named it as SHON. The human SHON-expressing plasmids were constructed by using a protocol previously described (22). The Bcl-2 P1 promoter reporter plasmid (23) and the NF-kB reporter plasmid (24) were previously described.

Tumor xenografts in nude mice

The experimental protocols followed are described in the Supplementary Materials and Methods.

Clinical samples, patient data analysis, and ethics statement

This study was approved by the Nottingham Research Ethics Committee 2. Immunohistochemical (IHC) staining was carried out using a cohort of breast tissue specimens from Nottingham, United Kingdom (25, 26). Detailed patient demographics and clinicopathologic characteristics and methodology about these samples can be found in the Supplementary Materials and Methods.

A full description of the materials and methods used for this work is described in Supplementary Materials and Methods.

Results

Expression of SHON in human normal tissues and carcinoma cell lines

Sequence homology searches using National Center for Biotechnology Information (NCBI) basic local alignment search tool (BLAST) revealed that SHON belongs to a hominoid-specific gene family with no known orthologs outside the primate lineage. SHON contains a predicted open reading frame of 282 bp encoding a 93-amino acid residue protein with a predicted molecular mass of 9.7 kDa (Fig. 1A). There is a possibility that an internal disulfide bond is formed between the two cysteines in the mature protein. SHON gene expression was detected in all 48 human tissues in a commercially available panel of cDNAs after 40 cycles of PCR amplification (Fig. 1B). SHON mRNA was also expressed in all cancer cell lines tested (Fig. 1C, top).

A rabbit polyclonal antibody against the C-terminal of the predicted mature SHON protein was produced and its specificity was validated (Supplementary Figs. S1 and S2). A specific band of the approximate expected size (12 kDa) was detected in Western blot analysis in all cancer cell lines tested (Fig. 1C, bottom). However, no SHON protein expression was detected in the normal human mammary epithelial cell line, MCF10A (Supplementary Fig. S2D).

SHON is a secreted protein

SHON is predicted to be a secreted protein with a leading signal peptide encompassing amino acid residues 1 to 21 by SignalP 3.0 (Fig. 1A; ref. 27). HIS-tagged SHON protein can be readily detected in both the whole cell lysate and the conditioned media by Western blotting (Fig. 1D).

Forced expression of SHON increases mammary carcinoma cell growth

MCF-7 cells were stably transfected with the SHON expression plasmid pRESneo3-SHON (designated MCF7-SHON) or with the empty vector pRESneo3 (MCF7-Vec; Fig. 2A). Forced expression of SHON also resulted in increased SHON protein in conditioned media (Fig. 2A).

Forced expression of SHON in MCF-7 cells significantly increased cell number under serum-replete (10% FBS: Fig. 2B) and under serum-reduced (0.2% FBS) conditions (Fig. 2C). Forced expression of SHON in MCF-7 cells not only decreased apoptotic cell death (Fig. 2D), but also increased entry into S-phase (Fig. 2E).
Forced expression of SHON promotes anchorage-independent growth

Forced expression of SHON in MCF-7 cells increased the total cell number in suspension culture relative to the control cell line, MCF7-Vec, over 8 days (Fig. 2F). In soft agar, forced expression of SHON increased the number of colonies formed compared with MCF7-Vec cells (Fig. 2G).

Forced expression of SHON enhances cell migration and invasion

SHON increased MCF-7 cell migration in MCF7-SHON cells versus the control cell line MCF7-Vec (Fig. 2H). The invasive capacity of MCF7-SHON cells over 48 hours was enhanced compared with the control cell line MCF7-Vec (Fig. 2H). Complete wound healing occurred within 72 hours for the MCF7-SHON cell line, but not in the MCF7-Vec cell line (Fig. 2I). Forced expression of SHON therefore enhanced the motility and invasive capacity of MCF-7 cells.

MCF7-Vec cells grew in clumps with prominent cell-to-cell contacts in 2D and 3D cultures, typical of an epithelial phenotype, whereas MCF7-SHON cells were scattered, displayed little intercellular contacts, and formed long cellular protrusions and filopodia, indicating an invasive phenotype (Fig. 2J).

SHON-stimulated oncogenicity in mammary carcinoma cells is mediated by BCL-2 and NF-κB

Quantitative PCR was performed to examine the effect of forced expression of SHON on the relative expression levels of key genes involved in cell proliferation, survival, oncogenic transformation and growth (Supplementary Table S3). Most notably SHON increased BCL-2 expression by 10.16-fold. SHON also regulated the expression of signaling molecules from several key signal transduction pathways such as NFKB1 (encodes NF-xB p105) and NFKB1A (encodes IkBα) from the NF-xB pathway, AKT1 (encodes AKT/PKB) and PIK3R1 [encodes phosphoinositide 3-kinase (PI3K) regulatory subunit...
We conducted further analyses to elucidate the roles of BCL-2 and NF-κB in SHON-mediated oncogenicity. Significantly higher luciferase activity of the Bcl-2 gene P1 promoter was observed in MCF7-SHON cells than in the MCF7-Vec control cells (Fig. 3A), indicating that SHON expression increased the transcriptional activation of the Bcl-2 gene. SHON expression also resulted in increased BCL-2 protein expression in MCF-7 cells relative to the control (Fig. 3B).

Figure 2. Forced expression of SHON increases the oncogenicity of mammary carcinoma cells. A, SHON expression in MCF-7 cells stably transfected with SHON-expressing plasmid (MCF7-SHON) or the control empty vector (MCF7-Vec) was determined by RT-PCR and Western blot (WB) analysis. B and C, total cell number assays. MCF7-SHON (SHON) and MCF7-Vec (Vector) cells were seeded with 10% FBS (Serum; B) or low serum (0.2%; Serum free; C). Cell number was determined at the indicated days. D, serum starvation-induced apoptosis was assessed with fluorescein isothiocyanate–conjugated Annexin V and propidium iodide. Apoptotic cells at both early (Annexin V-positive and propidium iodide-negative) and late (Annexin V- and propidium iodide-positive) stages are presented with the percentage. E, S-phase entry was assessed by BrdUrd incorporation assays. F, suspension culture. Cells were grown on the poly-HEMA–coated plates and cell number was determined at the indicated days. G, soft agar assays. Cells were seeded in 0.35% agarose and colonies formed were counted after incubation for 14 days. H, cell migration and invasion were determined by use of Transwell inserts as described in Materials and Methods. I, wound healing assays. Photographs were taken at the time indicated. Bar, 100 μm. J, morphology of stable MCF7-SHON (SHON) and MCF7-Vec (Vector) cells was examined in two-dimensional (2D) cultures and three-dimensional (3D) cultures in 4% growth factor–reduced Matrigel by visualization of actin filaments using fluorescein isothiocyanate–phalloidin labeling (green) and cell nuclei were stained with Hoechst 33258 (blue). Bar, 10 μm. **, P < 0.01; ***, P < 0.001.
Figure 3. Signaling pathways via which SHON mediates oncogenicity in MCF-7 cells. A, Bcl-2 P1 promoter reporter or NF-κB activation reporter plasmid, together with a β-galactosidase expression plasmid as a transfection efficiency control, were cotransfected into MCF7-Vec (Vector) and MCF7-SHON (SHON) stable cells. Luciferase activities were measured with a kit from Promega and normalized for β-galactosidase activity. B, Western blot analyses of key signaling molecules as indicated on the left with P representing the phosphorylated form. β-Actin was used as a cell lysate input control. C and D, soft agar colony formation assays of MCF7-Vec (Vector) and MCF7-SHON (SHON) stable cells treated with vehicle DMSO (Veh), BCL-2 inhibitor YC137 (C), or NF-κB inhibitor BMS-34554 (D) with indicated concentrations. The colony viability was measured by MTT assays. E and F, 3D Matrigel growth assays. MCF7-Vec (Vector) and MCF7-SHON (SHON) stable cells were grown in 4% growth factor–reduced Matrigel and treated with vehicle DMSO (Veh) or with 1 µmol/L of YC137 (E) or 10 µmol/L of BMS-34554 (F). The cell growth was measured using alamarBlue. G, nuclear translocation of NF-κB subunit p65. MCF7-Vec (Vector) and MCF7-SHON (SHON) stable cells were grown and treated with or without 10 ng/mL of TNF-α for 20 minutes before fixation. Cells were then prepared for immunostaining with a fluorescein isothiocyanate–conjugated p65 antibody to detect p65 (top; green) and Hoechst 33258 to delineate the nuclei of each cell (middle; blue). Bar, 10 μm; *, P < 0.05; **, P < 0.01; ***, P < 0.001.
inhibitor (28). YC137 not only reduced the colony formation of both MCF7-Vec and SHON cells in a dose-dependent fashion, but also largely attenuated the SHON-stimulated enhancement of the anchorage-independent growth of MCF7-SHON cells, indicative of BCL-2 dependence of SHON-stimulated soft agar colony formation (Fig. 3C). Furthermore, YC137 also largely abrogated the SHON-stimulated enhancement of MCF7-SHON cell growth in 3D Matrigel although significant inhibition of MCF7-Vec cell growth was also observed as expected (Fig. 3E).

SHON expression significantly increased NF-κB luciferase reporter activity (Fig. 3A), indicating the activation of NF-κB pathways. The NF-κB pathways include classic and alternative pathways using NF-κB precursor proteins, p105 (NF-κB1) and p100 (NF-κB2), respectively. SHON increased the expression of NF-κB1 (both p50 and its precursor p105) as well as its phosphorylated form (phospho-p105), but did not affect p100 (NF-κB2) expression (Fig. 3B), indicating SHON activated the classic NF-κB pathway. Activation of the classic pathway involves the release of p50–p65 (Rel-A) from the inhibitory protein IκB, as a result of phosphorylation of IκB by IκB kinase and degradation of IκB by the proteasome. SHON increased the expression of p65 and phospho-p65 (Fig. 3B). Activation of NF-κB is achieved primarily through the proteolytic degradation of IκB triggered by IκB phosphorylation at specific N-terminal residues. SHON increased the level of phospho-IκB although total IκB expression remained unchanged (Fig. 3B). NF-κB resides in the cytoplasm in an inactive form and once activated following IκB degradation, the NF-κB complex undergoes translocation to the nucleus. SHON expression resulted in a constitutive p65 translocation into the nuclei in MCF7-SHON cells, whereas a predominant cytoplasmic p65 localization was observed in the control cell line, MCF7-Vec (Fig. 3G). Both Akt and p44/42 (ERK1/2) mitogen-activated protein kinases (MAPK) play a crucial role in the activation of NF-κB (29, 30). SHON expression significantly increased the expression of total Akt and phosphorylated Akt (phospho-AKT) as well as total p44/42 and phosphorylated p44/42 (phospho-p44/42) MAPK (Fig. 3B).

BMS-345541 is an NF-κB–specific inhibitor and inhibits IκB kinase thereby blocking NF-κB activation (31). BMS-345541 demonstrated a dose-dependent inhibition of colony formation in soft agar in both MCF7-Vec and MCF7-SHON cells (Fig. 3D). At a concentration of 10 μmol/L, BMS-345541 largely abrogated the SHON-induced increase in colony formation of MCF7-SHON cells in soft agar (Fig. 3D) and the SHON-induced increase in MCF7-SHON cell growth in 3D Matrigel (Fig. 3F), compared with the control cell line, MCF7-Vec.

**SHON transforms the normal human breast epithelial cell**

We stably expressed SHON in MCF10A cells (Fig. 4A). Compared with the control cell line MCF10A-Vector, SHON-overexpressing MCF10A-SHON cells displayed a more scattered morphology with reduced cell-to-cell contact (Supplementary Fig. S3). When cultured in 3D Matrigel, MCF10A-Vector cells formed typical acini consisting of regular spherical structures with a hollow lumen. In contrast, MCF10A-SHON cells formed large disorganized multicellular structures with filled lumina (Fig. 4B). Furthermore, SHON dramatically enhanced anchorage-independent growth as indicated by colony formation in soft agar (Fig. 4C).

** Forced expression of SHON promotes MCF-7 tumor xenograft growth in vivo**

In a tumor xenograft mouse model, both MCF7-SHON and MCF7-Vec cells formed palpable and measurable tumors, but MCF7-SHON cells formed faster growing and larger tumors compared with the MCF7-Vec cells (Fig. 4D). Statistically significant enhancement of tumor volume was achieved after 1.5 weeks, and tumors formed by MCF7-SHON cells were larger in size by 36.0% compared with tumors formed by MCF7-Vec cells after 6.5 weeks. Tumors formed by MCF7-Vec cells were well differentiated and cellular, whereas those formed by MCF7-SHON cells were poorly differentiated and diffuse (Fig. 4E). The MCF7-SHON tumors exhibited a higher percentage of bromodeoxyuridine (BrdUrd)-labeled nuclei (Fig. 4F) and few terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL)–positive cells compared with the MCF7-Vec tumors (Fig. 4G).

**SHON mRNA is expressed in normal and cancerous breast tissues**

**SHON mRNA** was expressed in both normal and breast cancer tissues in a commercially available Breast Cancer cDNA Array (Fig. 5A). However, an increased SHON mRNA expression was observed in cancer tissues compared with the normal breast tissues. Moreover, the expression level of SHON mRNA was positively correlated with higher cancer stages (Fig. 5A, bottom).

**SHON expression is strongly correlated with hormone receptor status and BCL-2 expression in breast cancer tissues**

We next performed a large-scale IHC analysis of SHON protein in mammary carcinoma using tissue microarrays (25, 26). A total of 1,237 tumors were suitable for analysis of SHON expression. Sixty-two percent (766 of 1,237) of the tumors were SHON positive (Supplementary Table S5). The associations of SHON expression in breast cancer with clinicopathologic features are presented in Supplementary Table S7. Notably, SHON expression was highly positively correlated with hormone receptor status, i.e., ER+/PR+ and AR+ (androgen receptor–positive), and BCL-2 expression, whereas SHON was negatively correlated with both EGFR and HER2 overexpression and the triple-negative phenotype.

**Prognostic significance of SHON expression for response to endocrine therapy in ER+ high-risk breast cancer patients**

Multivariate Cox regression analysis revealed that SHON was an independent predictor for clinical outcome in the studied cohort of patients with breast cancer (Supplementary Table S8). Moreover, SHON protein expression was
found to be a prognostic marker for response to endocrine therapy for patients whose tumors were ER$^+$ and categorized as high risk (Nottingham Prognostic Index $C3/3.4$).

Endocrine therapy–treated patients with tumors negative for SHON expression had a 2-fold increase in risk of death [HR, $2.1$; 95% confidence interval (CI), $1.4–3.1$; $P < 0.0001$], recurrence (HR, $1.9$; 95% CI, $1.4–2.6$; $P < 0.0001$), and distant metastasis (HR, $1.8$; 95% CI, $1.2–2.5$; $P = 0.007$) at 10 years compared with patients whose tumors were positive for SHON expression (Fig. 5B). SHON expression was not significantly related to disease-free survival in the ER$^+$/C0 patient cohort with or without anthracycline treatment (Supplementary Fig. S7).

**SHON is an estrogen inducible gene**

E2 treatment of ER$^+$ MCF-7 cells resulted in increased SHON mRNA and protein expression (Supplementary Fig. S8A and S8B). Upon E2 treatment, a synergistic increase in cell number was observed in MCF7-SHON cells compared with MCF-Vec cells, whereas the ER pure antagonist ICI 182780 partially attenuated SHON-stimulated growth advantages in MCF-7 cells, indicating that SHON signaling is at least in part mediated by ER (Supplementary Fig. S8C).

**Depletion of endogenous SHON by siRNA significantly inhibits cell growth, anchorage-independent growth, and cell migration and invasion in MCF-7 cells**

A stable MCF-7 cell line (MCF7-siRNA) in which endogenous SHON expression was depleted by a SHON-specific siRNA plasmid and a control cell line (MCF7-CK) stably transfected by the negative control siRNA plasmid were established (Fig. 6A). SHON depletion also reduced SHON protein secreted into conditioned media. Depletion of endogenous SHON decreased MCF-7 cell number in serum-replete conditions (Fig. 6B) and significantly increased apoptotic cell death induced by serum deprivation (Fig. 6C).
Depletion of SHON significantly reduced the number of colonies formed in soft agar (Fig. 6D) and cell migration/invasion (Fig. 6E). SHON-depleted MCF7-siRNA cells also displayed a significant reduction in wound-induced migration (Fig. 6F). Moreover, the colonies formed by MCF7-siRNA cells in 3D Matrigel were much smaller than the MCF7-CK cells (Supplementary Fig. S9).

Concordantly, depletion of endogenous SHON in MCF-7 cells significantly decreased BCL-2 gene transcription and protein expression (Supplementary Fig. S10). Moreover, the transcriptional activity of NF-κB was decreased as a result of SHON depletion in MCF-7 cells (Supplementary Fig. S10A). Consistently, depletion of SHON expression decreased the protein expression of NF-κB1 (p50 and p105), p65, and IκB, as well as the phosphorylation of these key proteins in the NF-κB signaling pathway (Supplementary Fig. S10B).

We also established another SHON-depleted MCF-7 cell line using the second most effective siRNA, which also exhibited similar inhibitory effects on cell growth, anchorage-independent growth, and cell migration/invasion in MCF-7 cells (Supplementary Fig. S11).

Figure 5. SHON mRNA is expressed in normal and cancerous breast tissues and SHON protein expression predicts patient response to endocrine therapy in ER+ breast cancer. A, SHON mRNA in a Breast Cancer cDNA Array (OriGene) was examined by PCR. The array contained 48 samples covering 5-normal, 11-stage I, 8-IIA, 6-IIIB, 8-IIIA, 2-IIIB, 4-IIIC, and 4-IV. β-Actin was included as the cDNA input control. Relative expression of SHON was estimated by densitometric analysis using the ImageJ software (NIH), with β-actin as the normalization control. B, Kaplan-Meier survival curves demonstrating the rates of breast cancer specific survival (BCSS), disease-free survival (DFS), and distant metastasis-free survival (DMFS) of high-risk breast cancer (Nottingham Prognostic Index > 3.4/ER+ patients treated by endocrine therapy. Tumors were stratified into SHON positive (SHON+) versus negative (SHON-) subgroups. P values represent log-rank testing of the difference in survival.
Functional inhibition of SHON in MCF-7 cells using a polyclonal antibody reduces growth in 3D Matrigel and impairs cell invasion

Consistent with the effect of siRNA, a rabbit polyclonal antibody to SHON inhibited MCF-7 cell invasion in a dose-dependent manner (Fig. 6G). The 3D Matrigel growth of both MCF7-Vec and MCF7-SHON was significantly inhibited by the polyclonal antibody (Fig. 6H). Hence, SHON functions as a secreted protein.

Forced expression of SHON increases BCL-2 and NF-κB expression and promotes anchorage-independent growth and growth in 3D Matrigel of T47D cells

Forced expression of SHON in ER⁺ T47D cells resulted in a significant increase in the colony formation in soft agar (Supplementary Fig. S12B) and growth in 3D Matrigel (Supplementary Fig. S12D). Concordantly, depletion of SHON in T47D cells reduced colony formation in soft agar and growth in 3D Matrigel (Supplementary Fig. S12C and S12E). Furthermore, forced expression of SHON in T47D increased BCL-2 expression and activated NF-κB pathways (Supplementary Fig. S13A), which were shown to mediate the anchorage-independent growth of T47D as demonstrated in soft agar assays (Supplementary Fig. S13B and S13C).

Secreted SHON promotes the growth and migration of MCF-7 and T47D cells

As SHON is a secreted protein, coculture assays were used to determine whether SHON could function in a paracrine fashion. As shown in Fig. 7, SHON secreted from MCF7-SHON stable cells promoted growth of both MCF-7 and T47D cells and also served as a chemoattractant to enhance cell migration of both MCF-7 and T47D cells.

Discussion

Our results presented here have demonstrated that SHON is a novel hominoid-specific gene that enhances oncogenicity of mammary carcinoma cell lines. Clinically, expression of SHON...
in tumors could be a predictive biomarker for the response of ER\(^+\) patient to endocrine therapies.

SHON is expressed in both ER\(^-\) and ER\(^+\) breast cancer cell lines. However, we have demonstrated that SHON is an estrogen-regulated gene. A very significant correlation of SHON expression with ER expression was identified in breast cancer tissues and consequently SHON may be an important modulator of ER signaling. ER signal transduction involves a complex web of signaling interactions with other growth factor signaling pathways (32, 33). In addition to the classic genomic action associated with nuclear ER, nongenomic ER action has also been reported: cell membrane or cytoplasm-associated ER action associated with nuclear ER, nongenomic ER action has been reported in various human cancers and consequently SHON may be an important modulator of estrogen signaling. A very significant correlation of SHON expression increased phospho-AKT and phospho-p44/42 MAPK, PI3K/AKT/mTOR pathways and NF-κB transcriptional activation of BCL-2. Indeed, we observed synergistic increases in cell number when cells with forced expression of SHON were concomitantly treated with estrogen.

Acquired resistance to antiestrogens remains a major obstacle for the successful management of ER\(^+\) breast cancer. Several molecular mechanisms have been proposed to be responsible for antiestrogen resistance (7, 10). However, the precise mechanisms remain uncertain. The majority of ER\(^+\) tumors that become antiestrogen resistant retain ER expression (10). Hence, ER-regulated functions have been proposed to be more important. For example, several estrogen-regulated genes such as ESRI, PR, BCL-2, Survivin, SCUBE2, HOXB13, IL17BR, and CHDH have all been associated with endocrine resistance (42, 43). In this study, we have observed that SHON expression is a predictor for responsiveness to antiestrogen treatment in patients with ER\(^+\) tumors. We have demonstrated that SHON is an early estrogen responsive gene, and that SHON expression in breast tumors is positively correlated with ER, PR, and AR status. As both PR and AR are ER-targeted genes and we have shown that SHON is highly correlated to ER\(^-\)/PR\(^+\) subtype of breast cancer, SHON could therefore be an important modulator of estrogen signaling. It is therefore not surprising that SHON expression predicted a favorable outcome for tamoxifen-treated patients with ER\(^+\) breast cancer. BCL-2 is an important promoter of cell survival and is overexpressed in 50% to 70% of solid malignancies, including breast cancer (44). Although BCL-2 facilitates the survival of tumor cells, its expression has been consistently associated with a better prognosis in breast cancer (12). This has been partly attributed to its correlation with favorable prognostic features such as ER/PR positivity (45), low proliferation index, absence of HER2 amplification, and mutant p53 expression (46). We have observed that SHON increased the expression of BCL-2 and its upstream regulator NF-κB in vitro, and SHON expression is significantly and positively associated with BCL-2 expression in breast cancer. Therefore, SHON may exert its predictive value via the enhanced expression of BCL-2/NF-κB. However, the role of SHON and its mechanisms in endocrine therapy response needs further investigation. For example, forced expression of SHON in ER\(^+\) MCF-7 cells increased the transcriptional activation of NF-κB and activated the PI3K/AKT/mTOR pathway. However, increased NF-κB activity has been reported to be associated with antiestrogen resistance and inhibition of NF-κB activity restored the sensitivity of resistant MCF-7 cells to antiestrogens (47). In addition, extensive evidence suggests that the PI3K/AKT/mTOR pathway is linked to resistance to tamoxifen. In ER\(^-\)-positive breast cancers treated with tamoxifen, AKT activation was associated with poor survival (48) and patient relapse (49). In contrast, activation of AKT was reported to be associated with favorable outcomes in non–small cell lung cancer (49). Therefore, whether that compartmentalization of SHON may be important in determining its prognostic effects via activation of AKT requires further investigation.
We have demonstrated that SHON is a secreted protein and that the secreted protein is functional. The antibody neutralization and coculture experiments indicate that SHON can exert autocrine and/or paracrine effects. The paracrine effects may be of some relevance, given that IHC staining revealed both stromal and epithelial expression of SHON. The stroma and the factors that stromal cells secrete have been demonstrated to play pivotal roles in the growth and dissemination of mammary carcinoma. Cytoplasmic and nuclear immunolocalization of SHON also suggests that SHON may exert some cellular functions intracellularly like some other secreted factors such as growth hormone, EGF, and fibroblast growth factors, which are often detected in the nucleus of cancer cells (50). SHON may therefore possess both intercellular and/or intracellular functions. Nonetheless, whether SHON functions through binding to a specific cell surface receptor or serves as an extracellular or intracellular cofactor of other signaling molecules remains to be determined.

In conclusion, our results show that SHON expression is induced by estrogen in breast cancer cells and that SHON mediates the oncogenicity of breast cancer cells by modulating ER signaling through the activation of BCL-2, NF-kB, p44/42 MAPK, and PI3K/AKT/mTOR pathways. We further demonstrate that SHON expression is positively correlated with better survival outcomes for patients with ER+ tumors treated with antiestrogens. These results suggest that pharmacologic modulation of SHON activity, either by siRNA or antibody inhibition, may be clinically useful to treat breast cancer and/or that evaluation of SHON expression may predict the therapeutic effect of antiestrogens in patients with ER+ breast cancer.

Disclosure of Potential Conflicts of Interest

D.-X. Liu, T.M.A. Abdel-Fatah, J.K. Perry, J. Lu, B. Huang, S.Y.T. Chan, A.R. Green, and I.O. Ellis are named inventors on a PCT patent application PCT/ NZ/2013/000188 and patent applications NZ603056 and NZ616981; D.-X. Liu and R.Z. Ma are applicants for the applications PCT/NZ/2013/000188 and NZ616981; and D.-X. Liu is the applicant for the application NZ603056. No potential conflicts of interest were disclosed by the other authors.

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