Low Expression of the Snail Gene is a Good Prognostic Factor in Node-Negative Invasive Ductal Carcinomas

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**Background:** While Snail is a zinc-finger transcription factor that triggers the epithelial-mesenchymal transition, it has also been reported to be indirectly regulated by estrogen receptor α (ERα) and to be involved in the transcriptional repression of the aromatase gene. The aim of the present study was to examine the role of Snail expression in node-negative invasive ductal carcinomas.

**Methods:** We analyzed Snail mRNA expression levels in 86 node-negative invasive ductal carcinomas by real-time quantitative RT–PCR and studied whether Snail mRNA expression correlates with clinicopathological factors.

**Results:** No correlation was found between Snail mRNA expression and ERα protein expression levels. However, we observed that none of the 34 patients showing low Snail mRNA expression developed distant metastasis while 6 of 52 (12%) showing high expression of Snail mRNA did. The level of Snail mRNA expression was not found to be significantly correlated with clinicopathological factors. No inverse correlation was found between the Snail and aromatase mRNA expression levels in our series.

**Conclusion:** Our data show that low expression of Snail mRNA is a good prognostic factor in node-negative invasive ductal carcinomas. Snail expression is suggested to be involved in distant metastasis in node-negative invasive ductal carcinomas.

**Key words:** Snail – estrogen receptor – E-cadherin – breast cancer – aromatase

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**INTRODUCTION**

It is well established that estrogens are important not only for the normal growth and development of mammary glands but also for the initiation and progression of estrogen-dependent breast cancer. The effect of estrogens on breast carcinogenesis is believed to be mediated mainly through estrogen receptor α (ERα). Clinically, the ERα status of breast cancer tissues is widely used both as an indicator for endocrine therapy responsiveness and as a predictor of prognosis. However, ~30% of ERα-positive patients do not respond to endocrine therapy while ~10% of ERα-negative patients do. Thus, ERα status is an imperfect predictor of endocrine therapy response (1). For ERα-positive and node-positive breast cancers, chemotherapy is usually recommended in addition to endocrine therapy. On the other hand, endocrine therapy alone is often recommended for ERα-positive and node-negative breast cancers (2). If it were possible to identify the endocrine therapy-nonresponsive subset of ERα-positive and node-negative patients in advance, chemotherapy could be considered for recommendation to these patients. Unfortunately, at present we have no better predictive markers for endocrine therapy than ERα. It is therefore not easy to select truly endocrine non-responsive patients from all (ERα-positive and node-negative) breast cancer patients.

The Snail gene (GenBank: NM 005985) was identified through the genetic analysis of dorsoventral patterning in Drosophila. Snail zinc-finger protein is thought to be required zygotically for mesoderm formation (3,4). During embryonic development, Snail family members have been implicated in the triggering of epithelial-mesenchymal transition (EMT) in the precursors of the mesoderm and the neural crest, promoting their delamination and subsequent migration from the primitive streak and the neural tube, respectively (5–8). The induction of EMT by Snail is mediated by the direct transcriptional repression of the cell adhesion molecule E-cadherin. Snail has been shown to repress E-cadherin expression and trigger EMT associated with epithelial tumor progression (7,9). E-cadherin expression has been reported to be regulated not only by Snail.

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but also by Slug, SIP1 and E12/E47 (10–12). With respect to Snail, apart from the inverse correlation between its expression and that of E-cadherin, a direct correlation has been observed with the invasive and metastatic properties of mouse and human tumor cell lines such as skin, breast and colon (7,9).

It has recently been reported that MTA3 is an estrogen-dependent component of the Mi-2/NuRD transcriptional corepressor in breast epithelial cells and a key component of an estrogen-dependent pathway regulating growth and differentiation (13). Snail has been shown to be a direct regulatory target of MTA3 action, and the expression of MTA3 is reported to influence the Snail and E-cadherin expression levels in tumor cells (13). Therefore, Fujita et al. proposed that in ER-positive breast cancer cells estrogen indirectly up-regulates MTA3, which in turn represses Snail expression, and that Snail represses E-cadherin expression (13).

In postmenopausal women the concentration of estradiol in breast cancer tissue is reported to be higher than in plasma and normal breast tissue (14). This may be due to in situ synthesis of estrogen by breast tissues, which is believed to be catalyzed mainly by aromatase (15). Promoter I.3 is one of the major promoters that control the expression of aromatase in breast cancer tissue. Snail has been reported to down-regulate this promoter in normal breast tissues (16).

In the present report, we studied the correlation between Snail and other factors to examine the role of Snail expression in node-negative invasive ductal carcinomas, which are the most common type of human breast cancers.

**METHODS**

**PATIENTS AND TUMOR SAMPLES**

Primary invasive ductal carcinoma tissues were obtained during surgical excision from 86 patients at Nagoya City University Hospital between January 1992 and December 2000. Informed consent was obtained from all patients before the surgery. The tissues were placed in liquid nitrogen immediately after resection and stored at −80°C until RNA extraction. The histological grade was estimated according to the Bloom and Richardson method proposed by Elston and Ellis (17). As postoperative adjuvant treatment for node-negative patients, endocrine therapy alone was given to patients with ERα- and/or PgR-positive tumors and (a) tamoxifen alone for postmenopausal patients and (b) tamoxifen plus luteinizing hormone releasing hormone (LH-RH) agonist for premenopausal patients. For the patients with ERα- and PgR-negative tumors, the following chemotherapy regimens were administered: (a) CMF [cyclophosphamide (100 mg orally on day 1–14), methotrexate (40 mg intravenous days 1 and 8) and 5-fluorouracil (500 mg intravenous days 1 and 8)]; (b) AC (doxorubicin 60 mg and cyclophosphamide 600 mg, every 3 weeks); or (c) oral 5-fluorouracil. Since 1995, postoperative treatment has been performed with reference to the recommendation of Goldhirsch et al. (18). After recurrence, patients with ERα- and PgR-negative tumors were treated with CMF, AC and taxanes. Patients with hormone receptor-positive tumors and nonvisceral metastases were treated with endocrine therapy, such as anti-estrogens, aromatase inhibitors and medroxyprogesterone acetate. Patients were followed postoperatively every 3 months. The median follow-up period was 67 months (range, 26–128 months).

**RNA EXTRACTION AND REVERSE TRANSCRIPTION (RT)**

Total RNA from homogeneous breast cancer tissue was isolated from ~500 mg of frozen specimen. Total RNA was also isolated from one flask of the human hepatoma HepG2 cell line (kindly provided by Dr N. Harada) for use as a control and to generate a standard curve of aromatase mRNA expression. mRNA was isolated with TRIZOL reagent (Life Technologies, Inc., Tokyo, Japan) according to the manufacturer’s recommendations. RT was performed as described previously (19). Briefly, the 20 μl reaction mixture contained 1 μg of total RNA, buffer [10 mM Tris–HCl (pH 9.0), 50 mM KCl, 1.5 mM MgCl2], 1 μM of each deoxynucleoside triphosphate, 25 units of RNase inhibitor (Amersham Pharmacia Biotech Inc., Tokyo, Japan), 200 units of SuperScript II reverse transcriptase (Life Technologies, Inc.) and 100 ng of pd(N)6 random hexamer (Amersham Pharmacia Biotech Inc.).

**REAL-TIME RT–POLYMERASE CHAIN REACTION (PCR)**

Real-time quantitative RT–PCR of the Snail and aromatase genes was performed on a LightCycler (Roche Molecular Biochemicals, Mannheim, Germany) using hybridization probes in combination with the LightCycler DNA Master Hybridization Probes Kit (Roche Molecular Biochemicals). Blast searches (GenBank) were conducted to confirm the specificity of the nucleotide sequences chosen for the primers and probes and to confirm the absence of DNA polymorphism. To avoid spurious signals from contaminating genomic DNA, the primers were located at two different exons. One Snail probe (TCA AGA AGT ACC AGT GCC AGG CTT G) was labeled at the 5′ end with a LightCycler-Red fluorophore and modified at the 3′ end by phosphorylation. The other Snail probe (GCC CAC CTC CAG ACC CAC TCA GAT) was labeled with fluorescein. The PCR primers of Snail were as follows: forward primer, GCT GCT ACA AGG CCA TGT, and reverse primer, CGG ACT CTT GGT GCT TGT.

The primers and probes for aromatase gene were designed to examine total amount of aromatase mRNA expression while aromatase gene has several promoters such as I.3, I.4, PII and I.7. One aromatase probe (TGC TTC ATT ATG ACC ATA CTT GAG GAC) was labeled at the 5′ end with a LightCycler-Red fluorophore and modified at the 3′ end by phosphorylation. The other aromatase probe (CTG CCG AAT CGA GAG CTG TAA TGA TT) was labeled with fluorescein. The PCR primers of the aromatase gene were as follows: forward primer, TCT GGA TCT CTG GAG AGG AAA, and reverse primer, GCC TTT CTC ATG CAT ACC GA. The PCR
conditions were as follows: initial denaturation at 95°C for 1 min, followed by 50 cycles of 95°C for 0 s, 55°C for 5 s and 72°C for 5 s. To ensure the fidelity of mRNA extraction and reverse transcription, Snail and aromatase signals from all samples were normalized against glyceraldehyde-3-phosphate dehydrogenase (GAPDH), a constitutively expressed gene used as an internal control. The GAPDH primers were as follows: forward primer, AAA TCA AGT GGG GCG ATG CTG, and reverse primer, GCA GAG ATG ATG ACC CTT TTG. The sequences of the GAPDH probes for real-time RT–PCR by a LightCycler were AGA AGG CTG GGG TTG. The PCR conditions for amplification of GAPDH were as follows: initial denaturation at 95°C for 1 min, followed by 50 cycles of 95°C for 0 s, 60°C for 5 s and 72°C for 8 s.

For each PCR run, a standard curve was constructed with serial dilutions of cDNA obtained from a tumor tissue that expressed Snail at a relatively high level and from the HepG2 cell line, which expresses aromatase (20). The levels of mRNA expression of the Snail and aromatase genes were given as relative copy numbers normalized against GAPDH mRNA. Relative expression levels of the Snail and aromatase mRNAs were calculated by these formulas: (Snail/GAPDH)×1000 and (aromatase/GAPDH)×1000. A non-template negative control was included in each experiment. All experiments were performed in duplicate. All the tumor samples with a coefficient of variation for gene mRNA copy number data of 10% or more were retested with the method of Bieche et al. (21).

IMMUNOHISTOCHEMICAL STAINING OF ERα, PgR AND E-CADHERIN

Immunostaining of ERα, PgR and E-cadherin was performed as described previously (22). Briefly, 4 μm sections were cut from one representative tissue block from each case of breast cancer. The slides were incubated at a dilution of 1:100 with either anti-ERα primary antibody (ER1D5, DAKO, Kyoto, Japan) or anti-PgR primary antibody (PgR636, DAKO) and stained by the labeled polymer method (DAKO EnVision+TM, DAKO) according to the manufacturer’s instructions. The slides were also incubated at a dilution of 1:50 with monoclonal mouse anti-human E-cadherin antibody (NCH-38, DAKO) with the same method. The immunostaining of ERα, PgR and E-cadherin was subjectively assessed by two independent investigators [Z.Z. and H.I. (ERα and PgR) or T.T. and H.I. (E-cadherin)], and discordant results were resolved by consultation with a third investigator (H.Y.). The expression of ERα and PgR was scored by assigning a proportion score and an intensity score according to Allred’s procedure (23). In brief, the proportion of positive staining throughout the entire slide was assessed as 0 (negative), 1 (<1%), 2 (1–10%), 3 (10%–1/3), 4 (1/3–2/3) and 5 (>2/3), and the average staining intensity was logged as 0 (negative), 1 (weak), 2 (moderate) or 3 (strong) under light microscopy.

The immunohistochemical score of each slide (0 or 2–8) was obtained as the sum of the proportion and intensity. ERα and PgR status by immunohistochemistry was then assessed as negative (scores 0 and 2) or positive (scores 3–8). The expression of E-cadherin was scored by immunohistochemistry as 0 (no membrane staining whatever), 1 (rare, scattered positive cell membranes), 2 (moderate to strong membrane staining, but with some cells showing no membrane staining easily found) and 3 (strong membrane staining, uniformly present over the whole section, equivalent to or stronger than seen in normal breast epithelium) (24).

STATISTICAL ANALYSIS

Statistical calculations were performed with StatView-J 5.0 software (SAS Institute Inc., Cary, NC). Associations between Snail mRNA expression level and ERα or E-cadherin protein expression level were evaluated using the nonparametric Kruskal–Wallis test, while the relationship between Snail mRNA expression and clinicopathological factors was assessed by the χ² test and Fisher’s exact probability test. Distant disease-free survival and overall survival were evaluated using the Kaplan–Meier method. The hypergeometric distribution method was performed to calculate P-value of disease-free and overall survivals. The relationship between Snail and aromatase mRNA expression was assessed by regression analysis. Follow-up continued through 31 May 2004.

RESULTS

NO CORRELATION BETWEEN SNAIL mRNA AND ERα PROTEIN EXPRESSION LEVELS

We examined the level of Snail mRNA expression in 86 samples of node-negative invasive ductal carcinoma tissues by real-time quantitative RT–PCR using a LightCycler. The relative expression level of Snail, normalized to the expression level of GAPDH in these tissues, was 9.6 ± 9.0 (mean ± standard deviation).

As the expression of Snail is down-regulated by MTA3, which is a downstream gene of ERα, we examined the relationship between Snail mRNA and ERα protein expression levels. The latter was evaluated by the immunohistochemical score proposed by Allred et al. (23), which is based on the estimated proportion and intensity of ERα positive-staining tumor cells (range: 0–8). As shown in Fig. 1, we found no statistically significant correlation between Snail mRNA and ERα protein expression levels in our series. We also found no correlation between Snail mRNA and PgR protein expression levels (data not shown).

LOW mRNA EXPRESSION OF THE SNAIL GENE IS A GOOD PROGNOSTIC FACTOR IN NODE-NEGATIVE INVASIVE DUCTAL CARCINOMAS

To identify a cut-off point for Snail mRNA expression that is clinically meaningful with respect to prognosis, various levels
of Snail mRNA expression were tested using the Kaplan–Meier method. Using a cut-off point of 6, we observed that in 86 node-negative invasive ductal carcinomas, none of the 34 patients showing low Snail mRNA expression developed distant metastasis, while 6 of 52 (12%) showing high expression of Snail mRNA experienced distant metastases and poor prognoses (Fig. 2A and B). Our data suggest that patients with node-negative invasive ductal carcinomas showing low Snail mRNA expression have good prognoses. The level of Snail mRNA expression was not found to be significantly correlated with clinicopathological factors (Table 1).

AROMATASE AND SNAIL mRNA EXPRESSIONS IN HUMAN BREAST CANCERS

Snail has been reported to down-regulate the promoter I.3 activity of human aromatase gene in normal breast tissues (16). Therefore, we investigated the relationship between Snail and aromatase mRNA expression in node-negative invasive ductal carcinoma tissues. As shown in Fig. 3, however, no inverse correlation was found between the Snail and aromatase mRNA expression levels in our series.

CORRELATION BETWEEN SNAIL mRNA AND E-CADHERIN PROTEIN EXPRESSIONS

The induction of EMT by Snail is mediated by the direct transcriptional repression of E-cadherin. Snail has been shown to repress E-cadherin expression and trigger EMT associated with epithelial tumor progression in tumor cells (7,9). Only small studies (25,26) have been reported concerning the correlation between Snail and E-cadherin expression in human breast cancer specimens. Therefore, we studied the correlation between Snail mRNA expression and E-cadherin protein expression in node-negative invasive ductal carcinoma samples of which paraffin blocks were available. As shown in Fig. 4, a modest inverse correlation was found between Snail mRNA and E-cadherin protein expression levels (P = 0.03).

DISCUSSION

We hypothesized that Snail could play an important role in the tumorigenesis and the progression of ERα-positive invasive ductal carcinomas. However, the present study showed no correlation between Snail mRNA and ERα protein expression levels. While Snail has been shown to be a direct regulatory target of MTA3, ERα has not been found to activate MTA3 expression directly (13). This might be one reason that the level of Snail mRNA expression was not affected by ERα expression status in human breast cancer specimens.

Snail expression of breast cancer tissues was reported to correlate with lymph node metastasis (25), and Snail expression of effusions in metastatic breast cancer patients was reported to predict poor overall survival (27). These reports suggested that high level of Snail expression is correlated with...
poor prognosis in advanced breast cancers. Our data showed that low expression of Snail mRNA was a good prognostic factor in the node-negative invasive ductal carcinomas, despite the lack of correlation between Snail mRNA expression and ERα protein expression levels. This result suggests that Snail is a prognostic factor even in early-stage breast cancer patients.

While Snail has been known for several years as a transcriptional repressor for E-cadherin (7,9), only a modest inverse correlation was found between Snail mRNA expression and E-cadherin expression as in the previous reports (25,26), while other important roles for Snail in carcinogenesis and tumor progression have been recently reported (28–31).

Table 1. Patient characteristics and the relationship between the level of Snail mRNA expression and other clinicopathological factors

<table>
<thead>
<tr>
<th>Snail mRNA</th>
<th>Low expression</th>
<th>High expression</th>
<th>Total</th>
<th>P-value</th>
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<tr>
<td>Median age (years) (range)</td>
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<td>57.2 (35–80)</td>
<td>57.2 (35–80)</td>
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<td>Tumor size (cm)</td>
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<tr>
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<td>15</td>
<td>29</td>
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<td>32</td>
<td>49</td>
<td></td>
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<td>4</td>
<td>7</td>
<td></td>
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<td>1</td>
<td>1</td>
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</tr>
<tr>
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<td>13</td>
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<td>60</td>
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<tr>
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<td>10</td>
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<td>26</td>
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<td>3</td>
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</table>

Figure 3. Correlation between Snail and aromatase mRNA expressions in human breast cancers. Expression is shown as relative copy numbers normalized against GAPDH mRNA.

Snail has been shown to bind to a noncanonical E-box in the Na,K-ATPase β1-subunit promoter and suppress its promoter activity (28). While expression of E-cadherin alone did not induce tight junction formation, expression of both E-cadherin and Na,K-ATPase β1-subunit induced epithelial polarization including the formation of tight junctions and reduced invasiveness and cell motility in virus-transformed cells (32,33). Therefore, Snail would trigger EMT by the repression both of E-cadherin and Na,K-ATPase β1-subunit. Snail has also been demonstrated to repress vitamin D3 receptor (VDR) (29), which is a ligand-dependent transcription factor implicated in regulation of cell cycle, differentiation and apoptosis of both normal and transformed cells derived from mammary gland (34–38). Recent studies (39,40) have shown that 1,25-dihydroxyvitamin D3 [1,25(OH)2D3] and VDR, which is present in over 80% of human breast cancers (41,42), participate in negative growth regulation of mammary gland. Therefore, Snail expression might affect breast tumorigenesis and progression of breast cancer via reduction of VDR levels.

Figure 4. Snail mRNA and E-cadherin protein expression levels in breast cancer tissues. (A) Immunohistochemical staining of breast cancer tissue with a monoclonal antibody to E-cadherin. Strong membranous immunoreactivity for E-cadherin (score 3+) is shown in the left panel, and no immunoreactivity for E-cadherin (score 0) is shown in the right panel (×400). (B) Correlation between Snail mRNA and E-cadherin protein expression levels. Snail mRNA expression level is shown as relative copy numbers normalized against GAPDH mRNA.
Furthermore, down-regulation of Snail has been reported to suppress mouse colorectal tumorigenesis by modulation of apoptosis and proliferation (30). Snail has also been reported to increase cancer invasion by up-regulating the matrix metalloproteinase (MMP) family in hepatocellular carcinoma cells (31). Our results suggest that high expression of Snail would be involved in distant metastasis in node-negative invasive ductal carcinomas by the various possible mechanisms such as E-cadherin silencing, decreasing expression of Na,K-ATPase β1-subunit and VDR, and up-regulation of the MMP family.

Promoter I.3 is one of the major promoters that control the expression of aromatase in breast cancer tissue. Snail has been reported to down-regulate the promoter I.3 activity of human aromatase gene in normal breast tissues (16). If Snail represses the aromatase gene, it will inhibit ERα regulation by limiting the levels of estrogen. However, our data showed no inverse correlation between the Snail and aromatase mRNA expression levels. Aromatase gene has several promoters such as I.3, I.4, PII and I.7 (43). A recent report suggested that the usage of these promoters were different between breast cancer and normal breast tissues and that only I.3 but also I.4, PII and I.7 promoters of the aromatase gene play important roles in breast cancer tissues (43). Therefore, Snail may have little role to repress aromatase mRNA expression in breast cancer tissues.

Our data suggest that Snail could play an important role in distant metastasis in node-negative invasive ductal carcinomas. However, further study will be needed to resolve remaining unknown functions of Snail in breast cancer.

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References