Analysis of Normal Epithelial Cell Specific-1 (NES1)/Kallikrein 10 mRNA Expression by in Situ Hybridization, a Novel Marker for Breast Cancer

Sanjay Dhar, Rohit Bhargava, Michael Yunes, Biao Li, Jaya Goyal, Stephen P. Naher, David E. Wazer, and Vimla Band

Division of Radiation and Cancer Biology, Department of Radiation Oncology, New England Medical Center, Boston, Massachusetts 02111 [S. D., M. Y., B. L., J. G., D. E. W., V. B.]; Department of Biochemistry, Tufts University School of Medicine, Boston, Massachusetts 02111 [V. B.]; and Baystate Medical Center, Springfield, Massachusetts [R. B., S. P. N.]

ABSTRACT

Purpose: Normal epithelial cell specific-1 (NES1)/kallikrein 10 gene is expressed in normal mammary and prostate epithelial cells, but the expression of NES1 mRNA and protein is markedly reduced in established breast and prostate cancer cell lines although the NES1 gene is intact. Here, we wished to assess whether NES1 expression is down-regulated in primary breast cancers.

Experimental Design: We developed and used an in situ hybridization technique with an antisense NES1 probe to detect NES1 mRNA in sections of normal breast specimens, typical and atypical ductal hyperplasia, ductal carcinoma in situ, and infiltrating ductal carcinoma.

Results: All of the 30 normal breast specimens showed high NES1 expression. Notably, 18 (75%) of 24 typical and atypical breast hyperplasia specimens showed high NES1 expression, with weak-to-moderate expression in 6 (25%). Significantly, 13 (46%) of 28 ductal carcinoma in situ specimens lacked NES1 expression, and the remaining 15 (54%) showed weak-to-moderate expression. Finally, 29 of 30 (97%) infiltrating ductal carcinoma grades I-III samples lacked NES1 mRNA, with weak expression in the remaining one sample.

Conclusions: Our results demonstrate that NES1 mRNA is expressed in normal breast tissue and benign lesions, with loss of NES1 expression during tumor progression. We suggest that NES1 expression may serve as a molecular tool in the study of breast cancer progression. Studies with larger series of specimens should help assess whether NES1 expression can be a diagnostic and/or prognostic marker in breast and other cancers.

INTRODUCTION

Breast cancer is the second leading cause of cancer-related deaths in the United States. Although a number of genetic markers have been identified in recent years for hereditary breast cancer, much less progress has been made in defining molecular markers for sporadic nonhereditary breast cancer, which account for the vast majority of breast cancers (1). Using subtractive hybridization between 76R-30, a radiation-transformed breast epithelial cell line, and its isogenic mammoplasty-derived normal parental strain, 76N, we previously identified a gene NES1 that was expressed in normal but not in radiation-transformed mammary epithelial cells (2, 3). The predicted NES1 polypeptide showed strong homology with trypsin, kallikrein, and kringles families of serine proteases (3, 4). Furthermore, NES1 gene is located on chromosome 19q13.3 within the kallikrein locus (5, 6). On the basis of these findings, NES1 has been designated as KLK10 (6). However, using a large number of biochemical assays, we have been unable to show NES1 to be a functional protease (4). Importantly, NES1 mRNA as well as protein expression was dramatically down-regulated or completely lost in a majority of breast cancer cell lines (3). Additional analyses showed loss of NES1 mRNA expression in prostate tumor cell lines (5). Transfection of NES1 cDNA into a highly aggressive NES1-negative breast cancer cell line, MDA-MB-231, dramatically reduced the tumorigenic phenotype, as evidenced by decreased anchorage independence and inhibition of tumor formation in nude mice (5). These findings suggest that, in addition to providing a possible tumor marker, inactivation of the NES1 gene expression may be linked to oncogenesis.

The function of classical tumor suppressor genes (class I tumor suppressors) such as p53 and Rb, is typically lost through gene deletion or mutation (7). Recent studies have, however, brought into focus a large number of genes, the functional inactivation of which involves loss of expression as a result of gene silencing rather than of mutation/deletion (8, 9). Although NES1 expression is lost in most breast tumor cell lines, thus far we have not observed any deletion or mutation in the NES1 gene in these tumor cells (Ref. 3 and unpublished data). The loss of NES1 gene expression in tumor cells without evidence of gene

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2 To whom requests for reprints should be addressed, at Department of Radiation Oncology, Box No. 824, New England Medical Center, 750 Washington Street, Boston, MA 02111. Phone: (617) 636-4776; Fax: (617) 636-6205; E-mail: VBAND@lifespan.org.

3 The abbreviations used are: NES1, normal epithelial cell specific-1; IDC, infiltrating ductal carcinoma; DCIS, ductal carcinoma(s) in situ; KLK10, kallikrein 10; DH, ductal hyperplasia; ADH, atypical DH.

4 J. Goyal and V. Band, unpublished observations.

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deletion/mutation indicates that it is a class II tumor suppressor (7). “Class II tumor suppressors” has grown considerably and include prominent examples, such as retinoblastoma (Rb), a cyclin-dependent kinase inhibitor (CDKI) p16, BRCA1, retinoic acid receptor-β (RARβ), 14–3-3r, and cyclin D2 are frequently inactivated through loss of expression (10–15).

Given the in vitro data that NES1 mRNA and protein expression is lost in a majority of tumor mammary epithelial cell lines (3, 5), we wished to determine whether NES1 expression is lost in human breast cancers. Because NES1 is a secreted protein (3), analyses of NES1 protein expression with currently available polyclonal antibodies proved impractical. Therefore, in this study, we have developed an in situ hybridization technique to examine the NES1 mRNA expression in normal breast and tumor tissues.

MATERIALS AND METHODS

Specimens/Tumors. The study included archival specimens obtained during the period of 1998 to 2000 from 58 patients with diagnoses of breast carcinoma (28 DCIS and 30 IDC) and 48 patients with DH (24 typical and 24 atypical), together with 30 normal breast tissue specimens obtained by excisional biopsy, mastectomy, and reduction mammoplasty. The specimens were obtained as formalin-fixed, paraffin-embedded blocks from the archives of the Department of Pathology at Baystate Medical Center (Springfield, MA). Procurement of samples was done under institutional guidelines for human subjects and the applicable laws to protect the privacy of patients. For each specimen selected, sections were subjected to H&E staining and reviewed by two pathologists (S. P. N. and R. B.) to confirm the diagnosis, and the histological grade of invasive tumors according to the modified Bloom-Richardson scheme (Ref. 16; grades 1, 2, and 3 correspond to well-differentiated, moderately differentiated, and poorly differentiated tumors, respectively). The breast tissues were assigned categories of normal breast, DH, ADH, DCIS, and IDC.

Preparation of Sense and Antisense NES1 Probe. A 1069-bp NES1 cDNA, including 238 bp of 5’ and 3’ untranslated region and the entire protein coding region (nucleotides 82 to 912), was cloned into pBluescript (KS) vector (Stratagene, La Jolla, CA). To generate the antisense RNA probe (complementary to NES1 mRNA), the plasmid was linearized with HindIII, and the transcription reaction was carried out using polymerase T7. To generate the sense probe, the plasmid was linearized with XhoI and transcribed using the T3 polymerase. In vitro transcription reactions were carried out using the Maxiscript T7/T3 kit (Ambion, Austin, TX) in the presence of Biotin-16-UTP (Boehringer Mannheim, Mannheim, Germany) to generate biotin-labeled probes as described by supplier’s protocol. The probes were purified using G-50 spin columns (Amersham Pharmacia Biotech, Piscataway, NJ).

In Situ Hybridization. Formalin-fixed, paraffin-embedded tissue blocks were cut into 5-μm-thick sections in RNase-free water and were mounted on ProbeOn Plus slides (Fisher Scientific, Pittsburgh, PA). In situ hybridization was performed using the mRNA-locator-Hyb kit (Ambion). Briefly, the sections were deparaffinized by submerging the slides in xylene; rehydrated in decreasing concentrations of ethanol (100% three times followed by one time each of 90, 70, and 50%); and equilibrated, first in nuclease-free water and then in Tris buffer (pH 7.5). The tissue sections were subjected to proteinase K digestion [200 pg in 50 μl of Tris buffer (pH 7.5) per slide] at 37°C for 30 min in a humidified chamber. The slides were washed three times, 4 min each, with Tris buffer (pH 7.5). The probes were denatured at 65°C for 5 min and applied to slides at 10 ng of probe/slide in 50 μl of hybridization buffer (provided by Ambion). Hybridization was carried out at 50°C overnight in a humidified chamber. The slides were washed once with 2× in situ wash solution (provided by Ambion) for 10 min followed by three washes in 1× in situ wash solution for 10 min each.

Detection of Biotin-labeled Probes. Detection of in situ hybridization probes was performed using mRNAlocator-Biotin kit (Ambion). Briefly, the sections were incubated with streptavidin-alkaline phosphatase (50 μl of 1:3000 dilution/sample) at 37°C for 30 min in a humidified chamber. The sections were washed twice with Tris buffer (pH 7.5) for 4 min each. The color signals were developed by applying 50 μl of nitroblue tetrazolium chloride/5-bromo-4-chloro-3-indolylphosphate p-toluidine salt (NBT/BCIP) solution per slide and incubation at 37°C for 4 h in a humidified chamber. Color development was terminated by two washes of 4 min each in nuclelease-free water. The sections were dehydrated in ascending concentrations of ethanol (one time each in 50, 70, and 90% and two times in 100%), cleared in xylene, and permanently mounted with Permount (Fisher Scientific). A positive enzymatic reaction in this assay stained dark purple. Known positive specimens were used in each batch of hybridization. These consisted of five reduction mammoplasty specimens for which positive hybridization was reproducibly observed in pilot studies. Controls for endogenous alkaline phosphatase included treatment of the sample in the absence of the probe and use of chromogen alone. All of the samples were concurrently processed for in situ hybridization with antisense and sense probes using serial sections. Staining intensity was graded from 0 to 3+ as follows: no staining (0), weak staining (1+), moderate staining (2+), and intense staining (3+).

RESULTS

Our previous studies revealed that NES1 mRNA and protein is expressed in normal and nontumorigenic immortal mammary and prostate cells grown in vitro, but the expression is down-regulated in most established breast and prostate cancer cell lines (3, 5). These findings led us to investigate whether there is a progressive loss of NES1 mRNA expression during breast cancer progression in situ. Thus, we analyzed 30 normal breast tissues, 24 DH, 24 ADH, 28 DCIS, and 30 IDC for levels of NES1 mRNA expression by in situ hybridization. NES1 mRNA expression was visualized by in situ hybridization using a biotin-labeled, full-length NES1 antisense probes. A NES1 sense probe was used as a negative control for every specimen by hybridization on a parallel section.

Expression of NES1 in Normal Breast Specimens. To establish that NES1 mRNA is expressed in normal breast tissue in situ, we analyzed 30 histologically normal breast specimens to establish NES1 mRNA expression by in situ hybridization. The average age of women from whom the tissues were obtained was 52.0 ± 7.5 years (mean ± SD; median, 52 years; range, 31–77 years). All (100%) of the 30 normal breast tissue...
specimens showed strong staining with the NES1 antisense probe with only low background staining observed with the NES1 sense probe (Fig. 1 shows a representative example). NES1 mRNA expression was graded as 3+/H11001 (intense) in all of the cases. The most intense staining was obtained over the mammary epithelium. None of the normal breast specimens showed lack of NES1 expression (Table 1).

Expression of NES1 in DH. To assess NES1 mRNA expression in benign breast lesions, 24 DH specimens were analyzed for NES1 mRNA expression. The average age of patients was 44.7 ± 4.4 years (mean ± SD; median, 45 years; range, 40–67 years). None of the specimens showed lack of NES1 mRNA expression. Six (25%) of 24 DH specimens showed moderate mRNA expression, whereas 18 (75%) showed intense signals for NES1 expression (see Fig. 2 for a representative example and Table 1).

Expression of NES1 in ADH. To further assess the NES1 expression in various proliferative lesions of the mammary epithelium, 24 ADH specimens were examined. The average age of the patients was 65.4 ± 17.6 (median, 65 years; range, 15–83 years). Intense NES1 expression was observed in 18 (75%) specimens, whereas moderate expression was seen in the remaining 6 (25%) specimens (a representative example is in Fig. 3 and Table 1).

NES1 Expression in DCIS. We analyzed 28 DCIS specimens, which included comedo, micropapillary, cribriform, and solid-type patterns of tumors. The average age of patients was 53.77 ± 8.17 years (mean ± SD; median, 54 year; range, 31–82 years). Thirteen (46%) of 28 tumors showed loss of NES1 mRNA expression (see Fig. 4A for a representative example),

Table 1. NES1 signal intensity and distribution

<table>
<thead>
<tr>
<th>Tissues</th>
<th>n</th>
<th>Absent</th>
<th>Weak/Moderate</th>
<th>Intense</th>
<th>P*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal breast</td>
<td>30</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
<td>30 (100%)</td>
<td>0.0052</td>
</tr>
<tr>
<td>DH</td>
<td>24</td>
<td>0 (0%)</td>
<td>6 (25%)</td>
<td>18 (75%)</td>
<td>0.0052</td>
</tr>
<tr>
<td>ADH</td>
<td>24</td>
<td>0 (0%)</td>
<td>6 (25%)</td>
<td>18 (75%)</td>
<td>±0.0001</td>
</tr>
<tr>
<td>DCIS</td>
<td>28</td>
<td>13 (46%)</td>
<td>15 (54%)</td>
<td>0 (0%)</td>
<td>±0.0001</td>
</tr>
<tr>
<td>IDC</td>
<td>30</td>
<td>29 (97%)</td>
<td>1 (3%)</td>
<td>0 (0%)</td>
<td>±0.0001</td>
</tr>
</tbody>
</table>

*Ps were calculated by Fisher exact test. Level of significance in each category was determined by comparison with normal breast samples.

b Showed very weak expression signal.

Fig. 1. Normal breast lobule expressing a high level of NES1 mRNA. Top panel, H&E-stained tissue section; middle panel, absence of staining with NES1 mRNA sense probe; bottom panel, intense staining with NES1 mRNA antisense probe. NBT, normal breast tissue. ×100.

Fig. 2. DH expressing a high level of NES1 mRNA. Top panel, H&E-stained tissue section; middle panel, absence of staining with NES1 mRNA sense probe; bottom panel, intense staining with NES1 mRNA antisense probe. ×200.
that three (75%) of four had loss of NES1 mRNA expression with one tumor showing weak expression (Table 2B).

**Loss of NES1 Expression in IDC.** To determine whether NES1 mRNA expression was lost in breast cancers, we analyzed 30 specimens from patients with IDC. The average age of the patients was 53.5 ± 9.5 (mean ± SD; median, 53 years; range, 37–87 years). NES1 expression was lost in 29 cases (97%), as indicated by hybridization signals with antisense NES1 probe comparable with those observed with the sense probe (See Fig. 5 for a representative example). One (3%) of 30 specimens showed weak NES1 mRNA expression (Table 1). None of the tumor specimens showed the intense signals for NES1 mRNA expression comparable with that seen in normal breast tissue. Analysis of the NES1 expression in different grades of IDC showed a complete loss of NES1 mRNA expression in all 4 (100%) of the grade 1 tumors; 12 (92%) of 13 grade 2 tumors, with weak expression in 1 case (8%); and all 13 (100%) of the grade 3 tumors (Table 3).

**DISCUSSION**

The NES1 gene was identified through subtractive hybridization between normal and radiation-transformed mammary epithelial cells, and it was found that its expression was down-regulated in most breast and prostate tumor cell lines but not in nontumorigenic cells (3, 5). These characteristics suggested that a loss of NES1 expression may be linked to tumor progression. The studies reported here demonstrate that high levels of NES1 mRNA expression are seen in normal breast tissue, and its expression is lost in nearly all IDCs. Although, a relatively small set of specimens of each type was examined in this study, the results comparing normal breast tissue with IDC represent a dramatic contrast. In all of the 30 normal specimens, the in situ hybridization signals for NES1 were positive and of high intensity (3+) on a scale of 0 to 3+. In contrast, 29 of 30 IDC specimens showed signals with antisense NES1 probe that were no higher than those with the sense (negative control) probe; only in one case was a low level of expression observed. Thus, NES1 expression is nearly uniformly lost in primary mammary infiltrating carcinoma but is highly expressed in normal breast tissue, suggesting that NES1 expression is lost during tumor progression.

In an effort to further correlate the lack of NES1 expression with tumor progression in breast cancer, we also analyzed specimens of typical DH, ADH, and DCIS in direct comparison with normal breast and IDC specimens. Remarkably, the vast majority of benign and preneoplastic lesions (typical and atypical hyperplasia) expressed high levels of NES1 mRNA, as judged by in situ hybridization signals. In this regard, these proliferative lesions retained the NES1 expression pattern of normal breast tissue. In contrast, a large proportions (about 50%) of DCIS showed loss of NES1 expression. Thus, whereas normal tissue and benign breast lesions retain NES1 expression, its expression is lost incrementally during tumor progression (Fig. 6).

Several cases revealed normal breast tissue, proliferative lesions, and carcinomas concurrently on one histological section. Staining with NES1 mRNA antisense probe on these sections revealed a differential staining pattern with moderate-to-intense staining of normal ductal elements and proliferative lesions and weak-to-negative staining in carcinoma. These ob-
Observations further support the hypothesis that NES1 mRNA expression is lost during mammary oncogenesis. Because the patient data analyzed here are small and these samples were from patients treated from 1998 to 2000, the NES1 expression could not be correlated with clinical outcome. However, this study does suggest that DCIS fall into two groups: NES1-negative and NES1-positive. Future studies to assess whether NES1 expression may represent a prognostic marker will be of substantial interest. The in situ hybridization analyses carried out here used formalin-fixed tissues, which should substantially facilitate prospective as well as retrospective studies to determine whether there are differences in the progression of DCIS to IDCs based on NES1 expression studies. Our recent studies suggest that tumor-specific loss of NES1 expression may be caused by hypermethylation (17), which is likely to provide another marker to assess the role of NES1 status in oncogenesis. Whereas our present study has focused on proliferative lesions and carcinomas of the breast, NES1 expression may also be lost in other types of malignancies. It is notable that in addition to mammary tumor cell lines, we also observed loss of NES1 expression in prostate (5) and cervical (data not shown) tumor cell lines. Therefore, the methodology developed here should facilitate analyses of other cancers.

At present, it is not clear how NES1 might function as a tumor suppressor. The primary sequence of NES1 predicted a serine protease; however, we have been unable to detect such an activity using a variety of biochemical approaches.4 Because NES1 is a secreted protein, it is likely that it functions extracellularly as a regulator of cell growth and/or differentiation in an autocrine or paracrine manner. Whereas additional studies are mandated to define the biological function of NES1, our present studies support the notion that a lack of NES1 expression may be useful as a molecular tool in the study of breast cancer progression. Future studies, with larger series of tumor samples representing different stages of tumor progression, should provide an indication of whether NES1 expression status might provide a marker for early diagnosis and/or prognosis, either by itself or with other tumor markers.

Table 2 NES1 signal intensity and distribution in DCIS

<table>
<thead>
<tr>
<th>Grades/Types</th>
<th>n</th>
<th>Absent</th>
<th>Weak/Moderate</th>
<th>Intense</th>
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<tr>
<td>A. Distribution of NES1 mRNA expression in different grades of DCIS</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low grade</td>
<td>6</td>
<td>3</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>Intermediate grade</td>
<td>6</td>
<td>4</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>High grade</td>
<td>16</td>
<td>6</td>
<td>10</td>
<td>0</td>
</tr>
<tr>
<td>B. Distribution of NES1 mRNA expression in different types of DCIS</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Solid type</td>
<td>13</td>
<td>4</td>
<td>9</td>
<td>0</td>
</tr>
<tr>
<td>Comedo type</td>
<td>13</td>
<td>5</td>
<td>8</td>
<td>0</td>
</tr>
<tr>
<td>Cribriform type</td>
<td>19</td>
<td>8</td>
<td>11</td>
<td>0</td>
</tr>
<tr>
<td>Micropapillary type</td>
<td>4</td>
<td>3</td>
<td>1</td>
<td>0</td>
</tr>
</tbody>
</table>

Fig. 4 A, duct containing high-grade DCIS with comedo necrosis and calcification lacking NES1 mRNA expression. Top panel, H&E- stained tissue section; middle panel, absence of staining with NES1 mRNA sense probe; bottom panel, absence of staining with NES1 mRNA antisense probe. ×200. B, duct containing high-grade DCIS with comedo necrosis expressing a low level of NES1 mRNA. Top panel, H&E- stained tissue section; middle panel, absence of staining with NES1 mRNA sense probe; bottom panel, weak staining with NES1 mRNA antisense probe. ×200. C, duct containing an intermediate-grade DCIS expressing a moderate level of NES1 mRNA. Top panel, H&E- stained tissue section; middle panel, absence of staining with NES1 mRNA sense probe; bottom panel, moderate staining with NES1 mRNA antisense probe. ×200.
Loss of NES1 Expression in Primary Breast Cancers

In: Hamid Band for critical reading of the manuscript and Linda Miyashiro for help in some of the experiments.

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