Estrogen Receptor Mutations and Changes in Estrogen Receptor and Progesterone Receptor Protein Expression in Metastatic or Recurrent Breast Cancer

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To investigate the frequency of estrogen receptor (ER) gene mutation in metastatic or recurrent breast cancer, metastatic lymph nodes or recurrent breast cancer tissue from 35 patients with ER-positive primary tumors were screened for mutations in the hormone-binding domain of the ER gene by sequence analysis. Four missense mutations, Val316Ile, Gly344Val, Ala430Val and Gly494Val, were identified in these lesions. Second, to clarify whether there is any disparity in hormone receptor status between primary and metastatic or recurrent tumors, we immunohistochemically studied 117 specimens including the above 35 specimens obtained from metastatic or recurrent breast cancer patients using monoclonal anti-ER and progesterone receptor (PgR) antibodies. Although hormone receptor status, especially ER, was highly maintained through disease progression, negative change in PgR expression at relapse (33%) was identified more frequently than in metastatic lymph nodes (6.7%). Therefore, it was suggested that development of PgR-negative phenotype might correlate with disease progression in some breast cancer patients. These results suggest that ER mutations in metastatic or recurrent breast cancer may be more frequent than in primary lesions, irrespective of high maintenance of ER protein expression through disease progression.

Key words: Estrogen receptor — Mutation — Metastasis

It is well accepted that estrogen receptor (ER) and progesterone receptor (PgR) are important prognostic factors in human breast cancer, and their presence is correlated with a better response to endocrine therapy and a favorable clinical outcome. However, about 30–40% of ER-positive (ER+) tumors fail to respond to antiestrogen therapy and are considered to be tamoxifen-resistant. Moreover, the majority of ER+ tumors that initially respond to antiestrogen therapy will eventually develop resistance to this therapy without necessarily altering their ER profile. It has been reported that mutations in the ER gene occur at a low frequency and do not account for most estrogen-independent, tamoxifen-resistant breast tumors, and that the ER-negative (ER−) phenotype is not the result of mutations in the coding region of the ER gene in the majority of primary breast cancers. Taplin et al. reported that mutations of the androgen-receptor genes in metastatic prostate cancer are not uncommon and may provide a selective growth advantage after androgen ablation. Therefore, we hypothesized that ER gene alterations in ER+ breast cancer may be more frequent in metastatic sites than in primary tumors. There have been a few reports concerning ER mutations in metastatic breast cancer. Although treatment of metastatic breast cancer based on the ER status of the primary tumor builds on the assumption that the ER status is equivalent between the primary and the metastatic tumors, their correspondence is controversial. To gain further insight into ER expression and mutations in metastatic breast cancer, we performed a molecular-pathological study in 117 specimens obtained from metastatic or recurrent sites of breast cancer patients.

MATERIALS AND METHODS

Patients and tissues Tissue samples of primary breast cancer and axillary lymph nodes were obtained from 90 female Japanese patients who underwent mastectomy at the Sagara Hospital (Kagoshima). Patients showed three different hormone receptor statuses determined by the dextran-coated charcoal (DCC) separation method as follows: 54 ER+/PgR+, 15 ER+/PgR− or 21 ER−/PgR−. Twenty-seven biopsy samples were also obtained from recurrent breast cancer patients. Hormone receptor statuses of primary breast cancer determined by the DCC separation method were as follows: 13 ER+/PgR+, 14 ER−/PgR−. In 10 cases, the tumor represented a local recurrence in the breast, whereas in 17 cases the recurrence was at a different site (2 lymph node metastases, 9 skin nodules, 5 chest wall, and 1 muscle). All patients were histologically diagnosed as invasive ductal carcinoma according to the World Health Organization typing scheme for breast tumors. The presence of metastatic or recurrent breast cancer was confirmed by histological examination.

Microdissection and DNA extraction Pieces of axillary lymph nodes or recurrent breast cancer tissues were fixed in 10% neutral buffered formalin for 24 h, and embedded in paraffin. Microdissection and DNA extraction were performed as described previously.
Polymerase chain reaction (PCR), subcloning and sequencing

Oligonucleotide primers were synthesized using a 392 DNA synthesizer (Applied Biosystems Inc., Foster City, CA). Seven sets of PCR primers were designed for analysis of genomic DNA according to the intron/exon locations defined by Ponglikitmongkol et al. All hormone-binding domains were examined. ER− metastatic tumors with ER+ primary tumors by immunohistochemical analysis were screened with exons 2 and 3 besides hormone-binding domain. The primer sequences, expected sizes of the PCR products, and their locations within the ER gene are shown in Table I. Genomic DNA (0.2 µg) was amplified in 50-µl reaction mixtures containing 20 mM Tris-Cl (pH 8.8), 10 mM KCl, 2 mM MgCl2, 10 mM (NH4)2SO4, 0.1% Triton X-100, 100 µg/ml bovine serum albumin (BSA), 200 mM each deoxynucleotide triphosphates, Pfu polymerase (2.5 U, Stratagene, La Jolla, CA) and each oligonucleotide primer (1 µM). PCR conditions were as follows: initial denaturation at 94°C for 5 min and then 35 cycles of 1 min at 94°C for denaturation, 1 min at 50–57°C for annealing, and 2 min at 72°C for elongation using a thermal cycler (Astek, Fukuoka). Agarose gel-purified PCR products were subcloned into the EcoRV site of pBlueScript II SK(+) (Stratagene). Bacterial colonies containing the correct inserts were screened by direct colony PCR using both M13 forward and reverse primers, and multiple isolates from each patient were selected. Both strands of the insert were sequenced by the double-stranded DNA dyeoxy sequencing method using Sequenase (Amersham). Base changes were determined to be mutations rather than Pfu polymerase errors on the basis of their identification in multiple plasmids and their isolation after a second independent round of PCR amplification and analysis.

Immunohistochemistry

After blocking of endogenous peroxidase activity, deparaffinized sections (3 µm) were predigested in 10 mM citrate buffer (pH 6.0) by microwaving (500 W, full power) for 15 min. After cooling for 60 min, sections were incubated with monoclonal anti-human ER antibody 1D5 (Immunotech, France) or monoclonal anti-human PgR antibody (diluted 1:40, Novocastra, UK) overnight at 4°C in moist chambers. The sections were incubated with biotinylated goat anti-mouse immunoglobulin (diluted 1:150, Vector Lab., UK) and horseradish peroxidase-conjugated streptavidin complex (diluted 1:100, Zymed, CA). To visualize immunoreactivity, we used diaminobenzidine/H2O2 (1 mg/ml) in phosphate-buffered saline (PBS) as the substrate. Previously defined strongly ER+ or PgR+ tumors were used as positive controls. Negative control sections were processed immunohistochemically without the primary antibodies (replaced by PBS containing 1.0% bovine serum albumin). Non-metastatic lymph nodes in the same sections also served as negative controls.

Hormone receptor analysis

ER and PgR levels were determined by the DCC separation method (Biomedical Lab., Tokyo) with cut-off values of 14 and 13 fmol/mg protein, respectively. Assessment of immunostaining

Nuclear staining was interpreted as positive when more than 20% of nuclei were stained.

RESULTS

We first examined ER gene mutations and/or polymorphisms in the hormone-binding domain in genomic DNA extracted from 22 metastatic lymph nodes with ER+/PgR+...
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29 (15 samples) or \( \text{ER}^+/\text{PgR}^- \) (7 samples) primary tumors and 13 recurrent tumors with \( \text{ER}^+/\text{PgR}^+ \) primary tumors (Fig. 1). No deletions or insertions were found. Of 22 metastatic lymph nodes, two (9%) missense mutations were detected: Tumor #18, codon 430 in exon 6 \([\text{GCT}(\text{Ala})\rightarrow\text{GTT}(\text{Val})]\); Tumor #3, codon 494 in exon 7 \([\text{GGC}(\text{Gly})\rightarrow\text{GTC}(\text{Val})]\). Of 13 recurrent tumors, two (15%) missense mutations were detected: Tumor #48, codon 316 in exon 4 \([\text{GTC}(\text{Val})\rightarrow\text{ATC}(\text{Ile})]\); Tumor #59, codon 344 in exon 4 \([\text{GGC}(\text{Gly})\rightarrow\text{GTC}(\text{Val})]\). The primary tumors #18, #3, #48 and #59 contained only wild-type sequences in codons 430, 494, 316 and 344, respectively. Expression of \( \text{ER} \) and \( \text{PgR} \) was maintained between primary and metastatic or recurrent tumors in samples #18, #3, #48 and #59. Primary tumor #18 had a missense mutation in codon 434 in exon 6 \([\text{TCG}(\text{Arg})\rightarrow\text{TTG}(\text{Trp})]\). Neutral polymorphism in codon 325 in exon 4 \([\text{CCC}(\text{Pro})\rightarrow\text{CCG}(\text{Pro})]\) was detected in 18 (81%) metastatic lymph nodes and 13 (100%) recurrent tumors. In total, 90 metastatic lymph nodes and 27 recurrent tumors were examined immunohistochemically (Figs. 2 and 3). The results of immunostaining are summarized in Tables II and III. Among 59 metastatic lymph nodes with \( \text{ER}^+/\text{PgR}^+ \) primary tumors, 47 (88%) had the same status as the primary tumor. Only 4 (6.7%) tumors had changed to \( \text{ER}^- \). Among 10 metastatic lymph nodes with \( \text{ER}^+/\text{PgR}^- \) primary tumors, 8 (80%) had the same status as the primary tumor. Among 12 recurrent tumors with \( \text{ER}^+/\text{PgR}^+ \) primary tumors, 8 had the same status as the primary tumor, but 4 had become \( \text{PgR}^- \). All metastatic lymph nodes and recurrent tumors with \( \text{ER}^-/\text{PgR}^- \) primary tumors had the same status as the primary lesion.
DISCUSSION

Although ER-splicing variants have been shown to be ubiquitous in human breast cancer, the number of naturally occurring missense mutations identified in primary breast cancers to date is extremely low. As most reported mutations in the ER gene are present in the hormone-binding domain, we focused on this region. We used sequence analysis, since it is expected to be more sensitive than PCR-single strand conformation polymorphism analysis, which is estimated to be capable of detecting about 85% of gene mutations. Karnik et al. reported a single base pair deletion in exon 6 in one of five metastatic breast cancer tissues. Zhang et al. also found three missense mutations in 30 metastatic breast cancer patients. We identified 4 missense mutations in 35 metastatic or recurrent breast cancers. Although the number of cases we investigated in this study was small, our results suggested that ER mutations in those lesions may be more frequent than in primary lesions. Recently, an anti-ER antibody, ER1D5, has become available which performs well in formalin-fixed paraffin-embedded sections in combination with antigen retrieval techniques. It was reported that ER immunohistochemistry in paraffin-embedded tissues with ER1D5 predicted breast cancer endocrine response more accurately than did that with H222Spy in frozen sections or cytosol-based ligand-binding assays. However, in most previous studies concerning changes in ER expression, ER1D5 was not used in combination with antigen retrieval techniques. Although our immunohistochemical study using ER1D5 revealed that ER phenotype was highly preserved through disease progression, Johnston et al. reported that ER and PgR expression were significantly reduced at relapse using the same antibody and techniques. This discrepancy may have been due to the difference in the number of cases investigated, and therefore further studies with larger numbers of patients are needed. Smith et al. reported an ER mutation [CGA (Arg) to TGA (stop)] at codon 157 in exon 2, resulting in a severely truncated ER protein which may not be recognized by ER1D5 monoclonal antibody, because the epitope for this antibody lies in the N-terminal domain (A/B region). To exclude the possibility that negative changes in ER expression may be caused by such mutations, we performed sequence analysis of exons 2 and 3 besides the hormone-binding domain in 4 cases with negative changes in ER expression. However, we found no mutations in these regions. Therefore, the reason for the nega-

| Metastatic tumor in lymph nodes | Primary tumor |
|--------------------------------|--------------|
| ER+PgR+                        | ER+PgR+      | ER+PgR-      | ER-PgR-      |
| 52                             | 2            | 0            |
| ER+PgR-                        | 3            | 8            | 0            |
| ER-PgR+                        | 3            | 0            | 0            |
| ER-PgR-                        | 1            | 0            | 21           |

| Recurrent tumor | Primary tumor |
|----------------|--------------|
| ER+PgR+        | ER+PgR-      | ER-PgR-      |
| 8              | 0            | 0            |
| ER+PgR-        | 4            | 1            | 0            |
| ER-PgR-        | 0            | 0            | 14           |

Fig. 2. Recurrent breast cancer in the soft tissue showing nuclear staining with ER1D5 (×270).

Fig. 3. Metastatic breast cancer in the regional lymph node showing nuclear staining with anti-PgR monoclonal antibody (×380).
tive change in ER expression remains unknown. In our study, negative changes in PgR expression at relapse (33%) was identified more frequently than in metastatic lymph nodes (6.7%). ER+ tumors with PgR expression have been shown to be more likely to benefit from endocrine therapy.\(^9\) Therefore, it was speculated that tumors with negative changes in PgR expression might have an altered ER function which provides a growth advantage. Our next study will focus on the endocrine response in the group with negative changes in PgR expression in the metastatic lymph nodes. There have been two functional studies of naturally occurring ER mutations, Tyr537Asn and Asp351Tyr. The former mutant demonstrated potent, estradiol-independent transcriptional activity as compared to wild-type ER, and its constitutive activity was virtually unaffected by estradiol, tamoxifen, or the pure antiestrogenICI164,384.\(^6\) The latter mutant showed increased estrogendependency of (fr) 4-OH tamoxifen.\(^20\) As the ER mutations found in the present study have not been reported previously, functional analyses of these ER mutants are necessary. Treatments of metastatic breast cancer based on the ER status of the primary tumor build on the assumption that the ER status is equivalent between the primary and the metastatic tumors. Therefore, it might be useful to investigate the presence of ER mutants with altered hormone responses or negative changes in PgR expression in metastatic or recurrent breast cancer, in order to provide more information to aid the choice of adjuvant therapy.

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