additional multiple levels of immunohistochemistry leads to significant upgrading. For example, Freneaux et al reported upgrading in 47% of examined cases using four H&E sections and six additional levels of cytokeratin immunohistochemistry at intervals of 0.15 mm.[38]

Most metastatic foci detected only by immunohistochemistry will be either micrometastases or isolated tumour cells. There is a small possibility that cells other than metastatic carcinomas may be positive (false-positive staining), such as some macrophages. Benign transport of breast epithelium and pseudometastasis from noninvasive carcinomas have been reported.[39,40] To avoid pseudometastasis and to detect only clinically significant metastases, it is recommended that the number of immunohistochemistry-positive cells be quantified,[10] e.g. less than 10 cells, 10-100 cells, and more than 100 cells, as represented in two dimensions on a slide.

Molecular analysis

RT-PCR has been used for molecular analysis of SLNs. It is more sensitive than immunohistochemistry, but specific markers are lacking. The results of upgrading are still variable, and the procedure is not feasible in all pathology laboratories. At least currently, it is only used for research.[4]

Assessment of metastases detected in SLNs

The clinical significance of carcinoma metastases in SLNs is important because almost half of SLN-positive cases may have further metastases in non-sentinel nodes.[41] Extranodal invasion from the SLN, the size of the metastatic focus in the SLN, the number of positive SLN nodes, and the size and lymphovascular invasion of the primary tumour are correlated with non-SLN metastases.[41,42] Conversely, small primary tumours (i.e. T1a) and micrometastases are unlikely to have further metastases in non-SLNs.[20,42]

The negative predictive value of SLNs is considered good,[43] but the probabilities are significantly changed according to pathological procedures. Turner and colleagues analysed 1,087 non-sentinel nodes from 60 patients who were SLN-negative by H&E and immunohistochemistry. Only one node (one case) was positive for carcinoma, and the lesion was only detected by additional immunohistochemistry.[44] Thus, the probability of non-SLN metastasis will be less than 0.1% if SLN negativity is confirmed by both H&E and immunohistochemistry. In other words, isolated tumour cells in SLNs are unlikely to be associated with non-SLN involvement.

Finally, the clinical significance of micrometastases and/or isolated carcinoma cells has not been well elucidated. There are some studies,[4,27,45,46] but the real prognostic significance of micrometastases (i.e. detected by immunohistochemistry only) will only be clarified by future additional studies.

References

1. The Research Group of Population-based Cancer Registration in Japan. Cancer incidence and incidence rates in Japan in 1997. Estimates based on data from 12 population-based cancer registries. *Jpn J Clin Oncol* 2002;32:318-22.
2. Silverberg SG, Chitale AR. Assessment of significance of proportions of intraductal and infiltrating tumor growth in ductal carcinoma of the breast. *Cancer* 1973;32:830-7.
3. Osaka Cancer Registry (OCR), 65th issue. Osaka Prefecture, Japan: Osaka Cancer Registry, 2002. [In Japanese]
4. Cserni G, Amendoeira I, Apostolikas N, et al. Pathological work-up of sentinel lymph nodes in breast cancer. Review of current data to be considered for the formulation of guidelines. *Eur J Cancer* 2003; 39:1654-67.
5. Pikren JW. Significance of occult metastases. *Cancer* 1961;14:1266-71.
6. Carr I, Murari PJ, Pettigrew NM. Lymph nodes and spleen. In: Silverberg SG, ed. *Principles and Practice of Surgical Pathology*, 2nd edition. New York: Churchill-Livingstone, 1989:375-430.
7. Diaz LK, Hunt K, Ames F, et al. Histologic localization of sentinel lymph node metastases in breast cancer. *Am J Surg Pathol* 2003;27: 385-9.
8. Weaver DL. Sentinel lymph nodes and breast carcinoma. Which micrometastases are clinically significant? *Am J Surg Pathol* 2003;27: 842-5.
9. Greene FL, Page DL, Fleming ID, et al, eds. *AJCC Cancer Staging Manual*, 6th edition. New York: Springer, 2002:223-40.
10. Schwartz GF, Giuliano AE, Veronesi U, for the Consensus Conference Committee. Proceedings of the Consensus Conference on the Role of Sentinel Lymph Node Biopsy in Carcinoma of the Breast, April 19-22, 2001, Philadelphia, Pennsylvania. *Hum Pathol* 2002;33: 579-89.
11. Veronesi U, Paganelli G, Galimberti V, et al. Sentinel-node biopsy to avoid axillary dissection in breast cancer with clinically negative lymph-nodes. *Lancet* 1997;349:1864-7.
12. Strattmann SL, McCarthy TM, Kuhn J. Radiation safety with breast sentinel node biopsy. *Am J Surg* 1999;178:454-7.
13. Viale G, Bosari S, Mazzarol G, et al. Intraoperative examination of axillary sentinel lymph nodes in breast carcinoma patients. *Cancer* 1999;85:2433-8.
14. Canavese G, Gipponi M, Catturich A, et al. Sentinel lymph node mapping opens a new perspective in the surgical management of early-stage breast cancer: a combined approach with vital blue dye lymphatic mapping and radioguided surgery. *Semin Surg Oncol* 1998; 15:272-7.
15. Schneebaum S, Standler J, Cohen M, et al. Gamma probe-guided sentinel node biopsy - optimal timing for injection. *Eur J Surg Oncol* 1998:24:515-9.
16. Koller M, Barsuk D, Zippel D, et al. Sentinel lymph node involve-

ment - a predictor for axillary node status with breast cancer - has the time come? *Eur J Surg Oncol* 1998;24:166-8.
17. Imoto S, Fukukita H, Murakami K, et al. Pilot study on sentinel node biopsy in breast cancer. *J Surg Oncol* 2000;73:130-3.
18. Noguchi M, Tsugawa K, Miwa K, et al. Sentinel lymph node biopsy in breast cancer using blue dye with or without isotope localization. *Breast Cancer* 2000;7:287-96.
19. Motomura K, Inaji H, Komoike Y, et al. Intraoperative sentinel lymph node examination by imprint cytology and frozen sectioning during breast surgery. *Br J Surg* 2000;87:597-601.
20. Weiser MR, Montgomery LL, Susnik B, et al. Is routine intraoperative frozen-section examination of sentinel lymph nodes in breast carcinoma worthwhile? *Ann Surg Oncol* 2000;7:651-5.
21. Veronesi U, Paganelli G, Viale G, et al. Sentinel lymph node biopsy and axillary dissection in breast cancer: results in a large series. *J Natl Cancer Inst* 1999;91:368-73.
22. Moriya T, Manabe T, Irei I, et al. Significance of imprint/scrape cytology in intraoperative consultation of breast diseases. *Pathol Clin Med* 1994;12:719-23. [In Japanese]
23. Rubio IT, Korourian S, Cowan C, et al. Use of touch preps for intraoperative diagnosis of sentinel lymph node metastases in breast cancer. *Ann Surg Oncol* 1998;5:689-94.
24. Ratanawichitrasin A, Biscotti CV, Levy L, et al. Touch imprint cytological analysis of sentinel lymph nodes for detecting axillary metastases in patients with breast cancer. *Br J Surg* 1999;86:1346-8.
25. Henry-Tillman RS, Korourian S, Rubio IT, et al. Intraoperative touch preparation for sentinel lymph node biopsy: a 4-year experience. *Ann Surg Oncol* 2002;9:333-9.
26. Karamlou T, Johnson NM, Chan B, et al. Accuracy of intraoperative touch imprint cytologic analysis of sentinel lymph nodes in breast cancer. *Am J Surg* 2003;185:425-8.
27. Dowlatshahi K, Fan M, Bloom KJ, et al. Occult metastases in the sentinel lymph nodes of patients with early stage breast carcinoma: A preliminary study. *Cancer* 1999;86:990-6.
28. Turner RR, Ollila DW, Stern S, et al. Optimal histopathologic examination of the sentinel lymph node for breast carcinoma staging. *Am J Surg Pathol* 1999;23:263-7.
29. Nahrig J, Richter T, Kowolik J, et al. Comparison of different histopathological methods for the examination of sentinel lymph nodes in breast cancer. *Anticancer Res* 2000;20:2209-12.
30. Torrenga H, Rahusen FD, Meijer S, et al. Sentinel node investigation in breast cancer: detailed analysis of the yield from step sectioning and immunohistochemistry. *J Clin Pathol* 2001;54:550-2.
31. Czerniecki BJ, Scheff AM, Callans LS, et al. Immunohistochemistry with pancytokeratins improves the sensitivity of sentinel lymph node biopsy in patients with breast carcinoma. *Cancer* 1999;85: 1098-103.
32. Noguchi M, Bando E, Tsugawa K, et al. Staging efficacy of breast cancer with sentinel lymphadenectomy. *Breast Cancer Res Treat* 1999; 57:221-9.
33. Pendas S, Dauway E, Cox CE, et al. Sentinel node biopsy and cytokeratin staining for the accurate staging of 478 breast cancer patients. *Am Surg* 1999;65:500-5.
34. Kowolik JH, Kuhn W, Nahrig J, et al. Detection of micrometastases in sentinel lymph nodes of the breast applying monoclonal antibodies AE1/AE3 to pancytokeratins. *Oncol Rep* 2000;7:745-9.
35. Mann BG, Buchanan M, Collins PJ, et al. High incidence of micrometastases in breast cancer sentinel nodes. *Aust NZ J Surg* 2000;70: 786-90.
36. Wong SL, Chao C, Edwards MJ, et al, for the University of Louisville Breast Cancer Study Group. The use of cytokeratin staining in sentinel lymph node biopsy for breast cancer. *Am J Surg* 2001;182: 330-4.
37. Salem AA, Douglas-Jones AG, Sweetland HM, et al. Intraoperative evaluation of axillary sentinel lymph nodes using touch imprint cytology and immunohistochemistry. I. Protocol of rapid immunostaining of touch imprints. *Eur J Surg Oncol* 2003;29:25-8.
38. Freneaux P, Nos C, Vincent-Salomon A, et al. Histological detection of minimal metastatic involvement in axillary sentinel nodes. A rational basis for a sensitive methodology usable in daily practice. *Mod Pathol* 2002;15:641-6.
39. Carter BA, Jensen RA, Simpson JF, et al. Benign transport of breast epithelium into axillary lymph nodes after biopsy. *Am J Clin Pathol* 2000;113:259-65.
40. Page DL, Carter BA. Sentinel lymph nodes, breast cancer, and pseudometastases. *Breast Dis* 2002;12:362-3.
41. Turner RR, Chu KU, Qi K, et al. Pathologic features associated with nonsentinel lymph node metastases in patients with metastatic breast carcinoma in a sentinel lymph node. *Cancer* 2000;89:574-81.
42. Chu KU, Turner RR, Hansen NM, et al. Do all patients with sentinel node metastasis from breast carcinoma need complete axillary node dissection? *Ann Surg* 1999;229:536-41.
43. Liu GJ, Fan ZM, Tang Q, et al. Lymphatic mapping and sentinel lymph node biopsy in the patients with breast cancer. *Chin J Cancer Res* 2000;12:293-5.
44. Turner RR, Ollila DW, Krasne DL, et al. Histopathologic validation of the sentinel lymph node hypothesis for breast carcinoma. *Ann Surg* 1997;226:271-6.
45. Millis RR, Springall R, Lee AH, et al. Occult axillary lymph node metastases are of no prognostic significance in breast cancer. *Br J Cancer* 2002;86:396-401.
46. Lagios MD. Clinical significance of immunohistochemically detectable epithelial cells in sentinel lymph node and bone marrow in breast cancer. *J Surg Oncol* 2003;83:1-4.

[CANCER RESEARCH 64, 4670–4676, July 1, 2004]

Estrogen-Related Receptor α in Human Breast Carcinoma as a Potent Prognostic Factor

Takashi Suzuki,[1] Yasuhiro Miki,[1] Takuya Moriya,[1] Norihiro Shimada,[1] Takanori Ishida,[2] Hisashi Hirakawa,[3] Noriaki Ohuchi,[2] and Hironobu Sasano[1]

Departments of [1]Pathology and [2]Surgery, Tohoku University School of Medicine, Sendai, Japan; and [3]Department of Surgery, Tohoku Kosai Hospital, Sendai, Japan

ABSTRACT

Estrogen-related receptor α (ERRα) was identified as a gene related to estrogen receptor α (ERα) and belongs to a class of nuclear orphan receptors. ERRα binds to estrogen responsive element(s) (ERE) and is considered to be involved in modulation of estrogenic actions. However, biological significance of ERRα remains largely unknown. Therefore, we examined the expression of ERRα in human breast carcinoma tissues using immunohistochemistry ($n = 102$) and real-time reverse transcription-PCR ($n = 30$). ERRα immunoreactivity was detected in the nuclei of carcinoma cells in 55% of breast cancers examined, and relative immunoreactivity of ERRα was significantly ($P = 0.0041$) associated with the mRNA level. Significant associations were detected between ERα and ERE-containing estrogen-responsive genes, such as pS2 ($P < 0.0001$) and EBAG9/RCAS1 ($P = 0.0214$), in breast carcinoma tissues. However, no significant association was detected between ERα and pS2 ($P = 0.1415$) in the ERRα-positive cases ($n = 56$) or between ERα and EBAG9/RCAS1 ($P = 0.8271$) in the ERRα-negative group ($n = 46$). ERRα immunoreactivity was significantly associated with an increased risk of recurrence and adverse clinical outcome by both uni- ($P = 0.0097$ and $P = 0.0053$, respectively) and multi- ($P = 0.0215$ and $P = 0.0118$, respectively) variate analyses. A similar tendency was also detected in the group of breast cancer patients who received tamoxifen therapy after surgery. Results from our study suggest that ERRα possibly modulates the expression of ERE-containing estrogen-responsive genes, and ERRα immunoreactivity is a potent prognostic factor in human breast carcinoma.

INTRODUCTION

Estrogens are well known to contribute immensely to the development of hormone-dependent breast carcinomas (1, 2). Biological effects of estrogens are mediated through an interaction with estrogen receptor (ER) α and/or β (3). ERs activate transcription of various target genes (*i.e.*, estrogen responsive genes) in a ligand-dependent manner by direct DNA interaction through the estrogen-responsive element(s) (ERE) or by tethering to other transcription factors (4, 5). Therefore, antiestrogens such as tamoxifen, which blocks ER, have been mainly used as an endocrine therapy in breast carcinoma for many years.

Estrogen-related receptor (ERR) family belongs to nuclear hormone receptors, and consists of three closely related members (α, β, and γ; Refs. 6 and 7). ERRs share significant homology to ERα at the DNA-binding domain and recognize the ERE (8–10), which indicates that ERRs modulate the actions of ERs (11–13). However, ERRs are not activated by known natural estrogens and are therefore classified as orphan receptors (14). ERRs can also bind to steroidgenic factor 1 (SF1)-binding element within the promoter regions of various steroidogenic P450 genes including aromatase (15, 16).

Previous *in vitro* studies have demonstrated the mRNA expression of ERRα in breast cancer cell lines (17) and breast carcinoma tissues (18). ERRα activated the expression of pS2, one of the estrogen responsive genes (17), in breast cancer cells, and it has also been reported that ERRα regulated aromatase expression in breast fibroblasts (11). However, a detailed examination of ERRα expression, including at the protein level, has not been examined in human breast carcinoma tissues, and the biological significance of ERRα remains largely unclear. Therefore, in this study, we examined the immunolocalization of ERRα in 102 cases of human breast carcinoma tissues and correlated these findings with various clinicopathological factors including the clinical outcome. In addition, we also examined mRNA expression of ERRα in 30 cases of breast carcinoma tissues using real-time reverse transcription-PCR and analyzed the correlation with the ERRα immunoreactivity or aromatase mRNA expression.

MATERIALS AND METHODS

Patients and Tissues. One hundred and two specimens of invasive ductal carcinoma of the breast were obtained from female patients who underwent mastectomy from 1985 to 1990 in the Department of Surgery, Tohoku University Hospital, Sendai, Japan. Breast tissue specimens were obtained from patients with a mean age of 53.6 years (range 27–82). None of the patients examined used oral contraceptives. The patients did not receive chemotherapy or irradiation before surgery. Eighty-eight patients received adjuvant chemotherapy, and ten patients received tamoxifen therapy after the surgery. The mean follow-up time was 106 months (range 4–157 months). The histological grade of each specimen was evaluated based on the method of Elston and Ellis (19). All specimens were fixed with 10% formalin and embedded in paraffin wax.

Thirty specimens of invasive ductal carcinoma were obtained from patients who underwent mastectomy in 2000 in the Departments of Surgery at Tohoku University Hospital and Tohoku Kosai Hospital, Sendai, Japan. Specimens of adipose tissue adjacent to the carcinoma and non-neoplastic breast tissues were available for examination in 7 and 5 of these 30 cases, respectively. Specimens for RNA isolation were snap-frozen and stored at −80°C, and those for immunohistochemistry were fixed with 10% formalin and embedded in paraffin-wax. Informed consent was obtained from all patients before their surgery and examination of specimens used in this study.

Research protocols for this study were approved by the Ethics Committee at both Tohoku University School of Medicine and Tohoku Kosai Hospital.

Antibodies. Mouse monoclonal antibody for ERRα (2ZH5844H) was purchased from Perseus Proteomics Inc. (Tokyo, Japan). This antibody was produced by immunizing mice with a systemic peptide corresponding to amino acids 98–171 of ERRα (GenBank accession number; X51416), and the characterization was confirmed by immunoblotting analyses.[4] Rabbit polyclonal antibody for estrogen sulfotransferase (EST; *SULT 1E1* gene; PV-P2237; Ref. 20) was purchased from Medical Biological Laboratory (Nagoya, Japan). EBAG9/RCAS1 antibody was a rabbit polyclonal antibody (21, 22) and was kindly provided from Dr. S. Inoue (Department of Biochemistry, Saitama Medical School, Saitama, Japan). Monoclonal antibodies for ERα (ER1D5), progesterone receptor (PR; MAB429), Ki-67 (MIB1), pS2 (M7184), cyclin D1 (P2D11F11), and c-myc (1–6E10) were purchased from Immunotech (Marseille, France), Chemicon (Temecula, CA), DAKO (Carpinteria, CA), DAKO, Novocastra Laboratories (Newcastle, United Kingdom), and Cambridge Research Biochemical (Cambridge, United Kingdom), respectively. Rabbit polyclonal antibodies for ERβ (06–629) and human epidermal growth factor

Received 1/26/04; revised 4/1/04; accepted 5/4/04.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Requests for reprints: Takashi Suzuki, Department of Pathology, Tohoku University School of Medicine, 2-1 Seiryo-machi, Aoba-ku, Sendai, 980-8575, Japan. Phone: 81-22-717-8050; Fax: 81-22-717-8051; E-mail: t-suzuki@patholo2.med.tohoku.ac.jp.

[4] Perseus Proteomics Inc., unpublished data.

receptor 2 (HER2; A0485) were obtained from Upstate Biotechnology (Lake Placid, NY) and DAKO, respectively.

Immunohistochemistry. A Histofine kit (Nichirei, Tokyo, Japan), which uses the streptavidin-biotin amplification method, was used for the identification of ERRα, ERα, PR, EST, HER2, Ki-67, pS2, EBAG9/RCAS1, cyclin D1, and c-myc immunoreactivity, whereas EnVision$^+$ (DAKO) was used for ERβ immunohistochemical analysis. Antigen retrieval for ERRα, ERα, ERβ, PR, HER2, Ki-67, EBAG9/RCAS1, and cyclin D1 immunostaining was performed by heating the slides in an autoclave at 120°C for 5 min in citric acid buffer [2 mM citric acid and 9 mM trisodium citrate dehydrate (pH 6.0)], and similarly, antigen retrieval for EST and pS2 immunostaining was done by heating the slides in a microwave oven for 15 min in a citric acid buffer. Dilutions of primary antibodies used in this study were as follows: ERRα, 1:1000; ERα, 1:50; ERβ, 1:50; PR, 1:30; EST, 1:9000; HER2, 1:200, Ki-67, 1:50; pS2, 1:30; EBAG9/RCAS1, 1:20; cyclin D1, 1:40; and c-myc 1:600. The antigen-antibody complex was visualized with 3,3′-diaminobenzidine solution (1 mM 3,3′-diaminobenzidine, 50 mM Tris-HCl buffer (pH 7.6), and 0.006% H_2O_2) and counterstained with hematoxylin.

Human tissues of heart were used as positive controls for ERRα immunohistochemistry (23). As a negative control for ERRα immunohistochemistry, normal mouse IgG was used instead of the primary antibody for ERRα, and no specific immunoreactivity was detected in these sections.

Real-Time Reverse Transcription-PCR. Total RNA was carefully extracted with guanidinium thiocyanate followed by ultracentrifugation in cesium chloride. A reverse transcription kit (SUPERSCRIPT II Preamplification system; Life Technologies, Inc., Grand Island, NY) was used in the synthesis of cDNA.

The Light Cycler System (Roche Diagnositics GmbH, Mannheim, Germany) was used to semi-quantify the mRNA level of ERRα, aromatase, and ribosomal protein L 13a (RPL13A) by real-time reverse transcription-PCR (24). Settings for the PCR thermal profile were as follos: initial denaturation at 95°C for 1 min followed by 40 amplification cycles of 95°C for 1 s, annealing at 62°C (ERRα), 60°C (aromatase), or 68°C (RPL13A) for 15 s, and elongation at 72°C for 15 s. The primer sequences used in this study are as follows: ERRα [X51416; forward 5′-TGCTCAAGGAGGGAGTGC-3′ (cDNA position; 785–802) and reverse 5′-GGCGACAATTTCTGGTTCGGGTCAGGCATGGCATAG-3′ (cDNA position; 981–998)], aromatase [(X13589; Ref. 20; forward 5′-GTGAAAAAGGGGACAAACAT-3′ (cDNA position; 1286–1305) and reverse 5′-TGGAATCGTCTCAGAAGTGT-3′ (cDNA position; 1481–1500)], and RPL13A [(NM012423; 25; forward 5′-CCTGGAGGAGAAGAGGAAAGAGA-3′ (cDNA position; 487–509) and reverse 5′-TTGAGGACCTCTGTGTATTTGTCAA-3′ (cDNA position; 588–612)]. Oligonucleotide primers for ERRα were designed in different exons to avoid the amplification of genomic DNA or human ERRα pseudo-gene (U85258). To verify amplification of the correct sequences, PCR products were purified and subjected to direct sequencing. Human heart tissue was used as a positive control for ERRα, whereas human placental tissue was used as a positive control for aromatase. Negative control experiments lacked cDNA substrate to check for the possibility of exogenous contaminant DNA, and no amplified products were detected under these conditions. mRNA level for ERRα and aromatase in each case has been summarized as a ratio of RPL13A and subsequently evaluated as a ratio (%) compared with that of the positive controls.

Scoring of Immunoreactivity and Statistical Analysis. ERRα, ERα, ERβ, PR, and Ki-67 immunoreactivity was scored in >1000 carcinoma cells for each case, and the percentage of immunoreactivity, *i.e.*, labeling index (LI), was determined. In this study, cases that were found to have ERRα LI of >10% were considered ERRα-positive breast carcinomas, according to a report for ERα and PR by Allred *et al.* (26). Immunoreactivity of EST was classified into the following three categories: ++, >50% positive cells; +, 1–50% positive cells; and −, no immunoreactivity, according to a previous report (20).

Values for LIs for ERRα, ERα, ERβ, PR, Ki-67, ERRα mRNA level, patient age, and tumor size were summarized as a mean ± 95% confidence interval. The association between immunoreactivity for ERRα status and these parameters were evaluated using a one-way ANOVA and Bonferroni test. The association between ERRα and PR LIs, and the association between ERRα mRNA and ERRα LI or aromatase mRNA were performed using a correlation coefficient (r) and regression equation. Statistical difference between ERRα status and menopausal status, stage, lymph node status, histological grade, ERα status, EST, or HER2 status was evaluated in a cross-table using the χ^2 test. Overall and disease-free survival curves were generated according to the Kaplan-Meier method, and the statistical significance was calculated using the log-rank test. Univariate and multivariate analyses were evaluated by Cox proportional hazards model using PROC PHREG in our SAS software. Differences with Ps < 0.05 were considered significant.

RESULTS

Immunohistochemistry for ERRα in Breast Carcinoma Tissues. Immunoreactivity for ERRα was detected in the nuclei of invasive ductal carcinoma cells (Fig. 1*A*). A mean value of ERRα LI in the 102 breast carcinoma tissues examined was 23.0% (range 0–75%), and a number of ERRα-positive breast carcinomas (*i.e.*, ERRα LI ≥ 10%) was 56 of102 cases (54.9%). ERRα immunoreactivity was focally detected in epithelial cells of morphologically normal glands (Fig. 1*B*), whereas the stroma or adipose tissue was immunohistochemically negative for ERRα. A mean value of ERRα LI in non-neoplastic mammary epithelia was 14.6% (range 0–33%), and the number of cases showing higher ERRα LI in carcinoma cells than that in non-neoplastic mammary epithelia was 49 of 102 (48.0%). In positive control sections for ERRα immunohistochemistry, ERRα immunoreactivity was markedly detected in the nuclei of myocardial cells of the heart (Fig. 1*C*).

Associations between ERRα immunoreactivity and clinicopathological parameters in 102 breast carcinomas are summarized in Table 1. ERRα immunoreactivity tended to be positively associated with ERα status and ERα LI and negatively associated with EST; however the correlation did not reach a statistical significance ($P = 0.0848$, $P = 0.1485$, and $P = 0.1224$, respectively). No significant association was detected between ERRα immunoreactivity and the other clinicopathological parameters examined, including patient age, menopausal status, stage, tumor size, lymph node status, histological grade, ERβ LI, PR LI, HER2 status, and Ki-67 LI, in this study.

Influence of ERRα Status on the Association between ERα and Estrogen Responsive Genes. pS2, EBAG9/RCAS1, PR, cyclin D1, and c-myc are all well recognized as estrogen-responsive genes in human breast cancers. As shown in Table 2, a significant positive association was detected between ERα LI and the status of these immunoreactivity genes except for c-myc in the 102 breast cancer tissues examined ($P < 0.0001$ for pS2, $P = 0.0214$ for EBAG9/RCAS1, $P < 0.0001$ for PR LI, $P = 0.0002$ for cyclin D1, and $P = 0.9372$ for c-myc), which agrees well with previous immunohistochemical studies (22, 27–30). However, when the breast cancers were classified into two groups according to ERRα status, no significant association was detected between ERα LI and pS2 in the group of ERRα-positive breast carcinomas ($P = 0.1415$; $n = 56$) or between ERα LI and EBAG9/RCAS1 in ERRα-negative breast cancers ($P = 0.8271$; $n = 46$). On the other hand, significant association was detected between ERα LI and PR LI ($P < 0.0001$ in ERRα-positive cases; $P < 0.0001$ in ERRα-negative cases) or cyclin D1 ($P = 0.0126$ in ERRα-positive cases; $P = 0.0082$ in ERRα-negative cases), regardless of the ERRα status in the breast cancer cases examined.

No significant association was detected between ERRα LI and these estrogen-responsive genes regardless of ERα status in 102 breast carcinoma tissues (Table 3).

Correlation between ERRα Immunoreactivity and the Clinical Outcome of the Patients. ERRα immunoreactivity was significantly associated with an increased risk of recurrence ($P = 0.0071$, log-rank test; Fig. 2*A*). After univariate analysis by Cox proportional hazards model (Table 4), lymph node status ($P < 0.0001$), tumor size ($P < 0.0001$), EST ($P = 0.0035$), and ERRα immunoreactivity

Fig. 1. Immunohistochemistry for ERRα in invasive ductal carcinoma. *A*, ERRα immunoreactivity was detected in the nuclei of invasive ductal carcinoma cells. *ERR*, estrogen-related receptor α. *B*, in morphologically normal mammary glands, immunoreactivity for ERRα was weakly detected in the nuclei of epithelial cells. *C*, in the positive control for ERRα immunohistochemistry, ERRα immunoreactivity was detected in the nucleus of myocardial cells in the heart. *Bar* = 50 μm, respectively.

Table 1 *Association between ERRα immunoreactivity and clinicopathological parameters in 102 breast carcinomas*

	ERRα immunoreactivity		
	+ (n = 56)	− (n = 46)	P
Age (yrs)[b]	54.3 ± 1.6	52.8 ± 1.8	0.5271
Menopausal status			
Premenopausal	27 (26.5%)	20 (19.7%)	
Postmenopausal	29 (28.4%)	26 (25.5%)	0.6329
Stage			
I	14 (13.7%)	15 (14.7%)	
II	35 (34.3%)	26 (25.5%)	
III	7 (6.9%)	5 (4.9%)	0.6852
Tumor size (mm)[b]	25.6 ± 1.8	24.8 ± 1.8	0.7443
Lymph node status			
Positive	27 (26.5%)	19 (18.7%)	
Negative	29 (28.4%)	27 (26.5%)	0.4849
Histological grade			
1	14 (13.7%)	13 (12.7%)	
2	22 (21.6%)	14 (13.7%)	
3	20 (19.7%)	19 (18.6%)	0.6462
ERα status			
Positive	45 (44.1%)	30 (29.4%)	
Negative	11 (10.8%)	16 (15.7%)	0.0848
ERα LI[b]	47.5 ± 4.5	38.1 ± 5.2	0.1485
ERβ LI[b]	15.3 ± 2.4	14.6 ± 2.7	0.8493
PR LI[b]	45.6 ± 4.8	40.7 ± 5.1	0.4894
EST			
−	35 (34.3%)	24 (23.5%)	
+	10 (9.8%)	15 (14.7%)	
++	11 (10.8%)	7 (6.9%)	0.1224
HER2 status			
Positive	20 (19.6%)	15 (14.7%)	
Negative	36 (35.3%)	31 (30.4%)	0.7421
Ki-67 LI[b]	24.7 ± 2.0	27.4 ± 2.7	0.4045

[a] ERRα, estrogen-related receptor α; ERα, estrogen receptor α; LI, labeling index; EST, estrogen sulfotransferase; HER2, human epidermal growth receptor 2.
[b] Data are presented as mean ± 95% confidence interval. All other values represent the number of cases and percentage.

(P = 0.0097) were demonstrated as significant prognostic parameters for disease-free survival in 102 breast carcinoma patients. A multivariate analysis (Table 4), however, revealed that only lymph node status (P = 0.0015) and ERRα immunoreactivity (P = 0.0215) were independent-prognostic factors with relative risks over 1.0, whereas tumor size and EST were not significant.

Overall survival curve was demonstrated in Fig. 2*B*, and a significant correlation was detected between ERRα immunoreactivity and adverse clinical outcome of the patients (P = 0.0018, log-rank test). Using a univariate analysis (Table 5), lymph node status (P < 0.0001), tumor size (P = 0.0002), ERRα immunoreactivity (P = 0.0053), EST (P = 0.0065), HER2 status (P = 0.0175), adjuvant chemotherapy (P = 0.0233), and histological grade (P = 0.0310) turned out to be significant prognostic factors for overall survival in this study. Multivariate analysis revealed that lymph node status (P = 0.0085), ERRα immunoreactivity (P = 0.0118), and EST (P = 0.0382) were independent-prognostic factors with a relative risk over 1.0; however other factors were not significant in this study (Table 5).

Ten patients received tamoxifen therapy after surgery, and these cases were ERα-positive breast cancers. The disease-free and overall survival curves in these patients were summarized in Fig. 2, *C* and *D*. ERRα immunoreactivity was also markedly associated with an increased risk of recurrence and worse prognosis in the group of breast cancer patients who received tamoxifen therapy, although *P*s were not available because no patient had a recurrence or died in the group of ERRα-negative breast cancers. Association between ERRα immunoreactivity and clinical outcome of the patients was not significantly changed regardless of the status of adjuvant chemotherapy after surgery in this study (data not shown).

Table 2 *Correlation between ERα[a] and estrogen responsive gene immunoreactivities associated with ERRα status in 102 breast carcinomas*

	Total (n = 102)		ERRα positive (n = 56)		ERRα negative (n = 46)	
	ERα LI	P	ERα LI	P	ERα LI	P
pS2						
Positive	54.7 ± 4.0		54.3 ± 5.5		53.8 ± 6.5	
Negative	28.8 ± 5.1	**<0.0001**	38.5 ± 7.2	0.1415	14.8 ± 6.3	**<0.0001**
EBAG9/RCAS1						
Positive	46.7 ± 4.4		51.6 ± 5.9		40.8 ± 6.7	
Negative	27.9 ± 7.3	**0.0214**	17.4 ± 6.1	**0.0093**	45.0 ± 16.4	0.8271
PR LI		**<0.0001** (r = 0.590)		**<0.0001** (r = 0.515)		**<0.0001** (r = 0.675)
Cyclin D1						
Positive	57.3 ± 4.5		59.6 ± 6.0		54.5 ± 7.0	
Negative	32.2 ± 4.4	**0.0002**	37.2 ± 6.0	**0.0126**	26.5 ± 6.5	**0.0082**
c-myc						
Positive	43.7 ± 5.3		47.9 ± 6.8		37.4 ± 8.3	
Negative	44.2 ± 4.5	0.9372	48.4 ± 6.1	0.9583	39.8 ± 6.7	0.8321

Ps < 0.05 were considered significant, and described as boldface.
[a] ERα, estrogen receptor α; ERRα, estrogen-related receptor α; PR, progesterone receptor; LI, labeling index.

ERRα mRNA Expression in the Breast Carcinoma Tissues. mRNA expression for ERRα, aromatase, and RPL13A was detected as a specific single band (214, 215, and 126 bp, respectively) and was semi-quantified by real-time reverse transcription-PCR. Expression of ERRα mRNA was detected markedly in the breast carcinoma tissues (65.7 ± 9.0%) but was low in non-neoplastic breast tissues (25.4 ± 6.0%, P = 0.0448 *versus* carcinoma tissues) or adipose tissues adjacent to the carcinoma (12.6 ± 7.3%, P = 0.0174 *versus* carcinoma tissues; Fig. 3*A*). ERRα mRNA expression was closely correlated with the ERRα immunoreactivity evaluated as ERRα LI (P = 0.0041, r = 0.509) in 30 breast carcinoma tissues examined (Fig. 3*B*). However, mRNA expression of ERRα was not significantly associated with that of aromatase (P = 0.6441, r = -0.088) in this study (Fig. 3*C*).

DISCUSSION

In this study, ERRα immunoreactivity was detected in the nuclei of carcinoma cells in 55% of breast cancer tissues and was significantly associated with its mRNA level. ERRα mRNA expression was demonstrated previously in various human breast cancer cell lines, breast carcinoma tissues, and normal mammary epithelial cells (17, 18), and our present findings were in good agreement with these previous reports. Results in our present study also demonstrated that ERRα immunoreactivity tended to be positively or inversely associated with ERα or EST, respectively. The possible correlation between ERRα and ERα expression remains controversial. Ariazi *et al.* (18) reported that increased ERRα mRNA levels were associated with ER-negative and PR-negative tumor status in 38 breast cancer tissues and suggested a possible unfavorable marker in the breast cancers. However, Liu *et al.* (31) demonstrated that estrogens stimulate the expression of ERRα in the human breast cell lines, and suggested that ERRα is a downstream target of ERα. On the other hand, EST catalyzes estrogens to biologically inactive estrogen sulfates (32, 33) and is considered to diminish estrogen actions in the breast cancers (20). Therefore, our present results suggest that expression of ERRα is, at least in a part, associated with estrogenic actions.

In our present study, significant associations were detected between ERα and estrogen responsive genes such as pS2, EBAG9/RCAS1, PR, and cyclin D1, as was reported previously (22, 27–29). However, the significant association between ERα and pS2 or EBAG9/RCAS1 disappeared in the group of ERRα-positive or -negative breast cancers, respectively. On the other hand, correlation between ERα and PR, cyclin D1, or c-myc was not influenced by ERRα status in these breast cancer patients examined. Both *pS2* and *EBAG9/RCAS1* genes are induced by ERα through an ERE in the promoter region (34, 35). However, functional ERE has not been identified in *PR* (36) and *cyclin D1* (5), and these are considered to be induced by ER through the interaction between ER and other DNA-binding transcription factors. Considering that ERα and ERRα directly compete for binding EREs (13), our present data suggest that ERRα mainly modulates ERα-mediated ERE-dependent transcription and changes the expression pattern of estrogen-responsive genes in the breast cancer cells.

ERRα immunoreactivity was significantly associated with an increased risk of recurrence or adverse clinical outcome of the patients, and results of multivariate analyses demonstrated that ERRα immunoreactivity is an independent-prognostic factor. Estrogens induce

Table 3 *Correlation between ERRα[a] and estrogen responsive gene immunoreactivities associated with ERα status in 102 breast carcinomas*

	Total (n = 102)		ERα positive (n = 75)		ERα negative (n = 27)	
	ERRα LI	P	ERRα LI	P	ERRα LI	P
pS2						
Positive	23.5 ± 2.7		23.0 ± 2.9		27.7 ± 8.6	
Negative	22.4 ± 3.4	0.7981	27.7 ± 4.5	0.3777	16.3 ± 4.8	0.2776
EBAG9/RCAS1						
Positive	22.6 ± 2.6		24.2 ± 3.0		18.5 ± 4.9	
Negative	23.6 ± 5.8	0.8834	21.8 ± 6.8	0.7542	29.0 ± 11.3	0.5341
PR LI		0.5072 (r = 0.066)		0.9069 (r = 0.014)		0.9671 (r = 0.008)
Cyclin D1						
Positive	24.7 ± 3.4		24.8 ± 3.6		23.3 ± 11.0	
Negative	21.8 ± 2.8	0.5134	24.2 ± 3.4	0.9058	18.1 ± 4.7	0.6770
c-myc						
Positive	25.0 ± 3.3		25.8 ± 3.7		22.5 ± 7.7	
Negative	22.0 ± 2.8	0.4943	23.7 ± 3.4	0.6648	17.8 ± 5.3	0.6100

[a] ERRα, estrogen-related receptor α; LI, labeling index; ERα, estrogen receptor α; PR, progesterone receptor.

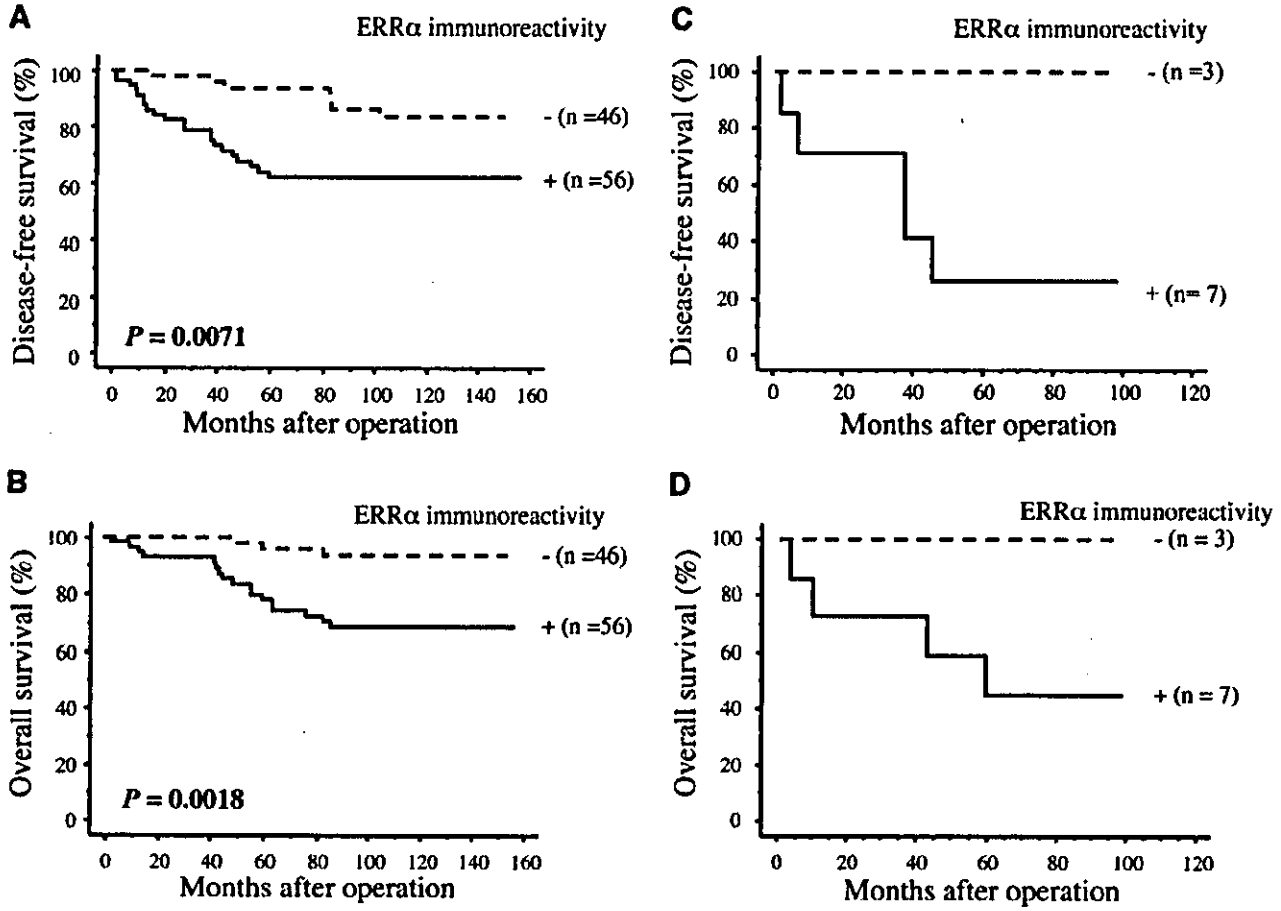

Fig. 2. *A* and *B*, disease-free (*A*) and overall (*B*) survival of 102 patients with breast carcinoma according to ERRα immunoreactivity (Kaplan-Meier method). ERRα immunoreactivity was significantly associated with an increased risk of recurrence ($P = 0.0071$, log-rank test; *A*, and worse prognosis ($P = 0.0018$, log-rank test). *C* and *D*, disease-free (*C*) and overall (*D*) survival of 10 patients received tamoxifen therapy after surgery according to ERRα immunoreactivity (Kaplan-Meier method). ERRα immunoreactivity was also associated with an increased risk of recurrence (*C*) and worse prognosis (*D*) in the group of patients who received tamoxifen therapy. *Ps* were not calculated, because no patient had a recurrence or died in the group of ERRα-negative breast cancer patients. *ERR*, estrogen-related receptor α.

Table 4 *Univariate and multivariate analyses of disease-free survival in 102 breast cancer patients examined*

Variable	*P*		Relative risk (95% CI[a])
	Univariate	Multivariate	
Lymph node status (pN_3-pN_0)[b]	**<0.0001[c]**	**0.0015**	2.593 (1.441–4.666)
Tumor size (75–7 mm)[b]	**<0.0001[c]**	0.3306	
EST (−/+, ++)	**0.0035[c]**	0.0613	
ERRα immunoreactivity (positive/negative)	**0.0097[c]**	**0.0215**	1.953 (1.116–3.149)
c-myc (positive/negative)	0.0581		
Adjuvant chemotherapy (no/yes)	0.1305		
Ki-67 LI (≥10/<10)	0.1795		
HER2 status (positive/negative)	0.2713		
Histological grade (3/1, 2)	0.2911		
ERα status (positive/negative)	0.4363		

[a] CI, confidence interval; EST, estrogen sulfotransferase; ERRα, estrogen-related receptor α; HER2, human epidermal growth factor receptor 2; LI, labeling index; ERα, estrogen receptor α.

[b] Data were evaluated as continuous variables in the uni- and multivariate analyses. All other data were evaluated as dichotomized variables.

[c] Data were considered significant in the univariate analyses, and were examined in the multivariate analyses.

various estrogen responsive genes in breast cancer cells, and these genes include not only activators of cell growth such as cyclin D1 (37) or c-myc (38) but also relatively good prognostic markers such as pS2 (29) or PR (39). ERRs display significant constitutive transcriptional activity (7, 9, 40). Therefore, poor clinical outcome in ERRα-positive breast cancer patients may be partly caused by constitutive modulation of the expression of estrogen-responsive genes, although we could not directly demonstrate such hypothesis from our present data, because of the lack of mechanistic examinations and the relatively limited number of cases examined in this study. Additional examinations are required to clarify the detailed mechanism of ERRα action in the breast cancer tissues.

Table 5 *Univariate and multivariate analyses of overall survival in 102 breast cancer patients examined*

Variable	*P*		Relative risk (95% CI[a])
	Univariate	Multivariate	
Lymph node status (pN_3-pN_0)[b]	**<0.0001[c]**	**0.0085**	2.414 (1.252–4.653)
Tumor size (75–7 mm)[b]	**0.0002[c]**	0.2675	
ERRα immunoreactivity (positive/negative)	**0.0053[c]**	**0.0118**	5.076 (1.217–21.173)
EST (−/+, ++)	**0.0065[c]**	**0.0382**	4.101 (1.027–19.705)
HER2 status (positive/negative)	**0.0175[c]**	0.4669	
Adjuvant chemotherapy (no/yes)	**0.0233[c]**	0.0635	
Histological grade (3/1, 2)	**0.0310[c]**	0.1458	
Ki-67 LI (≥10/<10)	0.1818		
c-myc (positive/negative)	0.2697		
ERα status (positive/negative)	0.7646		

[a] CI, confidence interval; ERRα, estrogen-related receptor α; EST, estrogen sulfotransferase; HER2, human epidermal growth factor receptor 2; LI, labeling index; ERα, estrogen receptor α.

[b] Data were evaluated as continuous variables in the uni- and multivariate analyses. All other data were evaluated as dichotomized variables.

[c] Data were considered significant in the univariate analyses, and were examined in the multivariate analyses.

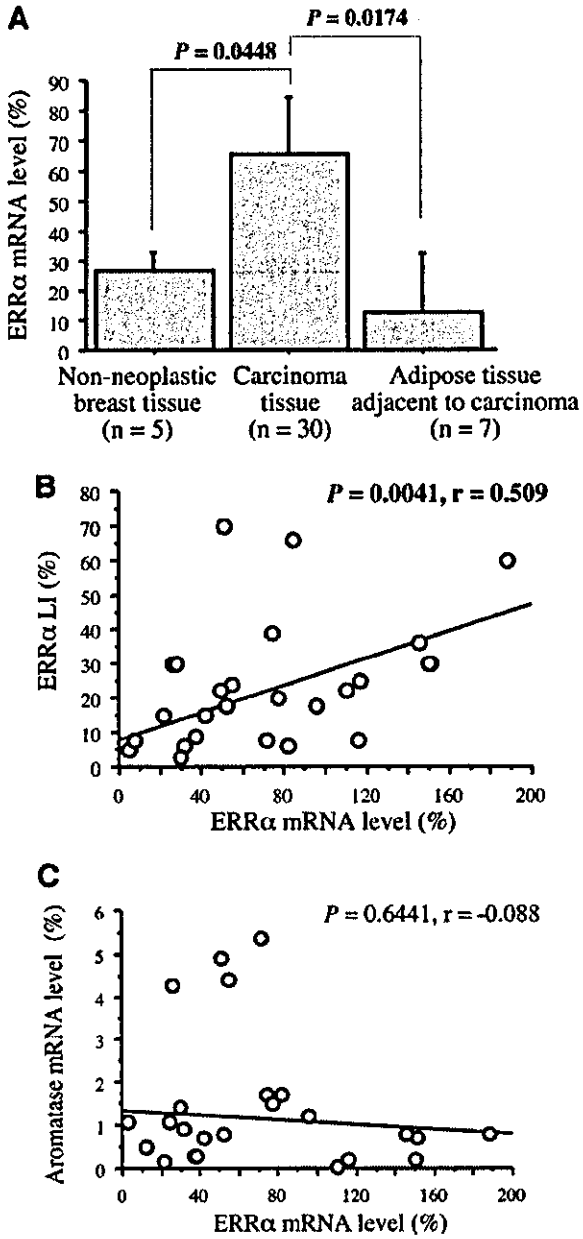

Fig. 3. Real-time reverse transcription-PCR for ERRα in the breast carcinoma. *A*, expression of ERRα mRNA was significantly higher in the breast carcinoma tissues (65.7 ± 9.0%, n = 30) than in non-neoplastic breast tissues [25.4 ± 6.0% (n = 5), P = 0.0448 *versus* carcinoma tissues] or adipose tissues adjacent to the carcinoma [12.6 ± 7.3% (n = 7), P = 0.0174 *versus* carcinoma tissues]. Data represent the mean ± 95% confidence interval. The mRNA level of ERRα in each specimen was evaluated as a ratio (%) of the positive control tissue (human heart tissue = 100%). *B*, association between the mRNA level and relative immunoreactivity (labeling index) of ERRα in 30 cases of breast carcinoma tissues. Significant positive association was detected (P = 0.0041, r = 0.509). *C*, association between ERRα and aromatase mRNA levels in 30 breast carcinoma tissues. No significant correlation was detected (P = 0.6441, r = −0.088). Aromatase mRNA expression in each case was evaluated as a ratio (%) of that in the human placental tissue. *ERR*, estrogen-related receptor α.

ERRα immunoreactivity was also associated with poor prognosis in the group of breast cancer patients who received tamoxifen therapy, which suggests that ERRα status is a possible predictive marker for tamoxifen therapy, although the number of cases examined was limited in this study. Previous *in vitro* studies demonstrated that both tamoxifen and 4-hydroxytamoxifen did not bind to ERRα or did not have any effects on the transcriptional activity of ERRα, whereas these are high-affinity ligands for ERRβ or ERRγ (41, 42). Therefore, ERRα may constitutively function independently of tamoxifen and result in tamoxifen resistance in ERRα-positive breast cancer patients.

Aromatase is a key enzyme in *in situ* estrogen biosynthesis in breast cancer tissue, and aromatase inhibitors are currently used in breast cancer patients as an endocrine therapy as well as antiestrogens. Aromatase is markedly activated by SF1 through an SF1-binding element within the promoter region (43). However, SF1 is not expressed in breast carcinoma tissues (11, 44). Previously, Yang *et al.* (11) reported the induction of aromatase expression by ERRα through a SF1-binding element in breast fibroblast, suggesting the possible importance of ERRα as a regulator of aromatase expression in breast cancer. However, in our study, we did not find ERRα immunoreactivity in the intra-tumoral stromal cells or adipocytes adjacent to the carcinoma, although these cells are well-known to express aromatase (45). Previous *in vitro* studies have shown the regulation of aromatase transcription in breast fibroblasts and/or adipocytes by various factors, including cytokines (46), prostaglandin E_2 (47), liver receptor homologue-1 (44) and CCAAT/enhancer-binding protein β (48).

In summary, ERRα immunoreactivity was detected in carcinoma cells in 55% of breast cancer tissues and was associated with its mRNA level. Association between ERα and ERE-containing estrogen-responsive genes was markedly altered according to ERRα status in the breast cancer tissues. ERRα immunoreactivity was associated with poor prognosis of the patients, and similar tendency was also detected in the group who received tamoxifen therapy. These findings suggest that ERRα possibly modulates the expression of ERE-containing estrogen responsive genes, and ERRα immunoreactivity is a potent prognostic factor, including a possible predictive marker for tamoxifen resistance, in human breast carcinoma.

ACKNOWLEDGMENTS

We thank Yoshiko Murakami, Chika Kaneko, and Yuko Hirakata (Department of Pathology, Tohoku University School of Medicine, respectively) for skillful technical assistance.

REFERENCES

1. Thomas DB. 1984 Do hormones cause cancer? Cancer (Phila) 1984;53:595–604.
2. Vihko R, Apter D. Endogenous steroids in the pathophysiology of breast cancer. Crit Rev Oncol Hematol 1989;9:1–16.
3. Kuiper GG, Enmark E, Pelto-Huikko M, Nilsson S, Gustafsson JA. Cloning of a novel receptor expressed in rat prostate and ovary. Proc Natl Acad Sci USA 1996; 93:5925–30.
4. Tsai MJ, O'Malley BW. Molecular mechanisms of action of steroid/thyroid receptor superfamily members. Annu Rev Biochem 1994;63:451–86.
5. Acconcia F, Marino M. Synergism between genomic and non genomic estrogen action mechanisms. IUBMB Life 2003;55:145–50.
6. Giguere V, Yang N, Segui P, Evans RM. Identification of a new class of steroid hormone receptors. Nature (Lond) 1988;331:91–4.
7. Hong H, Yang L, Stallcup MR. Hormone-independent transcriptional activation and coactivator binding by novel orphan nuclear receptor ERR3. J Biol Chem 1999;274: 22618–26.
8. Yang N, Shigeta H, Shi H, Teng CT. Estrogen-related receptor, hERR1, modulates estrogen receptor-mediated response of human lactoferrin gene promoter. J Biol Chem 1996;271:5795–804.
9. Vanacker JM, Bonnelye E, Chopin-Delannoy S, Delmarre C, Cavailles V, Laudet V. Transcriptional activities of the orphan nuclear receptor ERR alpha (estrogen receptor-related receptor-alpha). Mol Endocrinol 1999;13:764–73.
10. Zhang Z, Teng CT. Estrogen receptor-related receptor alpha 1 interacts with coactivator and constitutively activates the estrogen response elements of the human lactoferrin gene. J Biol Chem 2000;275:20837–46.
11. Yang C, Zhou D, Chen S. Modulation of aromatase expression in the breast tissue by ERR alpha-1 orphan receptor. Cancer Res 1998;58:5695–700.
12. Yang C, Chen S. Two organochlorine pesticides, toxaphene and chlordane, are antagonists for estrogen-related receptor alpha-1 orphan receptor. Cancer Res 1999; 59:4519–24.
13. Kraus RJ, Ariazi EA, Farrell ML, Mert JE. Estrogen-related receptor alpha 1 actively antagonizes estrogen receptor-regulated transcription in MCF-7 mammary cells. J Biol Chem 2002;277:24826–34.
14. Horard B, Vanacker JM. Estrogen receptor-related receptors: orphan receptors desperately seeking a ligand. J Mol Endocrinol 2003;31:349–57.

15. Tsukiyama T, Ueda H, Hirose S, Niwa O. Embryonal long terminal repeat-binding protein is a murine homolog of FTZ-F1, a member of the steroid receptor superfamily. Mol Cell Biol 1992;12:1286–91.
16. Ikeda Y, Lala DS, Luo X, Kim E, Moisan MP, Parker KL. Characterization of the mouse FTZ-F1 gene, which encodes a key regulator of steroid hydroxylase gene expression. Mol Endocrinol 1993;7:852–60.
17. Lu D, Kiriyama Y, Lee KY, Giguere V. Transcriptional regulation of the estrogen-inducible pS2 breast cancer marker gene by the ERR family of orphan nuclear receptors. Cancer Res 2001;61:6755–61.
18. Ariazi EA, Clark GM, Mertz JE. Estrogen-related receptor alpha and estrogen-related receptor gamma associate with unfavorable and favorable biomarkers, respectively, in human breast cancer. Cancer Res 2002;62:6510–8.
19. Elston CW, Ellis IO. Pathological prognostic factors in breast cancer. I. The value of histological grade in breast cancer Experience from a large study with long-term follow-up. Histopathology 1991;19:403–10.
20. Suzuki T, Nakata T, Miki Y, et al. Estrogen sulfotransferase and steroid sulfatase in human breast carcinoma. Cancer Res 2003;63:2762–70.
21. Tsuchiya F, Ikeda K, Tsutsumi O, et al. Molecular cloning and characterization of mouse EBAG9, homolog of a human cancer associated surface antigen: expression and regulation by estrogen. Biochem Biophys Res Commun 2001;284:2–10.
22. Suzuki T, Inoue S, Kawabata W, et al. EBAG9/RCAS1 in human breast carcinoma: a possible factor in endocrine-immune interactions. Br J Cancer 2001;85:1731–7.
23. Shi H, Shigeta H, Yang N, Fu K, O'Brian G, Teng CT. Human estrogen receptor-like 1 (ESRL1) gene: genomic organization, chromosomal localization, and promoter characterization. Genomics 1997;44:52–60.
24. Dumoulin FL, Nischalke HD, Leifeld L, et al. Semi-quantification of human C-C chemokine mRNAs with reverse transcription/real-time PCR using multi-specific standards. J Immunol Methods 2000;241:109–19.
25. Vandesompele J, De Preter K, Pattyn F, et al. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. Genome Biol 2002;3:RESEARCH0034.
26. Allred DC, Harvey JM, Berardo M, Clark GM. Prognostic and predictive factors in breast cancer by immunohistochemical analysis. Mod Pathol 1998;11:155–68.
27. Horwitz KB, McGuire WL. Estrogen control of progesterone receptor in human breast cancer. Correlation with nuclear processing of estrogen receptor. J Biol Chem 1978;253:2223–8.
28. Barbareschi M, Pelosio P, Caffo O, et al. Cyclin-D1-gene amplification and expression in breast carcinoma: relation with clinicopathologic characteristics and with retinoblastoma gene product, p53 and p21WAF1 immunohistochemical expression. Int J Cancer 1997;74:171–4.
29. Gillesby BE, Zacharewski TR. pS2 (TFF1) levels in human breast cancer tumor samples: correlation with clinical and histological prognostic markers. Breast Cancer Res Treat 1999;56:253–65.
30. Naidu R, Wahab NA, Yadav M, Kutty MK. Protein expression and molecular analysis of c-myc gene in primary breast carcinomas using immunohistochemistry and differential polymerase chain reaction. Int J Mol Med 2002;9:189–96.
31. Liu D, Zhang Z, Gladwell W, Teng CT. Estrogen stimulates estrogen-related receptor alpha gene expression through conserved hormone response elements. Endocrinology 2003;144:4894–904.
32. Aksoy IA, Wood TC, Weinshilboum R. Human liver estrogen sulfot 255 ransferase: identification by cDNA cloning and expression. Biochem Biophys Res Commun 1994;200:1621–9.
33. Falany CN, Krasnykh V, Falany JL. Bacterial expression and characterization of a cDNA for human liver estrogen sulfotransferase. J Steroid Biochem Mol Biol 1995; 52:529–39.
34. Stack G, Kumar V, Green S, et al. Structure and function of the pS2 gene and estrogen receptor in human breast cancer cells. Cancer Treat Res 1988;40:185–206.
35. Ikeda K, Sato M, Tsutsumi O, et al. Promoter analysis and chromosomal mapping of human EBAG9 gene. Biochem Biophys Res Commun 2000;273:654–60.
36. Petz LN, Ziegler YS, Loven MA, Nardulli AM. Estrogen receptor alpha and activating protein-1 mediate estrogen responsiveness of the progesterone receptor gene in MCF-7 breast cancer cells. Endocrinology 2002;143:4583–91.
37. Strauss M, Lukas J, Bartek J. Unrestricted cell cycling and cancer. Nat Med 1995; 1:1245–6.
38. Chan VT, McGee JO. Cellular oncogenes in neoplasia. J Clin Pathol 1987;40: 1055–63.
39. Foekens JA, Portengen H, van Putten WL, et al. Prognostic value of estrogen and progesterone receptors measured by enzyme immunoassays in human breast tumor cytosols. Cancer Res 1989;49:5823–8.
40. Sladek R, Bader JA, Giguere V. The orphan nuclear receptor estrogen-related receptor alpha is a transcriptional regulator of the human medium-chain acyl coenzyme A dehydrogenase gene. Mol Cell Biol 1997;17:5400–9.
41. Coward P, Lee D, Hull MV, Lehmann JM. 4-Hydroxytamoxifen binds to and deactivates the estrogen-related receptor gamma. Proc Natl Acad Sci USA 2001;98: 8880–4.
42. Tremblay GB, Bergeron D, Giguere V. 4-Hydroxytamoxifen is an isoform-specific inhibitor of orphan estrogen-receptor-related (ERR) nuclear receptors beta and gamma. Endocrinology 2001;142:4572–5.
43. Parker KL, Schimmer B. P Steroidogenic factor 1: a key determinant of endocrine development and function. Endocr Rev 1997;18:361–77.
44. Clyne CD, Speed CJ, Zhou J, Simpson ER. Liver receptor homologue-1 (LRH-1) regulates expression of aromatase in preadipocytes. J Biol Chem 2002;277:20591–7.
45. Sasano H, Harada N. Intratumoral aromatase in human breast, endometrial, and ovarian malignancies. Endocr Rev 1998;19:593–607.
46. Reed MJ, Purohit A. Aromatase regulation and breast cancer. Clin Endocrinol 2001;54:563–71.
47. Zhao Y, Agarwal VR, Mendelson CR, Simpson ER. Estrogen biosynthesis proximal to a breast tumor is stimulated by PGE2 via cyclic AMP, leading to activation of promoter II of the CYP19 (aromatase) gene. Endocrinology 1996;137:5739–42.
48. Zhou J, Gurates B, Yang S, Sebastian S, Bulun SE. Malignant breast epithelial cells stimulate aromatase expression via promoter II in human adipose fibroblasts: an epithelial-stromal interaction in breast tumors mediated by CCAAT/enhancer binding protein beta. Cancer Res 2001;61:2328–34.

Jpn J Clin Oncol 2005;35(3)149–153
doi:10.1093/jjco/hyi044

Case Reports

MRI Accurately Depicts Underlying DCIS in a Patient with Paget's Disease of the Breast Without Palpable Mass and Mammography Findings

Goro Amano[1,3], Mioko Yajima[2], Yasunori Moroboshi[1], Yoshiki Kuriya[1] and Noriaki Ohuchi[3]

[1]Department of Surgery and [2]Department of Pathology, Sakata Municipal Hospital, Sakata, Yamagata and [3]Department of Surgical Oncology, Tohoku University School of Medicine, Sendai, Japan

Received July 29, 2004; accepted November 2, 2004

Breast-conserving therapy must be carefully indicated among patients with Paget's disease of the breast, because the disease is often associated with an underlying *in situ* or invasive carcinoma, even when there are no palpable mass or mammography findings. We report a 52-year-old woman who complained of skin color change of her right nipple for 11 months. No mass was palpable in her breasts, and mammography did not show any density or calcification. Nipple biopsy revealed Paget's disease of the breast with ductal carcinoma *in situ* (DCIS) in the breast epithelium just beneath the nipple. Magnetic resonance imaging (MRI) of the breast demonstrated diffuse segmental enhancement in two different quadrants. According to the pattern of enhancement, the lesions depicted by MRI were diagnosed as an extensively spreading type of DCIS. Based on informed consent, the patient received a total mastectomy. The histopathological examination demonstrated non-invasive ductal carcinoma with comedo-necrosis. The histological mapping with subserial sectioning demonstrated an extent of the lesions that corresponded accurately to the lesions defined by MRI. We conclude that MRI may play an important role in selecting candidates for breast-conserving therapy out of those patients with mammary Paget's disease with no clinical evidence of an underlying breast carcinoma.

Key words: Paget's disease – breast carcinoma – MRI – ductal carcinoma in situ

INTRODUCTION

The treatment for patients with Paget's disease of the breast is controversial. The standard treatment has been mastectomy (1,2). However, some studies have proposed the use of breast-conserving therapy for patients with Paget's disease in whom an underlying breast cancer cannot be located (3,4).

Nevertheless, other investigators reported that wide local excision of the nipple–areola complex and underlying breast tissue (cone excision) would have been insufficient surgery in 40% of their cases with no palpable mass and a normal mammogram, because of the multicentricity of the disease (5). Therefore, candidates for breast-conserving therapy must be selected carefully on an individual basis among those patients who have Paget's disease of the breast (6–8).

Here we report a case of a mammary Paget's disease patient who did not present either palpable mass or mammography findings. Magnetic resonance imaging (MRI) of the breast was very useful for making a decision on the appropriate surgical procedure.

CASE REPORT

A 52-year-old woman visited our hospital in January 2004 for an 11 month history of skin color change of her right nipple. She had also worried about the exudates from the nipple for 3 months. Physical examination showed the flattening and scaling of the nipple (Fig. 1). No mass was palpable in her breasts, and mammography did not reveal any density or calcification. There was also no abnormal finding on ultrasonography. Exfoliative cytology of the nipple demonstrated Paget's cells. To determine the surgical procedure of choice, MRI of the right breast was investigated. There were diffuse segmental enhancements existing in the upper and lateral quadrants (Fig. 2). According to the pattern of enhancement,

For reprints and all correspondence: Goro Amano, Department of Surgery, Sakata Municipal Hospital, 2-3-20, Sengoku-cho, Sakata 998-8585, Japan. E-mail: gamano@hospital.sakata.yamagata.jp

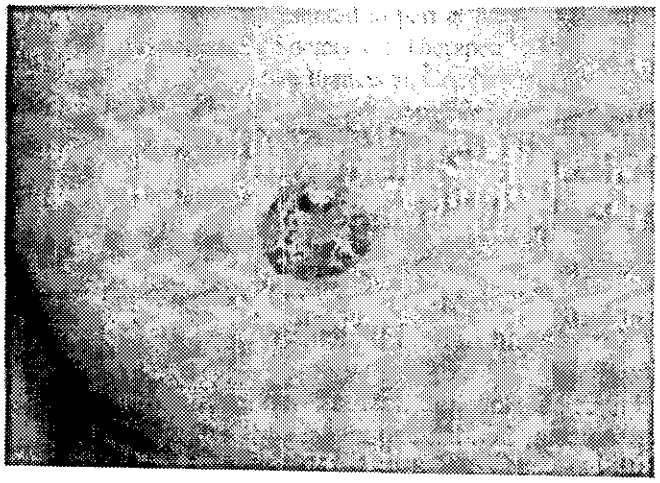

Figure 1. Flattening, scaling and color change of the right nipple were noted.

the lesions depicted by MRI were diagnosed as an extensively spreading type of ductal carcinoma *in situ* (DCIS). In an attempt to excise all the suspicious lesions, we recommended mastectomy to the patient, to which she gave her consent.

Prior to mastectomy, nipple biopsy was performed to confirm the histopathlolgical diagnosis of Paget's disease (Fig. 3A). Additional DCIS was also demonstrated in the breast epithelium just beneath the nipple (Fig. 3B).

On March 1, 2004, modified radical mastectomy was performed. The specimen was subserially sectioned in 7 mm thick slices, and every block was examined histopathologically. Non-invasive ductal carcinoma with comedo-necrosis was present (Fig. 4). Cytonuclear grade was grade 2 and estrogen/progesterone receptor status was negative. HER-2 was strongly (3+) immunoreactive. These cytological and immunohistochemical results were similar to those of Paget's cells in the nipple biopsy specimen. Lymph node metastasis was not

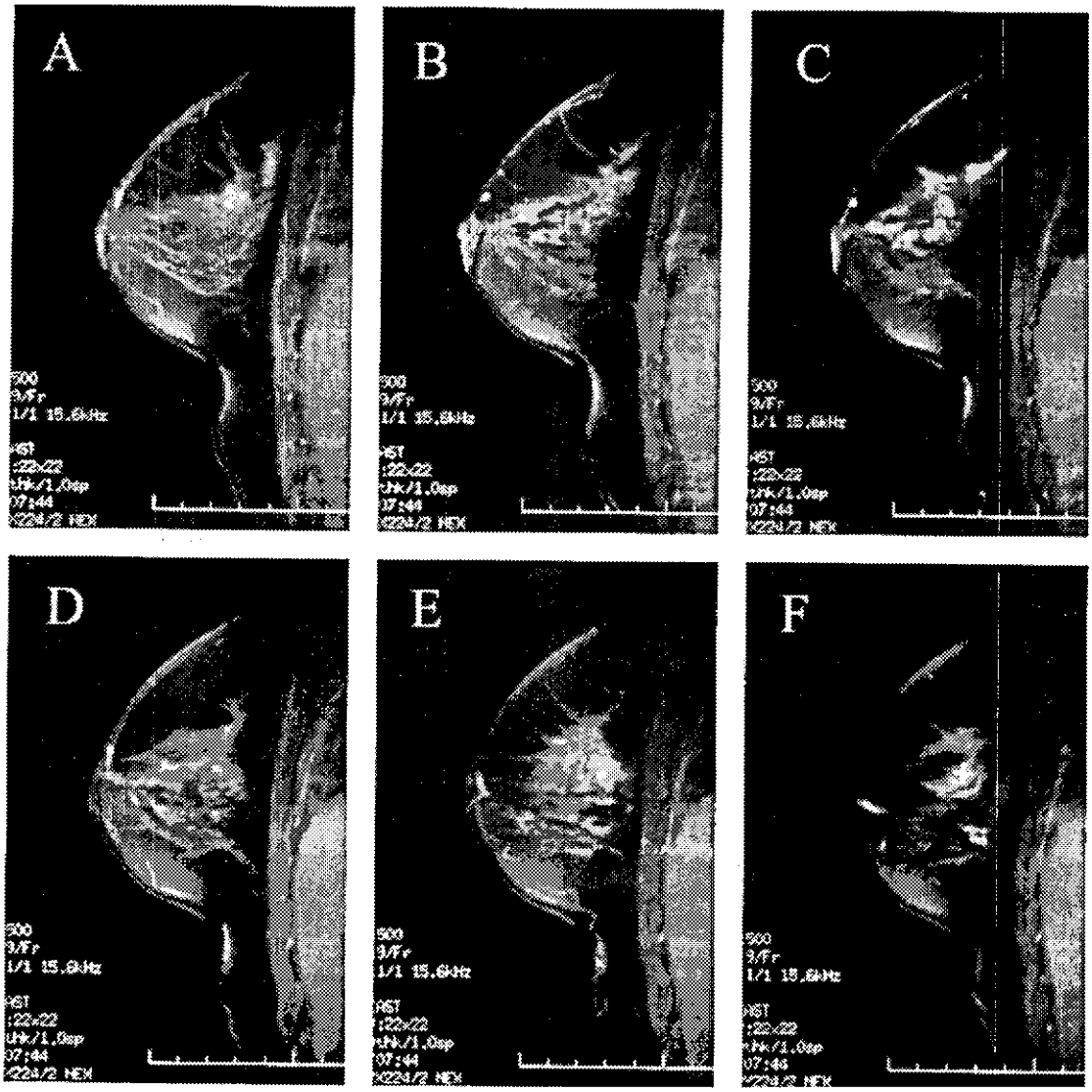

Figure 2. Fat-saturated, contrast-enhanced MRI of the right breast in the sagittal plane. From (A) to (F), a more lateral plane is shown at 6 mm intervals. (A–C) A diffuse segmental enhancement was apparent in the upper quadrant. (D–F) Another segmental enhanced lesion was shown in the lateral quadrant. The scale is graduated in centimeters.

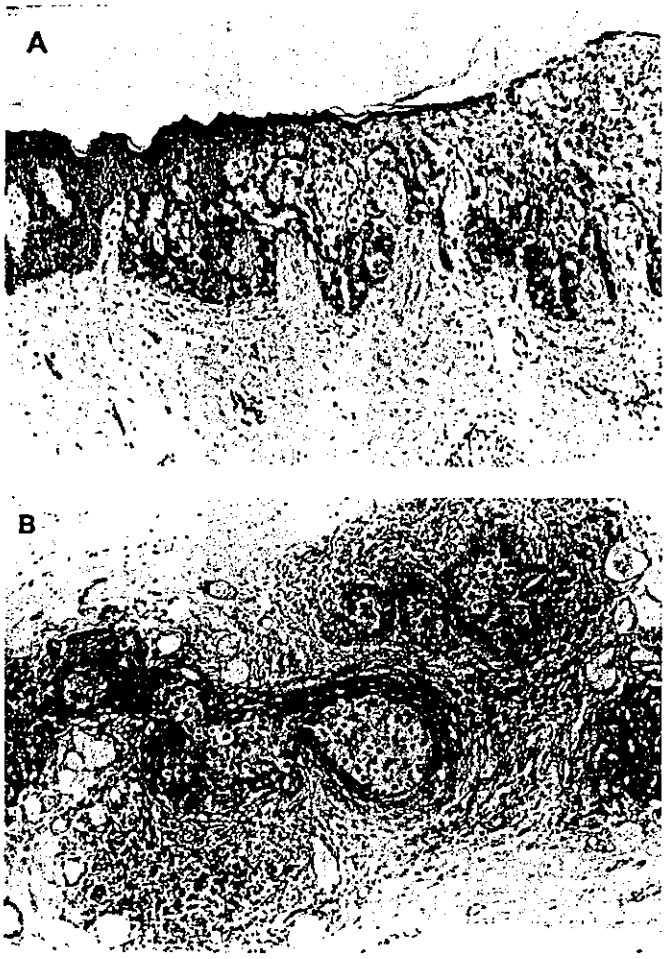

Figure 3. (A) A photomicrograph of a cross-section of the nipple biopsy specimen shows the Paget's disease. Large, round or ovoid intraepidermal Paget's cells with abundant clear cytoplasm and enlarged pleomorphic nuclei are present (H&E, objective: ×10). (B) A photomicrograph of a cross-section of the nipple biopsy specimen shows additional DCIS in the breast epithelium just beneath the nipple (H&E, objective: ×10).

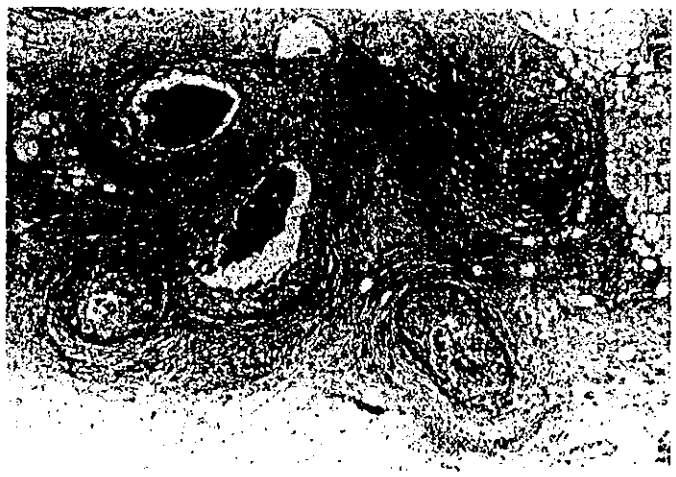

Figure 4. A photomicrograph of a cross-section of the mastectomy specimen shows non-invasive ductal carcinoma with comedo-necrosis (H&E, objective: ×4).

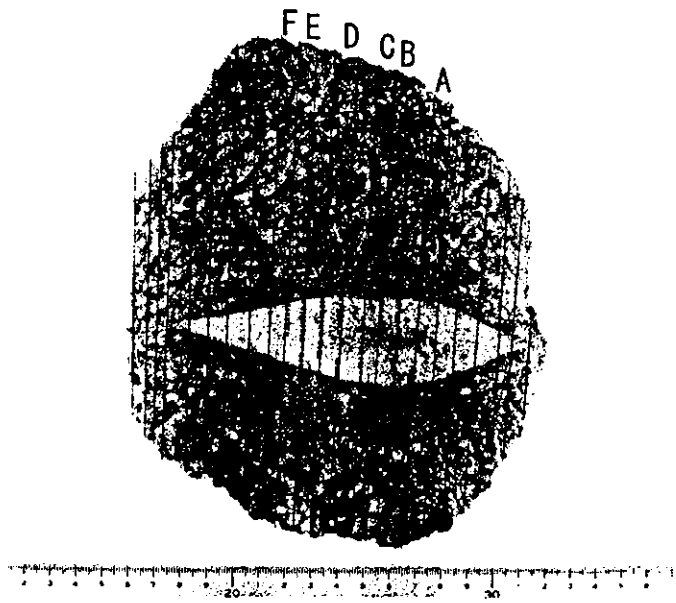

Figure 5. A histopathological cancer map of the mastectomy specimen, with the red marks denoting DCIS, reveals the extent of the lesions that are spreading in the upper and lateral quadrants. A–F indicate the location of the sliced specimen shown in Fig. 6. The scale is graduated in centimeters.

detected. According to the cancer map, DCIS were demonstrated extensively in the upper and lateral quadrants (Figs 5 and 6), accurately corresponding to the lesions shown by MRI (Fig. 2).

DISCUSSION

Paget's disease of the breast is a rare malignancy of the nipple–areola complex, comprising 0.5–5% of all breast cancer (1,7,9). It is manifested by progressive eczematoid changes of the areola with persistent soreness or itching (1,2,7). There have been debates about the histogenesis of this disease. According to the fact that this disease has been reported to be associated with an underlying breast carcinoma in 87–100% of cases (1–3,5–10), the epidermotropic theory, which postulates that Paget's cells are ductal cells that have migrated from an underlying breast carcinoma to the epidermis of the nipple, seems acceptable. The present case, where there existed underlying DCIS spreading extensively, is also compatible with the epidermotropic theory.

The treatment for patients with Paget's disease of the breast is controversial. Those patients with a palpable mass have a much greater incidence of invasive cancer, multifocal diseases, lymph nodal involvement and poor prognosis (1,2,6–8,10). Therefore, modified radical mastectomy is often the most appropriate treatment for patients with Paget's disease with a palpable lesion. On the other hand, for patients with Paget's disease who present no palpable mass, some investigators have proposed the use of breast-conserving therapy. Bijker et al. demonstrated that cone excision and radiotherapy is a feasible alternative for patients with Paget's disease and a limited extent of underlying DCIS (3). They reported a 5-year local

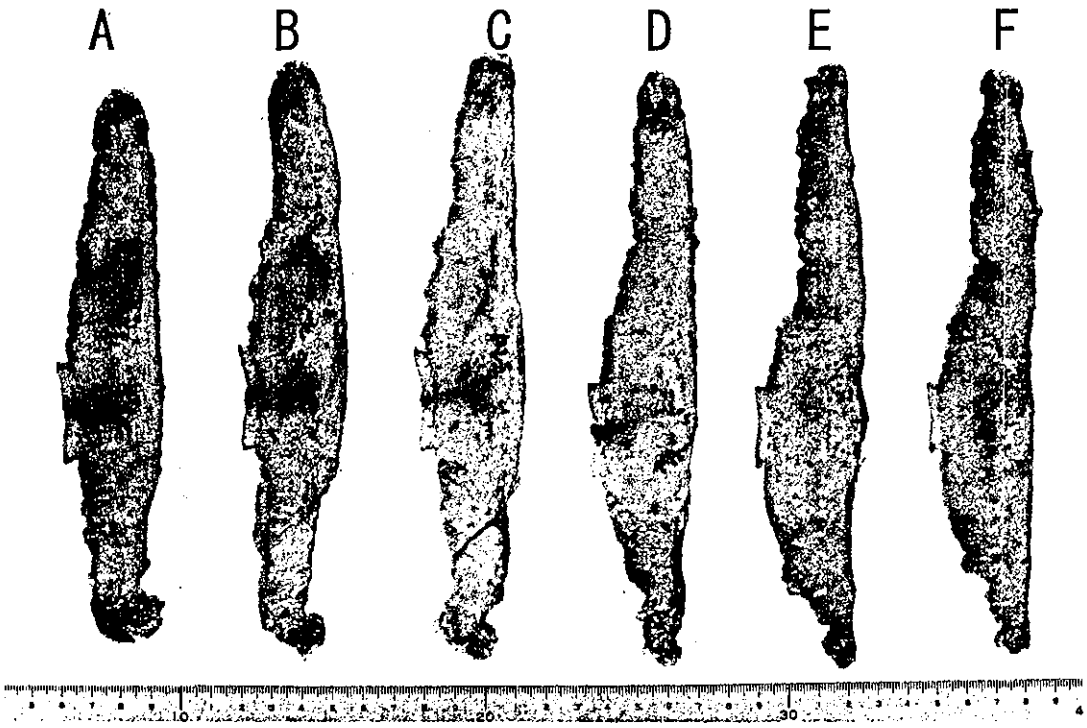

Figure 6. A more precise cancer map drawn on the cut surface of the mastectomy specimen. From (A) to (F), a more lateral plane is shown, and the location of the sliced specimen is indicated in Fig. 5. Note that the extent and distribution of DCIS, denoted by the red marks, correspond accurately to the enhanced lesions depicted by MRI in Fig. 2.

recurrence rate of 5.2%. Marshall et al. also recommended local excision and definitive breast irradiation as an alternative to mastectomy for patients with Paget's disease presenting no palpable mass or mammographic density (4). In their report, 5- and 10-year local control rates are 91 and 83%, respectively.

On the contrary, Kothari et al. warned against using breast-conserving therapy (5). They retrospectively reviewed the cases of 70 women with a clinical diagnosis of Paget's disease. Despite the fact that only one-third of women presented with a palpable mass, the malignancy was frequently extensive, being confined to the retroareolar region in only 25% of cases. They also demonstrated that the true extent of the disease was underestimated by mammography in 43% of cases. Of the 10 patients with no palpable mass and a normal mammogram, 40% had multicentric or multifocal carcinoma which would have been incompletely excised by cone excision of the nipple. Fu et al. described that of eight patients with no palpable mass who had been treated by quadrantectomy, two (25%) patients had recurrence (8). They concluded that even if the patient has no palpable mass, conservative surgery should be selected cautiously because of a higher recurrence rate and multifocal lesions.

In an era when breast-conserving surgery is sometimes recommended even for advanced infiltrating breast tumors, it seems quite reasonable to propose breast-conserving therapy for patients with Paget's disease with no definitive underlying breast cancer. However, as is already widely known, Paget's disease of the breast has a very high incidence of being accompanied by an underlying invasive or *in situ* carcinoma, even when there is no palpable mass (1–6,8–10). Mammography often fails to demonstrate the true extent of the disease (3–6,9). A more accurate and reliable imaging modality is necessary to select candidates for breast-conserving therapy more safely from among the patients who have Paget's disease of the breast.

Clinical utilization of MRI for breast cancer diagnosis has been under investigation since the late 1970s. With advances in surface coil technology and new imaging protocols using intravenously administered gadopentetate dimeglumine, MRI of the breast can now detect invasive cancer with 98–100% sensitivity (11,12). Amano et al. demonstrated that MRI can also detect the extensively spreading type of DCIS, that is often occult clinically and mammographically, as a pattern of diffuse segmental enhancement (13). In their study, the sensitivity of MRI to detect the extensively spreading type of DCIS was calculated to be up to 100%, and the specificity was estimated as high as 95%. The role of MRI in determining the extent of breast cancer is now well established (14). In the present case, no mass was palpable in her breasts, neither was there any abnormal findings on mammography or ultrasonography. There seemed a chance for her right breast to be treated conservatively, and for that reason we investigated it by MRI. There were diffuse segmental enhancements in the upper and lateral quadrants, strongly indicating the extensively spreading type of DCIS. Post-operative histopathological examination demonstrated non-invasive ductal carcinoma

with comedo-necrosis in the upper and lateral quadrants. The histological mapping with subserial sectioning demonstrated an extent of the lesions that corresponded accurately to the lesions defined by MRI. We conclude that MRI may play an important role in selecting candidates for breast conserving therapy out of those patients with mammary Paget's disease with no clinical evidence of an underlying breast carcinoma.

References

1. Ashikari R, Park K, Huvos AG, Urban JA. Paget's disease of the breast. *Cancer* 1970;26:680–5.
2. Paone JF, Baker RR. Pathogenesis and treatment of Paget's disease of the breast. *Cancer* 1981;48;825–9.
3. Bijker N, Rutgers EJ, Duchateau L, Peterse JL, Julien JP, Cataliotti L. EORTC Breast Cancer Cooperative Group. Breast-conserving therapy for Paget disease of the nipple: a prospective European Organization for Research and Treatment of Cancer study of 61 patients. *Cancer* 2001;91:472–7.
4. Marshall JK, Griffith KA, Haffty BG, Solin LJ, Vicini FA, McCormick B, et al. Conservative management of Paget disease of the breast with radiotherapy: 10- and 15-year results. *Cancer* 2003;97:2142–9.
5. Kothari AS, Beechey-Newman N, Hamed H, Fentiman IS, D'Arrigo C, Hanby AM, et al. Paget disease of the nipple: a multifocal manifestation of higher-risk disease. *Cancer* 2002;95:1–7.
6. Yim JH, Wick MR, Philpot GW, Norton JA, Doherty GM. Underlying pathology in mammary Paget's disease. *Ann Surg Oncol* 1997;4:287–92.
7. Sakorafas GH, Blanchard K, Sarr MG, Farley DR. Paget's disease of the breast. *Cancer Treat Rev* 2001;27:9–18.
8. Fu W, Mittel VK, Young SC. Paget disease of the breast: analysis of 41 patients. *Am J Clin Oncol* 2001;24:397–400.
9. Dixon AR, Galea MH, Ellis IO, Elston CW, Blamey RW. Paget's disease of the nipple. *Br J Surg* 1991;78:722–3.
10. Sheen-Chen SM, Chen HS, Chen WJ, Eng HL, Sheen CW, Chou FF. Paget disease of the breast—an easily overlooked disease? *J Surg Oncol* 2001;76:261–5.
11. Kaiser WA. MR Mammography (MRM). Berlin: Springer-Verlag, 1993;37–66.
12. Heywang-Kobrunner SH. Contrast-enhanced magnetic resonance imaging of the breast. *Invest Radiol* 1994;29:94–104.
13. Amano G, Ohuchi N, Ishibashi T, Ishida T, Amari M, Satomi S. Correlation of three-dimensional magnetic resonance imaging with precise histopathological map concerning carcinoma extension in the breast. *Breast Cancer Res Treat* 2000;60:43–55.
14. Esserman L, Hylton N, Yassa L, Barclay J, Frankel S, Sickles E. Utility of magnetic resonance imaging in the management of breast cancer: evidence for improved preoperative staging. *J Clin Oncol* 1999;17:110–9.

Breast Cancer
Vol. 11 No. 4 November 2004

Review Article

Ductal Carcinoma *in situ* and Related Lesions of the Breast: Recent Advances in Pathology Practice

Takuya Moriya*[1], Hisashi Hirakawa*[2], Takashi Suzuki*[3], Hironobu Sasano*[1,3], and Noriaki Ohuchi*[4]

*[1]*Department of Pathology, Tohoku University Hospital,* *[2]*Department of Surgery, Tohoku Kousai Hospital,* *[3]*Department of Pathology, Tohoku University Postgraduate School of Medicine, and* *[4]*Department of Surgery, Tohoku University Postgraduate School of Medicine, Japan.*

The incidence of ductal carcinoma in situ (DCIS) of the breast has increased significantly in Japanese women. It comprises 14.1% (172/1216) of all primary breast cancers at our institute, and nowadays this histological type is familiar to the surgeons and pathologists of any institute.

Several subclassifications have been published recently. Most based on nuclear atypia and the presence of comedonecrosis, and sometimes on the structures of the involved glands. These classifications are correlated with the biological behavior, tumor extent and the risk for local recurrences. The diagnostic accuracy of minimally invasive procedures (aspiration biopsy cytology/core needle biopsy) may differ between subclasses.

Atypical ductal hyperplasia (ADH) and microinvasive ductal carcinomas are lesions which resemble but deviate from the DCIS spectrum. The incidence of ADH seems to be lower than in Western countries. Patients with ADH may have a risk for subsequent breast cancer, because ADH is frequently associated with contralateral breast carcinomas. Microinvasion should be treated with caution, but we could not find any metastatic foci in microinvasive ductal carcinomas (T1mic). Tentatively, ADH may be treated similarly to non-comedo (low-grade) DCIS cases, according to our limited clinical experience.

Breast Cancer 11:325-333, 2004.

Key words: Breast, Ductal carcinoma *in situ*, Atypical ductal hyperplasia, Intraepithelial neoplasia, Microinvasive carcinoma

Recently, the incidence of ductal carcinoma *in situ* (DCIS) of the breast has increased, probably because of the early detection of cancer, especially by screening mammography. Nowadays, it is well known that DCIS is a heterogeneous group of diseases, both morphologically and biologically. Thus, further subclassification is recommended after this tumor is diognosed. Moreover, recent advances in less invasive diagnostic procedures has increased the chances for diagnostic pathologists to diagnose problematic intraductal lesions. These include questionable lesions suspicious for carcinoma, based on either fine needle aspiration cytology (FNAC) or core needle biopsy (CNB) results, and lesions that are definitely carcinoma of ductal origin, the invasiveness of which however cannot be determined by CNB. Additionally, the concept of intraductal proliferative lesions has been advanced to stratify lesions that pose different risks for the development of subsequent invasive carcinoma[1]. We will review recent advances in the field and the current situation in Japanese women.

Reprint requests to Takuya Moriya, Department of Pathology, Tohoku University Hospital, 1-1 Seiryo-machi, Aoba-ku, Sendai 980-8574, Japan.
E-mail: moriya@patholo2.med.tohoku.ac.jp

Received December 26, 2003

Incidence of DCIS

DCIS was not frequent several decades ago. Since the 1980's its incidence has progressively increased, especially in western countries. In the USA, CIS (most of them were DCIS) incidence rates increased between 1973 and 1997 (under 50 years old, white 146%, black 283%; and over 50 years old, white 308%, black 349%)[2]. In 1997 CIS accounted for 16.4% of all breast carcinomas in white women, and 18.6% in black women[3]. In Los Angeles County, the average annual age-matched

incidence of CIS increased more than 5-fold between 1972-1981 and 1997-1998 (3.8 to 17.7/100.000 women), and the ratio of increase was higher than that of invasive carcinomas (86.1 to 111.5). The ratio of CIS among all breast carcinomas has increased from 4.2% (1972-1981) to 15.0% (1997-1998)[4]. National surveillance in Japan from 1980 to 1990 revealed that the ratio of CIS was between 2 to 4%, and had gradually increased[4]. The ratio of CIS among non-palpable breast carcinomas was more than 50% in most of series in Japan[4]. Considering the development of mammographic detection of breast carcinoma during the past two decades, it is not surprising that the ratio of DCIS has increased substantially.

It is necessary to note the methods of pathologic examination used to diagnose DCIS, because more precise examination may detect a minute focus of invasive carcinoma. Invasiveness is associated with the ability to metastasize. It is desirable to examine the lesions as closely as possible. However, this precision will be limited in routine practice. If the diagnoses of DCIS is made, the manner of the pathological examination is very important. We usually examined resected specimens from lumpectomy or larger operations by serial sectioning every 3-5 mm of the whole specimen (for cases of breast conserving surgery) and parenchyma (for cases of total mastectomy). By these methods, we detected 172 DCIS cases (14.1%) among 1,216 breast carcinomas between December 1998 and March 2002. The significance of DCIS diagnosed by CNB will be discussed later.

Subclassification of DCIS

DCIS represents a spectrum of disease, and the main purpose of subclassification is to stratify the risk of subsequent invasive carcinoma and/or local recurrence[5]. A rare exception is the classification by growth pattern (microfocal, diffuse, and tumor forming) according to mammographic or gross findings[6].

Previously, the microarchitecture was thought to be the most reliable feature[7,8]. Any architectural pattern may present with any nuclear grade, with or without necrosis. The simplest ways of subclassification is separating comedo from non-comedo lesions. Comedo type DCIS cases may have higher nuclear grade (by definition in many articles), larger tumor size, aggressive biological marker expression, and a higher risk of the (micro) invasion[9-13]. Close to 90% of palpable, pre-mammographic DCISs were reported as high grade comedo type lesions. In contrast, nearly 60% of mammographically detected lesions are the non-comedo subtype[14]. The incidence of the latter group is increasing. Architectural patterns other than comedo included cribriform, papillary, micropapillary (low papillary), solid, and mixed subtypes[5,10].

One of the problems using the term "comedo" DCIS is that the definition is not uniform. Variable criteria have been employed according to the proportion of comedonecrosis, architecture, nuclear grade, and a combination of these characteristics for the same category[16]. Additionally, it is not easy for pathologists to fit each DCIS into an architectural classification. In such cases, the term mixed subtype may be used, but many DCIS cases may therefore be classified as mixed. Thus, the employment of architectural subdivision may not always be reliable. Various pathologic subclassifications, using other than architectural features, have since been proposed.

Both nuclear grade and necrosis are thought to be more reliable predictive factors than architecture by some authors[9,10,13], and some of the new subclassifications employ mainly nuclear atypia (nuclear grade), or a combination of nuclear atypia and necrosis[17-20]. Some studies enhanced interobserver reproducibility by using subclassifications devoid of architecture[21-25]. However, the architecture may be correlated with nuclear atypia, and some classifications still recommend describing the architecture along with stypia. Table 1 shows the relationship between architecture, nuclear atypia, and other findings of DCISs published previously[9], and the current van Nuys classification[16]. The predominant architectural patterns may correlate relatively well with the new grading system, although it is devoid of architecture. Additionally, there is some evidence that the micropapillary architectural type, when present in its pure form, is more commonly associated with more extensive, multifocal and multicentric disease[7,15].

One of the classifications employing an architectural description is the proposed classification of DCIS by the study group of the Japanese Breast Cancer Society (Table 2)[5]. The new WHO classification described three tier grading (low/intermediate/high grade), mainly according to nuclear features. The presence and absence of necrosis,

Table 1. Predominant Architecture of DCIS and their Comparison with the van Nuys Classification

Predominant architecture	COM	C + N	M + N	S + N	CRB	MCP	SOL	OTH	Total (or average)
No. of cases	7	3	7	6	22	19	9	12	85
NG 3	7								7
2		3	7	6	8	6	8	9	47
1					14	13	1	3	31
van Nuys Group	3	2	2	2	1	1	1	any	
mitotic counts; marked	4/7	2/3	1/7	2/6	0/22	2/19	1/9	2/12	17/85
mean No. of duct profiles involved	846	71	156	34	53	88	133	108	140
Maximum diameter (cm)	1.6	0.8	1.1	0.6	0.6	1.1	1.1	0.9	0.9

Van Nuys Group 3: high grade nuclei, Group 2: non-high grade nuclei with necrosis, Group 1: non-high grade nuclei without necrosis, COM: comedo, defined by high-grade nuclei, with solid nests and central necrosis, C + N: cribriform with necrosis, M + N: micropapillary with necrosis, S + N: solid with necrosis, CRB: cribriform, MCP: micropapillary, SOL: solid, OTH: others or mixed types

Table 2. Intraductal Proliferative Lesions: Different Terminology used in the Different Classification and their Relationships

Traditional terminology	Proposal by the study group, Japanese Breast Cancer Society, 2000	WHO classification 2003
Usual ductal hyperplasia (UDH)	Proliferative ductal lesions without atypia (mild/moderate-florid)	Usual ductal hyperplasia (UDH)
Flat epithelial atypia	Proliferative ductal lesions with atypia	Ductal intraepithelial neoplasia, grade 1A (DIN 1A)
Atypical ductal hyperplasia (ADH)	Proliferative ductal lesions with atypia (including ADH)	Ductal intraepithelial neoplasia, grade 1B (DIN 1B)
Ductal carcinoma in situ, low grade (Grade 1)	DCIS, HG 1 (low grade)	Ductal intraepithelial neoplasia, grade 1C (DIN 1 C)
Ductal carcinoma in situ, intermediate grade (Grade 2)	DCIS, HG 2 (intermediate grade)	Ductal intraepithelial neoplasia, grade 2 (DIN 2)
Ductal carcinoma in situ, high grade (Grade 3)	DCIS, HG3 (high grade)	Ductal intraepithelial neoplasia, grade 3 (DIN 3)

HG; histological grade

architectural feature, size of the lesions, and other characteristic features are also explained together[1]. If the different grade lesions are admixed within the same tumor, the description of their proportion is recommended. In any classification, the three-tier subdivision is always used, and the interrelationships between classifications are obvious. We employ the van Nuys classification currently, but we believe that it could be translated directly into the WHO classification in most cases.

Table 3 shows our recent experience of 82 cases of DCISs. The operative procedures consisted of 21 total mastectomies, 12 quadrantectomies, 37 wide excisions, 4 duct-lobular segmentectomies and 8 local excisions. All the cases were diagnosed by pathological examination of the whole tumor using 3-5 mm slices. By definition, all of the high-nuclear grade cases were classified into Group 3. The Group 1 cases were either of low or intermediate nuclear grade, but all of the Group 2 cases showed intermediate nuclear grade. Characteristically, the Group 3 cases showed a lower incidence of positive hormone receptor status ($p < 0.001$), and a higher incidence of HER-2 positivity ($p < 0.001$) compared with non-Group 3 cases. The results imply that nuclear grade will correlate well with hormone receptor/HER-2 neu status in DCIS cases. Additionally, although some authors reported a few cases (incidence 1-2%) of node-positive DCIS[15], we did not encounter any in our

Table 3. van Nuys Classification of DCIS and the Relationships between other Clinicopathological Features (Tohoku University Hospital 2002.6-2003.11)

Van Nuys Group	1	2	3	Total
Definition	non-high grade nuclei without necrosis	non-high grade nuclei with necrosis	high grade nuclei with/without necrosis	
No. of cases	39	30	13	82
NG 1/2/3	20/19/0	0/30/0	0/0/13	20/49/13
Age (average)	33-78 (54.4)	42-79 (54.1)	40-75 (59.2)	33-79 (55.0)
ER positive cases	33/34 (97.1%)	23/27 (85.2%)	2/10 (20.0%)*	58/71
PR positive cases	31/34 (91.2%)	22/27 (81.5%)	4/10 (40.0%)**	57/71
HER2 positive cases	2/32 (6.3%)	5/26 (19.2%)	7/10 (70.0%)***	15/68
Cases with lymph node positive	0/14	0/17	0/6	0/37

NG: Nuclear grade, ER: estrogen receptor, PR: progesterone receptor
*, **: significantly less frequent than non-Group 3 cases ($p < 0.001$)
***: significantly more frequent than non-Group 3 cases ($p < 0.001$)

series.

The unusual, rare subtypes include apocrine, mucinous, signet-ring cell, solid & papillary, spindled, neuroendocrine, Pagetoid, squamous, and clear. Most of these are classified according to their characteristic cell differentiation, rather than their architecture. Flat type DCIS, previously called clinging DCIS, may be a unique variant, which may resemble blunt duct adenosis on scanning magnification[26]. These lesions are malignant based on their genetic alteration[27], but are practically very difficult to diagnose accurately, especially low-grade lesions. More experience as well as further investigations will be necessary.

Differential Diagnoses of DCIS and Benign/Atypical Lesions

There are several lesions confused with DCIS in routine practice. They range from benign or borderline (atypically proliferating) intraductal lesions to minimally (micro-) invasive ductal carcinoma. Lobular neoplasia (atypical lobular hyperplasia and lobular carcinoma *in situ*) is another consideration.

Minimal Requirement to Diagnose DCIS

Low grade DCIS should be differentiated from benign and borderline (atypically proliferating) intraductal lesions. There have been several studies and proposals, including two famous studies by Page and colleagues, and Tavassoli[28, 29]. The new WHO classification employed both morphological and size criteria, probably according to these articles[1]. Morphologically, a monotonous cell population, high nuclear/cytoplasmic ratio, round nuclei, and hyperchromasia are necessary, combined with some architectural patterns. The evaluation of size has not been universally accepted. The entire involvement of 2 spaces, or cross section(s) exceeding 2 mm, are used for the minimal size.

The pathologist should pay great attention in routine practice to differentiate low-grade DCIS from intraductal hyperplasia or atypical ductal hyperplasia (ADH). A consensus conference on the classification of DCIS at Philadelphia in 1997[15] did not mention the precise distinction between DCIS and ADH, because they said it is difficult. Interobserver variability is sometimes problematic when intraductal lesions are diagnosed[30-32].

Intraductal Proliferative Lesions

Previously, proliferative disease of the breast was a form of epithelial hyperplasia usually seen with fibrocystic changes. Recently, the significance of these lesions has been enhanced, because they are related to carcinomas (whether directly or indirectly has not been proved, however), and early detection of low-grade carcinoma by mammography has also raised the incidence of precancerous proliferative lesions as well.

Currently, a new concept has emerged that describes intraductal proliferative lesions as a continuous disease entity, ranging from benign through atypical (ADH) to malignant disease

Table 4. Expression of Various Immunohistochemical Markers for Various Lesions (Including Intraductal) of the Breast

	UDH	ADH	DCIS		IDC	
			low grade	high grade	low grade	high grade
Average age	44.0	42.8	51.3	56.8	52.7	50.4
ER	5.7	6.7	6.4	5.0	5.8	3.3
PR	4.3	6.2	4.6	1.8	4.5	2.8
Ki-67 LI	3.7	4.5	9.5	9.4	21.3	35.9
p53	0/22	0/26	7/40			
c-erbB-2	0/22	0/26	8/40			

ER: estrogen receptor, PR: progesterone receptor, LI: labeling index, UDH: usual ductal hyperplasia, ADH: atypical ductal hyperplasia, DCIS: ductal carcinoma *in situ*, IDC: invasive ductal carcinoma (ER and PR were scored according to Allred DC et al. Mod Pathol 11: 155-168, 1998)

(DCIS)[29]. Even the concept of intraepithelial neoplasia (mammary intraepithelial neoplasia, MIN) has been adopted by some investigators[30]. The common loss of heterozygosity that occurrs with synchronous atypical ductal hyperplasia (ADH), DCIS and invasive ductal carcinoma (IDC) may suggest a stepwise progression from ADH to IDC[33]. However, smaller lesions are often removed by excision, and their real natural history is unknown. In practice, they are generally accepted to confer an increased risk for the subsequent development of invasive carcinoma, the magnitude of which varies according to the degree of proliferation and/or atypia[1]. In Japan, the study group of the Japanese Breast Cancer Society examined the interval to subsequent invasive carcinoma in the same quadrant of the breast after biopsy, and this was shorter in the cases showing a higher degree of intraductal proliferative lesions[5]. Table 2 shows the classification proposed by the the study group and its relationship between the new WHO classification[1, 5]. Table 4 shows the expression of some biomarkers analyzed immunohistochemically[33, 34].

Atypically proliferative lesions (atypical ductal hyperplaia-ADH/proliferative disease with atypia) are ductal proliferative lesions, which should be differentiated from DCIS histologically by the presence of structural and/or cytological atypia along with proliferative disease without atypia. This category may include the lesions with increased relative risk for subsequent invasive carcinomas, but their biological behavior and clinicopathological significance is uncertain, at least currently. Thus the diagnosis of "atypia" should be made with caution, and not used so frequently. If one uses this word on the pathological report, the reasons for the term "atypia" should be mentioned. For example, a description of the extent of the lesions, degree of epithelial proliferation, structural atypia, nuclear atypia or number of mitoses is recommended[5].

Atypical Ductal Hyperplasia (ADH)

ADH probably comprises the majority of "atypical" lesions but is also relatively rare. ADH may be diagnosed when one suspects but hesitates to diagnose DCIS, because of incompleteness of monotony (either structural or cytological) or limited extent. In any case, sufficient discussion with surgeons, and close follow-up is necessary.

At least in Japan, the diagnosis of ADH has not been widely accepted. We use this terminology in routine practice, according to the criteria of Page and colleagues[28]. They said that almost 3.5% of the biopsy specimens are diagnosed as ADH, however, we think that the incidence is much lower. This may be due to differences between Japanese and western populations or interobserver variability. We had a chance to review a biopsy series in which fibrocystic change was initially diagnosed, and found that the incidence of ADH was 1.2% by re-examination[35]. Table 5 shows the clinicopathological features of ADH cases in our laboratory. Only 21 cases were diagnosed as solitary ADH out of almost 1,000 primary breast cancer cases (cases with synchronous, ipsilateral carcinomas were eliminated, and consultation cases were not included). The patients were relatively young, as shown in Table 5. Interestingly, at least 5 cases

Table 5. Atypical Ductal Hyperplasia (ADH): Experience at The Pathology Department of Tohoku University Hospital from December 1998 to June 2002

- 21 case (cases with synclonous, ipsilateral carcinomas were eliminated)
- Background: 995 primary breast cancers, including 206 DCISs during the same period
- Age & gender: 28-56 (average 46.2) years old, all female
- Contralateral breast cancers: At least 5 cases (2 synclonous, 3 subsequent, follow-up period up to 5 years)
- Associated lesions: 3 were intermingled with papilloma, 1 with mucocele-like lesion, 1 within fibroadenoma
- Fine needle aspiration cytology: negative 5, indeterminate 6, suspicious for malignancy 3
- Diagnostic procedure: Local excision 15, Duct lobular segmentectomy 5, Core needle biopsy 1

showed contralateral breast cancer. This implies that ADH may be a relative risk for developing invasive breast carcinoma even in a population with a low incidence of ADH.

Microinvasive Ductal Carcinoma

The upper end of the DCIS spectrum is the borderline between DCIS and carcinoma with minimal stromal invasion. If invasion exists, there is a chance for metastasis. Variable definitions for "microinvasion" have been proposed previously. The cases with an invasive focus less than 1% of the total[1], or an invasive focus less than 1 mm (T1mic)[37] are relatively widely accepted to represent microvasion. They will show an apparent foci of infiltration into "interlobular" stroma. We have encountered 28 T1mic cases among 1,216 primary breast cancers (2.3%), and about 1/6 of DCIS cases (172 cases during the same period)[38]. Most were composed of small cell nests, or of single cells, but tongue-like projections with reactive stroma may be seen. There may be multiple foci (1-7 foci, average 3, in our series). These cases may show higher nuclear grade, tend to be associated with comedonecrosis, and more severe stromal reactions (lamellar fibrosis and/or chronic inflammatory cells) around the intraductal carcinomas.

Microinvasive carcinomas express a relatively low risk for lymph node metastases, and the prognosis is considered to be extremely good[39, 40]. None in our series expressed axillary lymph node metastases on serial sectioning of the whole carcinoma[38]. However, follow-up data using universally accepted procedures and/or criteria will be necessary to reach the final conclusions.

Diagnosis of DCIS and ADH by Minimally Invasive Procedures

Core needle biopsy (CNB) under stereo- or ultrasound guided procedures has been widely accepted. Thus, there has been an increased chance to diagnose earlier carcinomas (including low-grade DCIS) and borderline lesions. One of the problems of using this method is the specimen does not always include minute foci of invasion. If DCIS was diagnosed by CNB, there is still a chance for invasive carcinoma in the residual parenchyma. About 30% of DCIS diagnosed by CNB were truly invasive carcinoma in one study using a 14-gauge core[41], but the incidence fell to 10% with an 11-gauge vacuum-assisted procedure[42]. Similarly, if ADH is seen by core needle biopsy (CNB), 12-33% of the cases showed DCIS on excisional biopsy[43]. Some of the cases may be DCIS with invasion (IDC) but this situation is usually related to the number of foci (4 or more) of ADH on CNB[44].

Ultrasound-guided fine-needle aspiration biopsy cytology (FNABC) for dilated ducts may be performed. Any intraductal proliferative lesions, if correctly sampled, may show abundant, three-dimensional epithelial cell nests[45]. Our experiences revealed that the diagnostic accuracy of DCIS was 62.5%, lower than that of invasive ductal carcinomas (more than 80%)[46]. The cytological diagnosis of atypical hyperplasia is much more difficult, because most are small (less than 2 mm) and require sampling of the appropriate cells[17]. Some authors used the grading/scoring system for benign and malignant intraductal proliferative lesions[47, 48]. The author would like to recommend that if the dilated ductal lesion can be detected by ultrasound, US-guided FNABC may be performed, however, if the lesion is mammographically calcified and thought to be an intraductal lesion, CNB is recommended[49].

Pathological Factors of DCIS other than Grading, and their Significance

In addition to the methods for evaluating and grading DCIS, the extent of the lesions (size) and the surgical margins (if breast conserving surgery is performed) should be described. The Van Nuys