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Estrogen Receptor, c-*erb*B-2 and *nm*23/NDP Kinase Expression in the Intraductal and Invasive Components of Human Breast Cancers

Shunzo Kobayashi,[1,3] Hirotaka Iwase,[1] Yukashi Itoh,[1] Hideki Fukuoka,[1] Hiroko Yamashita,[1] Tatsuya Kuzushima,[1] Hiroji Iwata,[1] Akira Masaoka[1] and Narimichi Kimura[2]

[1]*Second Department of Surgery, Nagoya City University Medical School, 1, Kawasumi, Mizuho-cho, Mizuho-ku, Nagoya 467 and* [2]*Department of Biochemistry, Tokyo Metropolitan Institute of Gerontology, 35-2, Sakae-cho, Itabashi-ku, Tokyo 173*

Expression of the c-*erb*B-2 oncoprotein (*Erb*B-2) and the *nm*23 anti-metastatic gene product (nucleoside diphosphate [NDP] kinase) was examined in the intraductal and invasive components of 63 fresh human breast cancer tissues. The expression of estrogen receptor (ER) as a marker of hormone dependency and the Ki-67 protein as a proliferative cell marker was also examined. *Erb*B-2 and ER were positive in 77.8% (28/36) and 64.7% (22/34) of the intraductal components, and in 43.6% (27/62) and 57.1% (36/63) of the invasive components, respectively. NDP kinase was positive in 58% (18/31) of intraductal, and in 30.9% (17/55) of invasive areas. The average Ki-67-positive cell rates were 5.9% in the intraductal, and 10.7% in the invasive components. Thus, the cells within the intraductal component of breast cancer appear to have different characteristics from the invasive component, not only in markers of proliferative ability, but also in the expression of oncogenes and hormone receptors.

Key words: Breast cancer — Intraductal cancer — Estrogen receptor — c-*erb*B-2 — *nm*23

The recurrence of breast cancer is not rare, even in patients without axillary node involvement at the time of primary surgery. Therefore, additional reliable predictive markers of recurrence have been sought. Lack of estrogen receptor (ER) suggests a loss of hormone dependency, and may imply a poor prognosis.[1,2] Aneuploidy or percent of S-phase fraction seen on a DNA histogram,[3,4] amplification of specific genes,[5,6] and expression of specific proteins, such as the epidermal growth factor (EGF) receptor,[7] the c-*erb*B-2 protein,[8–12] and phosphoprotein p53,[13,14] are also predictive of recurrence. Among these, the expression of the c-*erb*B-2 oncogene is a newly adopted, powerful prognostic marker. NDP kinase is the product of the *nm*23-H1 anti metastasis gene, and the loss of its expression correlates with the recurrence of breast cancer.[15–21]

Regardless of whether or not metastases are present, the major problem with conservative surgery for breast cancer is the higher incidence of local recurrence.[22,23] The intraductal component of breast cancer often grows widely through the duct system, and an area of intraductal carcinoma is likely to be left within the conserved breast tissue after lumpectomy. Despite the non-invasive characteristic of intraductal carcinoma, expression of c-*erb*B-2 is often observed.[9,10] Therefore, a study of the expression of *Erb*B-2 in the intraductal and invasive components of breast cancer specimens was performed, along with an evaluation of hormonal dependency and proliferative ability, through expression of ER and Ki-67. The human nuclear antigen Ki-67 is expressed in all phases of the cell cycle except G0 and early G1.[24] It may therefore be a marker for active proliferation. Although the value of NDP kinase expression as a marker of metastatic potential has not been confirmed, it was also examined as a suppressive oncogene.

MATERIALS AND METHODS

Sixty-four fresh human breast cancer specimens were obtained by mastectomy or surgical biopsy, and stored at −80°C with or without dipping in "Tissue-Tek, OCT Compound" (Miles, Inc., Elkhart, IN) until use. Immunohistochemical examinations were performed on frozen sections of these specimens. The intraductal component of each breast cancer specimen was defined as an area of cancer cells completely surrounded by the basement membrane of the mammary duct. The cells within the ducts often showed a solid or papillary/cribriform pattern. In some cases, only a small number of cancer cells were observed within the duct. The actual number of cases examined with each special stain varied from 55 to 64, due to the different sizes of the tumors.

c-*erb*B-2 protein Expression of *Erb*B-2 was detected immunohistochemically by the streptavidin-biotin method, using a commercial kit (SAB-PO kit, Nichirei Co., Inc., Tokyo), and a monoclonal anti-*Erb*B-2 mouse antibody specific for the intracellular domain of the protein (mAb1, Triton Diagnostics Co., Inc., Alameda, CA). Expression was evaluated as positive when specific membranous staining was observed in a majority of cancer

[3] To whom requests for reprints should be addressed.

cell nests. Western blot analysis of *Erb*B-2 was performed on membrane and cytosol fractions of selected cases. Specimens were homogenized in a 0.3 *M* sucrose solution, and centrifuged at 1800*g* for 15 min to remove nuclei, and then at 135,000*g* for 60 min to obtain membrane and cytosol fractions. Immunoreaction with a specific polyclonal rabbit antibody to the intracellular domain of the protein was performed after electrophoresis in 7.5% polyacrylamide gel in 0.375 *M* Tris HCl buffer, pH 8.8, and transblotting of protein to a nitrocellulose membrane.

ER Immunohistochemical detection of ER was performed with a commercial kit (ER-ICA kit, Dynabott Co., Inc., Tokyo). Peroxidase-antiperoxidase complex was reacted via a bridging antibody after primary reaction with specific monoclonal anti-human ER antibody. ER-positive cell rates were calculated after counting 500 cancer cells in both the intraductal and the invasive components of the specimen. If there were fewer than 500 cells, all of them were counted.

Ki-67 Expression of Ki-67 protein was detected immunohistochemically by the streptavidin-biotin method, using a SAB-PO kit and a specific monoclonal anti-Ki-67 mouse antibody (DAKO-PC, DAKOPATTS Co., Inc., Glostrup, Denmark). The positive cell rate was calculated as for ER staining.

NDP kinase Expression of NDP kinase was also detected by the indirect immunoperoxidase method.[18] After blocking of nonspecific binding of antibody by preincubation with normal swine serum, a primary polyclonal rabbit anti-rat liver NDP kinase antibody, and peroxidase-conjugated anti-rabbit IgG swine antibody were used. Staining was done with diaminobenzidine in the presence of H_2O_2. The expression of NDP kinase was evaluated according to the staining intensity, compared to normal breast duct epithelium, and classified into three categories: negative to weak, moderate, and strong. This classification almost corresponds with that of Hirayama.[18]

The evaluation of all stained specimens was performed independently by two or three investigators, and adjusted after simultaneous re-examination of the specimens.

RESULTS

Among the 64 carcinomas examined, 28 had only an invasive component and one had only an intraductal component by hematoxylin-eosin staining. The remain-

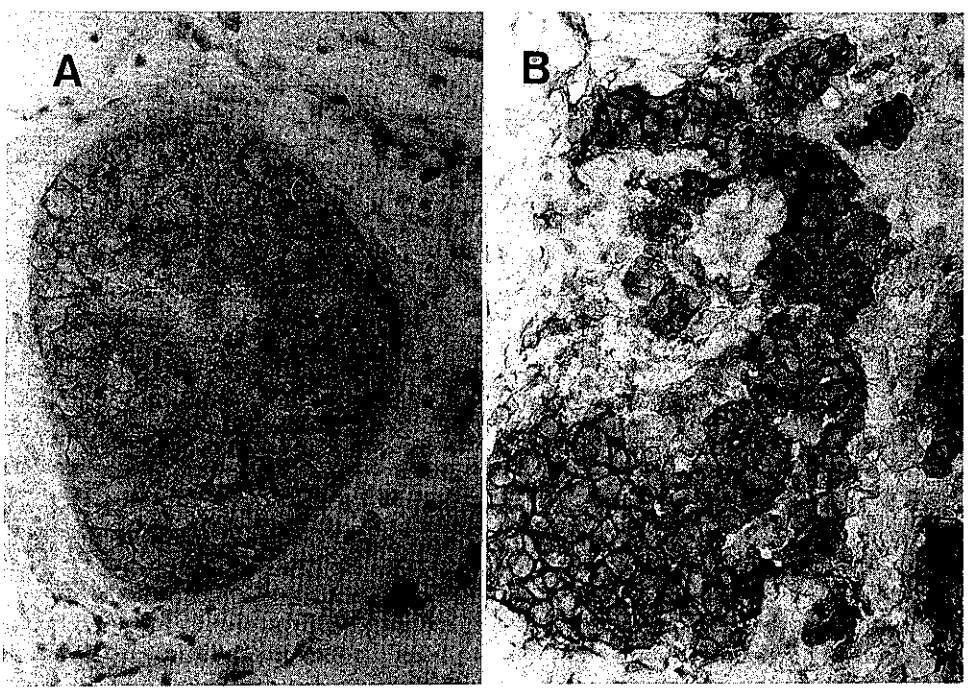

Fig. 1. Immunohistochemical staining of c-*erb*B-2 protein. A: Solid-type intraductal component with minor central necrosis showing very strong network-like membranous expression of c-*erb*B-2 protein (×100). B: Moderate grade expression of c-*erb*B-2 protein in the invasive component of the same case (×100).

ing 35 contained both components. Of the 36 with intraductal components, 18 showed solid and 18 showed the papillary/cribriform types of proliferation. Central necrosis was observed in 4 and 5 of the cases of solid and papillary/cribriform types, respectively.

*Erb*B-2 was positive at the cell membrane in 77.1% (27/35) and 54.3% (19/35) of intraductal and invasive areas of specimens containing both components, respectively. This difference was statistically significant. The difference was more clearly observed in the analysis of

Table I. Positive Case Rates of c-*erb*B-2 Protein and Estrogen Receptor in Intraductal and Invasive Components

	Intraductal		Invasive		*P*
	n[a)]	positive (%)	n	positive (%)	
*Erb*B-2[b)]	36	28 (77.8)	62	27 (43.6)	<0.01
ER[c)]	34	22 (64.7)	63	36 (57.1)	NS[d)]

a) n: Number of cases.
b) *Erb*B-2: Immunostaining of c-*erb*B-2 protein.
c) ER: Immunostaining of estrogen receptor.
d) NS: Not significant.

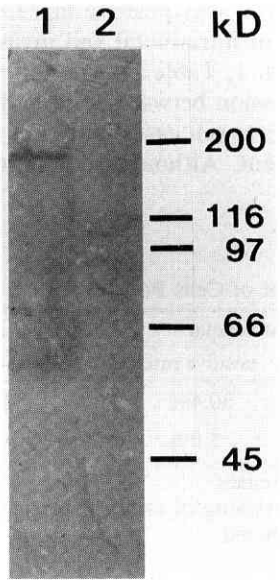

Fig. 2. Western blot of c-*erb*B-2 protein in a case which showed membranous staining only in frozen sections of the specimen. Lane 1: Membranous fraction with specific 185-kD band. Lane 2: Cytosolic fraction.

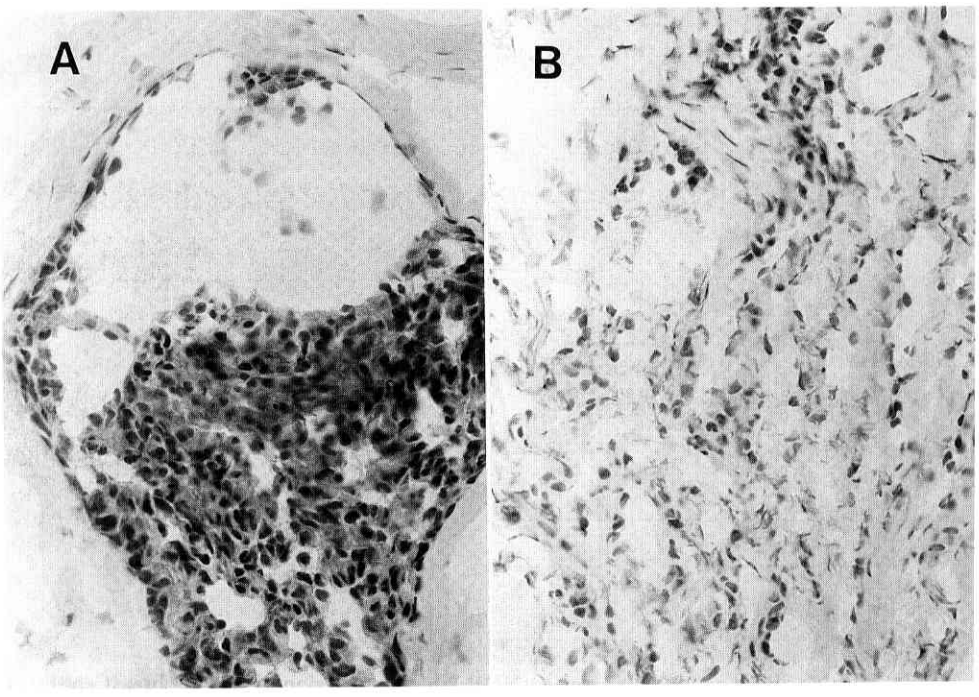

Fig. 3. Immunohistochemical staining of estrogen receptor. A: Papillary/cribriform-type intraductal component with obvious estrogen receptor expression. The positive cell rate was 84.8% (×50). B: Invasive component of the same case. The positive cell rate was 40.6% (×50).

total cases. *Erb*B-2 was positive in 77.8% (28/36) and 43.6% (27/62) of intraductal and invasive components, respectively (Fig. 1, Table I). The difference in the rate of *Erb*B-2 expression between solid and papillary-cribriform types, or between cases with and without necrosis was not significant. Although cytoplasmic staining was frequently observed, the specific 185-kD *Erb*B-2 protein was only detected in the membrane fraction by Western blotting (Fig. 2).

ER staining was positive in 21 cases each of intraductal and invasive components in 33 specimens containing both components. There were 3 cases having ER-positive cells only within one component. It was positive in 64.7% (22/34) and 57.1% (36/63) of intraductal and invasive components, respectively for total cases (Fig. 3, Table I). Concerning ER-positive cases, the ER-positive cell rates ranged from 2% to 84.8%, with an average of 30.4% in the intraductal components, and from 1.3% to 56.7%, with an average of 20.7%, in the invasive component (Table II). Although the difference between the two components was not statistically significant, both the incidence of ER-positive cases and the ER-positive cell rates tended to be higher in the intraductal components than in the invasive components.

Table II. Percent of Cells Positive for ER and Ki-67

	Intraductal		Invasive		*P*
	n[a)]	positive rate	n	positive rate	
ER[b)]	34	30.4%	63	20.7%	NS[c)]
Ki-67	35	5.9%	63	10.7%	<0.01

a) n: Number of cases.
b) ER: Immunostaining of estrogen receptor.
c) NS: Not significant.

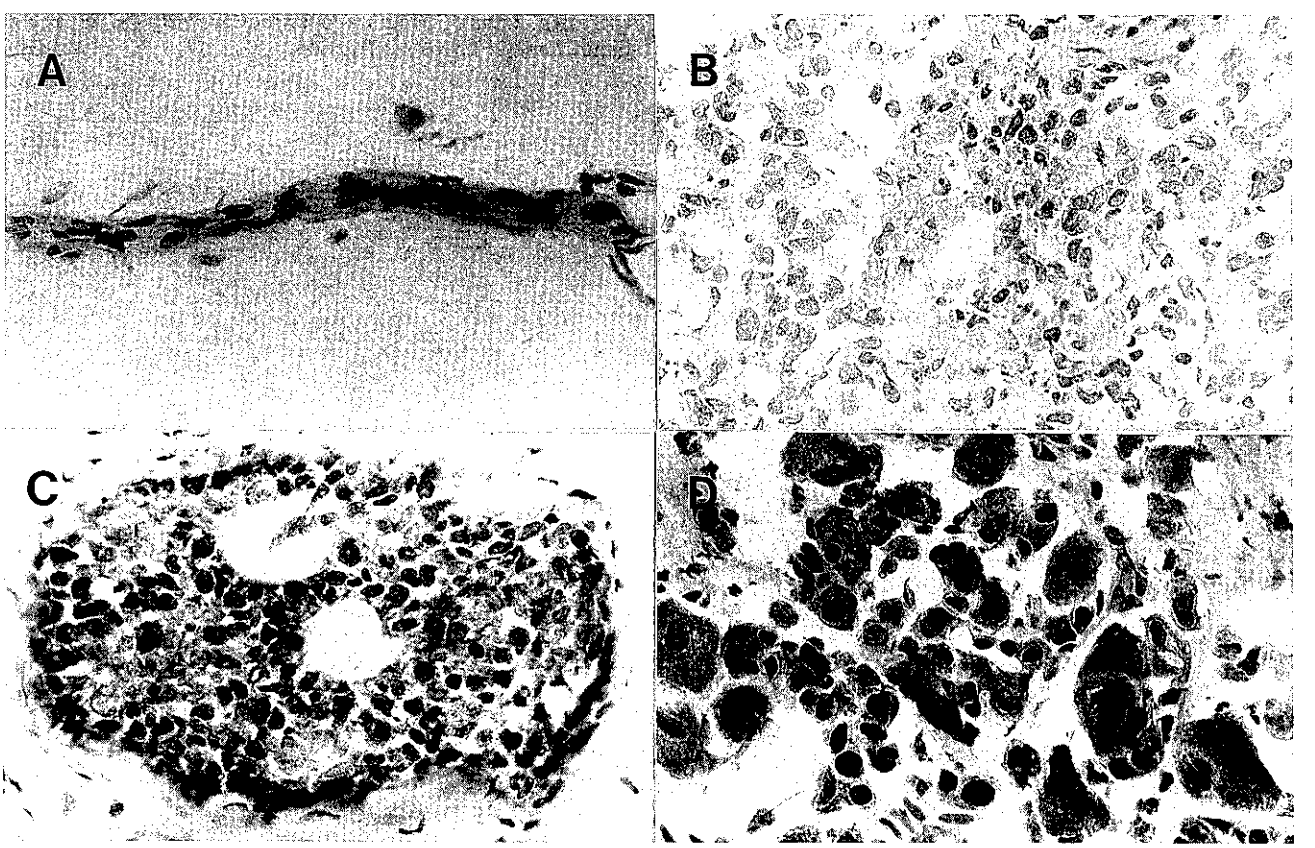

Fig. 4. Immunohistochemical staining of NDP kinase. A: NDP kinase expression in normal breast epithelium. Both nuclei and cytoplasm are moderately stained (×100). B: Weak or almost negative staining of NDP kinase in invasive component (×100). C: Strong staining in an intraductal component (×100). D: Strong staining in invasive component (×200). In the majority of cases the expression of NDP kinase was stronger than the normal level in the intraductal components. In contrast, it was moderate or decreased in the invasive components.

Table III. Staining Intensity of NDP Kinase in Intraductal and Invasive Components

	Intraductal				Invasive				*P*
	n[a]	W[b]	M[c]	S[d]	n	W	M	S	
NDP[e]	31	1	12	18	55	14	24	17	
(%)	(100)	(41.9)		(58.1)	(100)	(69.1)		(30.9)	<0.01

a) n: Number of cases.
b) W: No staining, or weaker staining than normal epithelium.
c) M: Moderately stained, almost equal to normal epithelium.
d) S: Stronger staining than normal epithelium.
e) NDP: Immunostaining of NDP kinase.

Ki-67 protein-positive cell rates varied from 0.5% to 24.6%, with an average of 5.9% in the intraductal components. They were from 0% to 46.8% with an average of 10.7% in the invasive components. The difference between the two components was statistically significant (Table II).

The staining intensity of NDP kinase varied widely throughout the tissues (Fig. 4). The distribution patterns of intensity, however, were clearly different between the two components. The majority (58%) of the intraductal components expressed NDP kinase more strongly than did normal breast epithelium, while 69.1% of the invasive components expressed NDP kinase at levels equal to or less than did normal epithelium. There was only one case (3.2%) with weak expression of NDP kinase in the intraductal components, and 14 (25.5%) such cases were observed out of 55 invasive components. The difference between the two components was statistically significant (Table III). The distribution of staining intensity was not very different even within specimens having both components. While 56.7% (17/30) of intraductal components expressed NDP kinase strongly, 66.7% (20/30) of invasive components expressed it at lower levels in those specimens.

DISCUSSION

Both the EGF receptor itself and *Erb*B-2 are phosphorylated after binding of EGF to the EGF receptor.[25] Although the role of *Erb*B-2 in growth regulation is not yet elucidated, the homology of *Erb*B-2 with the EGF receptor suggests that *Erb*B-2 may function as a growth factor receptor. In 1990, a 30-kD glycoprotein was reported as the ligand of *Erb*B-2, and autophosphorylation of an *Erb*B-2 tyrosine residue was observed after binding of the ligand.[26] Many retrospective studies have shown that recurrence of breast cancer is more frequent in patients with tumors expressing *Erb*B-2.[8–12] Excess *Erb*B-2 expression in invasive breast cancer tissues has been observed in about 20% to 30% of cases using formalin-fixed, paraffin-embedded specimens. We also obtained a similar positive rate using formalin-fixed, paraffin-embedded specimens (data not shown). The distinct but weak staining observed in this study using fresh frozen materials often disappeared after fixation, suggesting that immunoreactivity for membranous *Erb*B-2 in breast cancer tissues may be lost in part during the routine handling of specimens. Cytoplasmic *Erb*B-2 staining was also frequently observed in this study. Because the specific 185-kD protein was not detected by Western blot analysis in the cytoplasmic fraction of the specimens tested, immature or degradation products retained under fresh-frozen conditions might account for the observed cytoplasmic staining.

*Erb*B-2 was expressed by cells of the intraductal component at a higher rate than in invasive cells. This has also been reported by Gusterson and co-workers.[9] They observed a 41.7% positive rate in intraductal carcinoma, which was more than three times that of invasive ductal carcinoma. If the expression of *Erb*B-2 correlates with tumor growth, a higher growth rate of intraductal carcinoma cells compared to invasive carcinoma cells should be observed. However, the positive rate of Ki-67, a presumed marker of cellular proliferation, was lower in the intraductal component than in the invasive area.

The expression of ER was relatively higher in the intraductal component of invasive cancers than in the invasive component itself. The incidence of ER-positivity in invasive breast cancer in Japanese women has been reported to be about 55% or greater.[27] As an equal incidence was observed in the invasive component of this series, the incidence of ER expression within the intraductal component is also acceptable. Therefore, the hormonal dependency of cells within the intraductal component appears to be equal to or greater than that of the invasive component.

*nm*23 was identified from melanoma cell lines as a gene producing an antimetastatic factor.[15] Recently, the product of *nm*23 gene has been identified as NDP kinase.[16–18] Decreased expression of *nm*23 m-RNA,[20,21] and protein[18] correlates with both recurrence of, and poor prognosis in breast cancer. NDP kinase has at least two isoforms, originating from the *nm*23-H1 and *nm*23-H2 genes. According to a report by Stahl *et al.*, the expression of *nm*23-H1 is more distinctly decreased in human breast cancer tissues and cell lines having high metastatic ability than that of *nm*23-H2.[28] Recently, a positive relationship between NDP kinase immunoreactivity and metastatic ability in human tissues has also been reported.[29] In that paper, the enzyme was undetectable in normal tissues, including breast epithelium, and was expressed in neoplastic tissues, especially in carcinomas, including breast cancer. That study used NDP kinase A, which is identical to the product of the *nm*23-H1 gene,

obtained from human erythrocytes, as an immunogen. Hirayama and one of the present authors (N.K.) have reported a positive correlation between increased expression of NDP kinase and good prognosis of breast cancer patients.[18] The significance of these contradictory reports remains unclear.

In this study we obtained information about the different biological characteristics of intraductal cells of invasive human breast cancer from invasive cells of the same tumor. The cells within the intraductal component seemed to retain hormonal dependency equal to or rather higher than that of the invasive cells. It is also suggested that they have different abilities to express both oncogene and suppressive oncogene from invasive cells. However, the actual proliferation, as indicated by Ki-67 staining, is less than that in the invasive component.

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