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p53 Mutations and c-*erb*B-2 Amplification in Intraductal and Invasive Breast Carcinomas of High Histologic Grade

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In order to clarify the clinical significance of mutations of the p53 gene and amplification of the c-*erb*B-2 gene in breast carcinoma, these gene alterations were examined in 101 invasive, seven predominantly intraductal and 10 intraductal breast carcinomas by single-strand conformation polymorphism-direct sequencing or Southern blot-hybridization analysis. p53 mutations were detected in 32 (32%) of the invasive cases and two (12%) of the 17 intraductal/predominantly intraductal cases, whereas c-*erb*B-2 amplification was detected in 14 (14%) of the invasive and six (35%) of the intraductal/predominantly intraductal cases. Irrespective of differences in the positions and types of the mutations, cases carrying p53 mutations were almost always Grade 3 histologically and with a low hormone-receptor value. Since p53 mutations as well as c-*erb*B-2 amplification were detected almost selectively in Grade 3 cases but were not associated with lymph nodal status in invasive breast cancer, these two gene alterations could be indicators of prognosis of disease independent of lymph nodal status. Even in intraductal/predominantly intraductal carcinoma, these gene alterations were almost always detected in tumors of higher histologic grade. Thus, it is suggested that these gene alterations occur in breast cancers showing a high proliferation rate irrespective of the presence of invasion, and that other molecular alterations are involved in the process of breast cancer invasion.

Key words: Breast cancer — Histologic grade — Gene alteration — Intraductal carcinoma

The histologic features of cancer cells and tissues provide abundant information about their origin and the characteristics of their clinical behavior. In human breast carcinoma, the histologic grade, assessed on the basis of structural atypia, nuclear atypia and number of mitotic figures, has been shown to be an important prognostic factor of the disease.[1–5] The histologic grade of breast carcinoma is strongly suggested to be an indicator of biological aggressiveness rather than the extent of tumor spread, because multivariate analysis has shown that histologic grade is a prognostic factor independent of tumor size or lymph nodal status.[4, 5]

Recently, using molecular biological techniques, alterations in cancer cell DNA have been examined extensively, and many gene alterations have been detected in breast cancer, including amplification of oncogenes[6, 7] and mutation of tumor-suppressor genes.[8–13] These studies have revealed that breast carcinomas showing amplification of oncogenes and overexpression of their proteins, and nuclear immunoreaction for p53 protein, which appears to be due to mutation of the p53 gene, are predominant in groups with a high histologic grade and poor prognosis.[6, 7, 14–16] Therefore, in breast carcinoma, there seems to be a strong relationship among gene alterations, histological grade and aggressive clinical behavior.

Abbreviations used: PCR, polymerase chain reaction; SSCP, single-strand conformation polymorphism; ER, estrogen receptor; PgR, progesterone receptor

Breast carcinoma can be classified histologically into three groups according to the presence and extent of tumor invasion: intraductal carcinoma, invasive carcinoma with a predominant intraductal component (predominantly intraductal carcinoma)[17] and invasive carcinoma. In the former two groups, the cancer tissue is mostly or entirely limited to within the mammary duct,[17] usually does not show lymph node metastasis and is curable by surgical resection; it is generally considered to represent an early stage of breast cancer development.[18, 19] On the other hand, invasive carcinoma is regarded as a more advanced form.

In the present study, in order to clarify the clinical significance of p53 mutation and c-*erb*B-2 amplification in breast cancer, the association of these gene alterations with clinical and histologic findings was examined in both invasive and intraductal/predominantly intraductal carcinoma groups.

MATERIALS AND METHODS

Patients, tissue specimens and DNA isolation Tissue samples of primary breast cancer and non-cancerous skin were obtained from 118 Japanese patients who underwent mastectomies at the National Cancer Center Hospital between June 1990 and April 1992. For all cases, staging was performed in accordance with the TNM system. The number of metastatic lymph nodes and presence of invasion were examined microscopically. The specimens

included 10 intraductal carcinomas, seven predominantly intraductal carcinomas, in which the amount of invasive carcinoma was less than one-fourth of that of the intraductal component,[17] and 101 invasive carcinomas. Histologic grading into three categories, Grade 1, 2 or 3, was performed for all cases including intraductal/predominantly intraductal cases.[4] The amount of estrogen receptor (ER) and progesterone receptor (PgR) in tumor tissue were measured using ER-EIA and PgR-EIA kits, respectively (Abbott Laboratory, North Chicago, IL).

High-molecular-weight DNA was isolated from 118 paired specimens of fresh cancer and non-cancerous tissue by phenol:chloroform extraction and dialysis.[20] To exclude false-negative results, fresh cancer tissue was embedded in OCT compound (Miles Inc., Elkhart, IN), cut into 5-μm-thick sections, and observed microscopically to confirm that it contained cancer cells predominantly (>50%). In predominantly intraductal cases, DNA was isolated from the intraductal portion.

Detection of p53 mutations and c-*erb*B-2 amplification

Using the polymerase chain reaction (PCR) and single-strand conformation polymorphism (SSCP) analysis, mutations of the p53 gene were examined in 118 cancer DNAs and 34 noncancerous tissue DNAs from cases in which mutation was detected in the cancerous tissue. Exons 4 through 9 were examined because they contain almost all the reported mutations.[21] Primers encompassing exon 4, 5, 6, 7, 8 or 9, identical with those used by Yamada *et al.*,[22] were synthesized using a 391 DNA Synthesizer (Applied Biosystems Japan, Tokyo). PCR, SSCP and direct DNA sequencing were performed according to the methods described previously.[23]

Five micrograms of DNA was completely digested with *Bam*HI enzyme (Toyobo, Kyoto), electrophoresed in 0.8% agarose gel, denatured, neutralized, and blotted onto Nitroplus filters (MSI, Westboro, MA) by Southern blotting. The filters were hybridized with [^{32}P]dCTP-

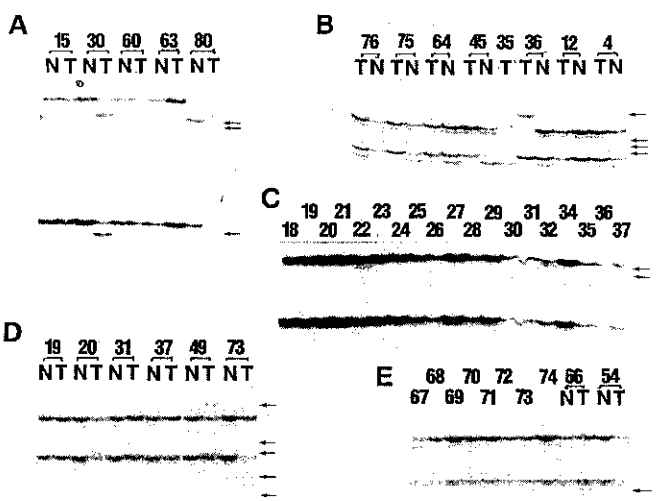

Fig. 1. Detection of p53 mutations in breast cancer by PCR-SSCP analysis for: A, exon 4; B, exon 5; C, exon 6; D, exon 7; and E, exon 8. At the top of each lane, the case number is shown. T, tumor DNA; N, non-tumor DNA. Arrows indicate mobility shifts in cancer DNA: Cases 15, 30 and 80 in A, Cases 4, 12, 36, 35, 45 and 64 in B, Cases 22, 29, 32 and 37 in C, Cases 19, 20, 31, 37, 49 and 73 in D, and Case 66 in E.

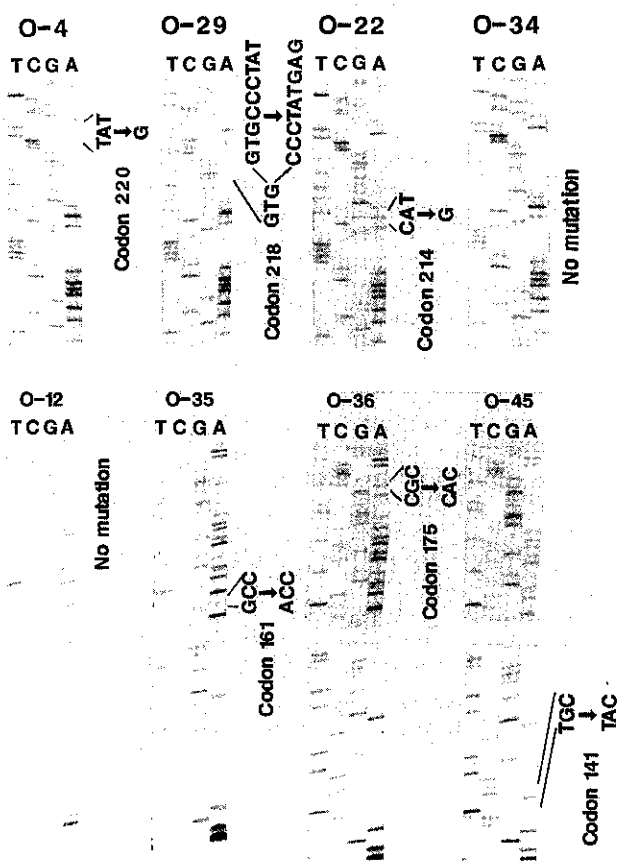

Fig. 2. Identification of p53 mutations in breast cancer by direct sequencing. At the top of each lane, the case number is shown. (Upper), mutations in exon 6. In Cases 4 and 22, base substitutions from TAT to TGT at codon 220 and from CAT to CGT at codon 214 are shown, respectively. In Case 29, deletion of three bases, GTG, in codon 216, 217 or 218 is shown. Case 34 did not reveal mutation. (Lower), mutations in exon 5. In Cases 35, 36 and 45, base substitutions from GCC to ACC at codon 161, CGC to CAC at codon 175 and from TGC to TAC at codon 141 are shown, respectively. In Case 12 mutation is not detectable. In Cases 4, 29, 22 and 35, not only mutant sequences but also contaminating wild-type sequences are evident.

Table I. Invasive Breast Cancer Cases Showing p53 Mutations

Cases No./Age	Stage	Nodal status[a]	Grade	Mutation pattern	
				Codon	Base change (amino acid)
A. G:C to A:T transitions					
O- 45/42F	III	19/19	3	141	TGC (Cys) → TAC (Tyr)
106/43F	III	30/39	3	156	CGC (Arg) → TGC (Cys)
35/49F	III	18/28	3	161	GCC (Ala) → ACC (Thr)
36/26F	III	0/41	3	175	CGC (Arg) → CAC (His)
104/67F	II	0/19	3	175	CGC (Arg) → CAC (His)
129/54F	III	8/16	3	175	CGC (Arg) → CAC (His)
101/38F	II	15/34	3	216	GTG (Val) → ATG (Met)
49/44F	II	5/21	3	237	ATG (Met) → ATA (Ile)
31/50F	II	0/29	3	241	TCC (Ser) → TTC (Phe)
19/41F	II	1/17	3	248	CGG (Arg) → TGG (Trp)
169/45F	I	0/9	1	273	CGT (Arg) → CAT (His)
B. A:T to G:C transitions					
O-37/45F	III	0/22	3	205	TAT (Tyr) → TGT (Cys)
22/38F	II	0/15	2	214	CAT (His) → CGT (Arg)
4/35F	II	28/37	3	220	TAT (Tyr) → TGT (Cys)
32/61F	III	0/23	3	220	TAT (Tyr) → TGT (Cys)
143/53F	III	26/26	3	220	TAT (Tyr) → TGT (Cys)
73/29F	II	0/19	3	234	TAC (Tyr) → TGC (Cys)
C. Small deletion/insertion					
O-136/47F	III	4/15	3	47–48	4 bp(GGAC) deletion
107/61F	III	0/32	3	78–82	13 bp deletion
80/42F	II	7/14	3	81	1 bp deletion (ACA → AA)
30/37F	II	0/27	3	108–109	5 bp (GGTTT) deletion
29/54F	II	3/29	3	216(7 or 8)	3 bp (GTG) deletion
20/47F	II	4/16	3	245(6)–249(50)	13 bp deletion
37[b]				251	4 bp (CTCA) insertion
115/45F	II	4/39	3	279	1 bp deletion (GGG → GG)
66/60F	II	9/17	3	293–297	15 bp deletion
D. Transversions					
O-15/40F	II	12/24	3	110	CGT (Arg) → CCT (Pro)
64/28F	II	1/31	3	179	CAT (His) → CAG (Gln)
63/41F	II	1/17	3	194	CTT (Leu) → CGT (Arg)
144/55F	III	4/6	3	208	GAC (Asp) → GTC (Val)
151/60F	I	0/22	3	240	AGT (Ser) → ATT (Ile)
158/45F	III	0/23	3	242	TGC (Cys) → AGC (Ser)
E. Undetermined Case 12 (Exon 5)					

a) Nodal status, number of metastatic lymph nodes/number of lymph nodes resected.
b) In Case 37, two mutations were identified.

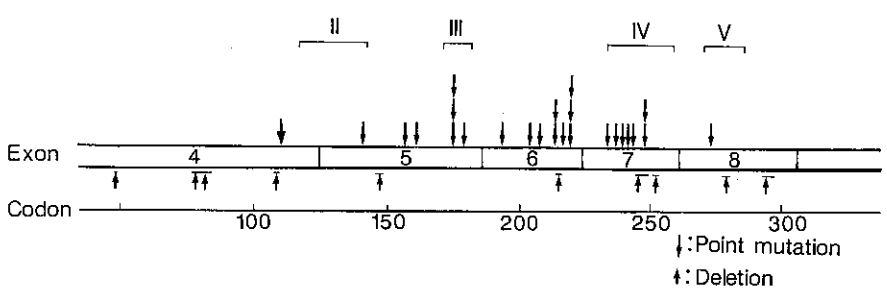

Fig. 3. Positions of p53 mutations in breast cancer. At the top, evolutionally conserved regions (II to IV) are indicated.[26] Downward arrows represent cases carrying a point mutation of p53, and upward arrows cases carrying a small deletion in the p53 gene.

labeled pCER204 probe (4.7 kb c-*erb*B-2 cDNA)[24] washed and autoradiographed.[7] After removal of the pCER204 probe, the filters were rehybridized with pRMU1 probe[25] on 17q24, which was used as the internal control for one copy per haploid DNA.[7] The c-*erb*B-2 gene was judged to be amplified if the signal intensity of the cancer DNA was two times or more higher than that of the non-cancerous DNA densitometrically when the signal intensity of pRMU1 was the same in both cancer and noncancerous DNAs.[7]

Statistical analysis Association of the gene alterations with clinical stage, histologic grade, lymph node status, ER and PgR values, age and menstrual status of the patients was analyzed by using the chi-squared test.

RESULTS

Positions and patterns of p53 mutations In total, 34 (29%) of the 118 breast carcinomas revealed 35 mobility shifts by SSCP. No case showed p53 mutation in normal tissue DNA. Except for one case (Case 12), the position and pattern of the p53 gene mutation were identified by DNA sequencing (Figs. 1 and 2) as listed in Tables I and III. With regard to position, the mutations were distributed between codons 47 and 297 (Fig. 3). The incidence was highest in exons 6 (10 cases), 5 (9 cases) and 7 (8 cases), followed by exons 4 (5 cases) and 8 (3 cases). With regard to type, the mutations were point mutations in 24 cases (11 transitions from G:C to A:T, 7 transitions from A:T to G:C and 6 transversions), small deletions in 10 and a small insertion in one. The most frequent patterns of mutation were G:C to A:T transitions in exons 5 (6/8) and 7 (3/8), A:T to G:C transitions in exon 6 (6/9), and small deletions in exons 4 (4/5) and 8 (2/3).

Amplification of the c-*erb*B-2 gene was detected in 20 (17%) of the 118 breast carcinomas examined. Copy numbers of the c-*erb*B-2 gene were 2 to 4 in seven cases and 5 or more in 13 cases.

Table II. Association between Clinical and Pathological Parameters of Invasive Breast Cancer and Gene Alterations

	Total	Number of cases: positive (%)		
		p53 mutation	c-*erb*B-2 amplification	Any of the two
A. Clinical stage				
I	16	2 (13)	1 (6)	3 (19)
II	53	18 (34) NS[a]	7 (13) NS	19 (36) NS
III	32	12 (37)	6 (19)	16 (50)
B. Number of metastatic lymph nodes				
0	37	12 (32)	7 (19)	13 (35)
1–3	21	4 (19) NS	1 (5) NS	4 (19) NS
≥4	43	16 (37)	7 (16)	21 (49)
C. Histologic grade				
1	10	1 (10)	0 (0)	1 (10)
2	37	1 (3) $P<0.001$	0 (0) $P<0.005$	1 (3) $P<0.001$
3	54	30 (56)	14 (26)	36 (67)
D. Estrogen receptor (fmol/mg protein)				
≥13	65	11 (17) $P<0.001$	5 (8) $P<0.005$	14 (22) $P<0.001$
<13	25	20 (80)	9 (36)	23 (92)
Not examined	11	1 (9)	0 (0)	1 (9)
E. Progesterone receptor (fmol/mg protein)				
≥10	54	11 (20) $P<0.001$	7 (13) NS	14 (26) $P<0.001$
<10	36	20 (56)	7 (19)	23 (64)
Not examined	11	1 (9)	0 (0)	1 (9)
F. Age of patients (years old)				
21–30	4	3 (75)	1 (25)	3 (75)
31–40	10	5 (50)	1 (10) NS	6 (60)
41–50	40	14 (35) $P<0.005$	7 (18)	18 (45) $P<0.005$
51–60	20	7 (35)	3 (15)	8 (40)
61–70	21	3 (14)	2 (10)	3 (14)
71–80	6	0 (0)	0 (0)	0 (0)
G. Menstrual status				
Premenopausal	51	22 (43) $P<0.05$	7 (14) NS	25 (49) $P<0.05$
Postmenopausal	50	10 (20)	7 (14)	13 (26)

a) Statistical significances were calculated by using the chi-squared test.
NS, not significant.

p53 and c-*erb*B-2 alterations in invasive breast carcinomas Thirty-three p53 mutations were detected in 32 invasive breast carcinomas (Table I). The p53 mutations were more frequent in cases at stage III (12/32, 37%) and stage II (18/53, 34%) than in those at stage I (2/16, 13%), and were more frequent in cases where the tumor size was >2.0 cm (29/83, 35%) than in cases of ≤2.0 cm (3/18, 17%), but were not associated with lymph nodal status (Table II, A and B).

Thirty (94%) of the 32 cases showing p53 mutations were Grade 3 histologically. The mutations were detected in 30 (56%) of 54 Grade 3 cases, but in only one (3%) of 37 Grade 2 and one (10%) of 10 Grade 1 cases. p53 mutations were also significantly more frequent in groups with lower ER or PgR values in the tumor, and in younger and premenopausal patients (Table II, C–G). In particular, the mutations were detected in 3 (75%) of 4 patients aged between 26 and 30 years. All four of these patients carried histologic Grade 3 tumors.

Amplification of the c-*erb*B-2 gene was detected in 14 (14%) of 101 invasive cases. It was confirmed that amplification of the c-*erb*B-2 gene was not significantly associated with clinical stage or lymph node status, but was more frequent in tumors of high histologic grade and with a low ER value (Table II). There was no association of c-*erb*B-2 amplification with age or menstrual status.

When p53 and c-*erb*B-2 aberrations were combined, at least one of these two alterations was detected in 38 (38%) in total, and in 36 (67%) of 54 Grade 3 cases, 23 (92%) of 25 ER-negative (<13 fmol/mg protein) cases and 23 (64%) of 36 PgR-negative cases of invasive breast carcinoma.

p53 and c-*erb*B-2 alterations in intraductal/predominantly intraductal carcinoma The clinical and histological profiles and presence of gene alterations examined are shown in Table III. Among these 17 cases, mutation of the p53 gene was detected in two (12%), and amplification of the c-*erb*B-2 gene in six (35%). p53 mutation was detected in one of two Grade 3 cases, and one of four Grade 2 cases but in none of 11 Grade 1 cases. Amplification of the c-*erb*B-2 gene was detected in one of two Grade 3 cases, and three of four Grade 2 cases, but only

Table III. Intraductal/Predominantly Intraductal Carcinomas Examined in the Present Study and Gene Alterations

Case No./Age	Histology	Grade	Size (cm)[a]	p53 mutation	c-*erb*B-2 amplification (copies)	ER status[b]
O-161/77	Predominantly intraductal	1	0.8 (2 mm)	−	−	+
53/47	Predominantly intraductal	1	1.3 (2 mm)	−	−	ND
100/38	Intraductal	1	1.5	−	−	ND
172/76	Intraductal	1	1.5	−	−	+
55/46	Intraductal	1	2.0	−	−	ND
84/36	Intraductal	1	3.2	−	−	ND
78/40	Predominantly intraductal	1	3.5 (2 mm)	−	−	ND
150/67	Intraductal	1	3.9	−	−	+
81/40	Predominantly intraductal	1	4.8 (2 mm)	−	−	+
140/43	Predominantly intraductal	1	6.0 (2 mm)	−	−	ND
13/37	Intraductal	1	10	−	+ (3)	+
88/39	Intraductal	2	3.0	Codon 147 1 bp del (GTT→GT)	−	ND
113/56	Intraductal	2	3.8	−	+ (5)	+
23/42	Predominantly intraductal	2	6.2 (10 mm)	−	+ (3)	+
93/42	Intraductal	2	10	−	+ (5)	+
174/66	Intraductal	3	1.8	Codon 214 CAT (His)→CGT (Arg)	+ (3)	−
117/45	Predominantly intraductal	3	4.0 (2 mm)	−	+ (8)	+

a) Size of intraductal component. Parentheses, size of invasive components.
b) ND, not done; +, ≥13 fmol/mg; −, <13 fmol/mg.

in one of 11 Grade 1 cases. In total, at least one of these two gene alterations was detected in all six Grades 2 and 3 cases examined, but in only one of 11 Grade 1 cases.

DISCUSSION

As reported previously,[9–12, 21] mutations of the p53 gene were confirmed to be frequent in invasive breast cancer. Most point mutations were distributed within exons 5, 6 and 7, whereas small deletions were distributed evenly from exons 4 to 8 (Fig. 3). Three regions showing mutations most frequently were codon 175 in exon 5 (3 cases), codons 214–220 in exon 6 (6 cases) and codons 234–251 (8 cases). Mutations were detected not only in the binding sites with simian virus 40 large T antigen, suggested to be important in malignant transformation of cells,[26, 27] but also outside these regions, such as exons 4 and 6. The pattern of mutation also varied: the majority of mutations were point mutations of the transition type, but transversions, small deletions and an insertion were also seen. Irrespective of the difference in their mutation positions or patterns, tumors carrying the p53 mutation were almost always of high histologic grade and had a low ER value. Because the histologic grade of cancer cells is known to be an indicator of aggressive biological behavior of invasive breast cancer,[4] p53 mutations of any pattern at any position were considered to be associated with aggressive biological behavior as well as an aggressive phenotype of breast cancer.

In invasive carcinomas, both p53 mutations and c-*erb*B-2 amplification were associated with high histologic grade and low ER value. In previous studies, amplification of the c-*erb*B-2 gene was detected in only one-third of Grade 3 cases.[4] When these two genetic alterations were combined, two-thirds of histologic Grade 3 cases were detected. Therefore, a combination of these two gene alterations could be a better indicator of aggressive invasive breast carcinomas than examination of the c-*erb*B-2 gene alone. Presence of at least one of p53 mutation and c-*erb*B-2 amplification can be a powerful indicator of poor prognosis in invasive breast carcinoma cases. Furthermore, this kind of study can be useful to select prognosis-poor patients among the cases without lymph node metastasis. In order to clarify its clinical utility, not only prospective study but also retrospective study seems to be necessary.

Different histologic grades were present among intraductal/predominantly intraductal carcinomas. The Grade 1 group roughly corresponds to the papillary or cribriform subtype,[28–30] whereas Grades 2 and 3 roughly correspond to the comedo subtype.[28–30] Meyer[31] showed that the comedo subtype was associated with a high rate of proliferation as determined by the thymidine labeling index, in comparison with other subtypes of intraductal carcinoma. Thus, the speed of proliferation of cancer cells was suggested to differ among intraductal carcinomas of different histologic grades.

Even in intraductal/predominantly intraductal breast cancers, p53 mutation and c-*erb*B-2 amplification were confirmed to occur, but detection of these gene alterations was almost always limited to cases of higher histologic grade. p53 mutations have been reported to occur in intraductal carcinoma,[10] and overexpression of the c-*erb*B-2 protein has been demonstrated frequently in intraductal carcinoma of the comedo subtype.[28–30] Thus, irrespective of the presence of invasion, both p53 mutations and c-*erb*B-2 amplification occurred almost exclusively in breast carcinomas of higher histologic grade, and, accordingly, showing rapid proliferation. In these intraductal carcinomas showing p53 mutation and/or c-*erb*B-2 amplification, other molecular events seem to be necessary for progression to invasive carcinoma.

Liu *et al.*, using differential PCR methods, showed that 48% of intraductal carcinomas carried an amplified c-*erb*B-2 gene.[32] We also demonstrated a high incidence of c-*erb*B-2 amplification in intraductal/predominantly intraductal carcinomas (35%), especially those of a higher histologic grade (5/6, 83%), by careful sampling and Southern blot analysis. In contrast, in invasive carcinomas, the total incidence of c-*erb*B-2 amplification was only 14%, and it was only 26% even in Grade 3 cases. As described by Liu *et al.*,[32] there seem to be two typical pathways of breast cancer development: in one pathway, the tumor invades the stroma relatively soon after carcinogenesis and is detected clinically as invasive carcinoma, while in the other pathway, the tumor remains within the duct for a long period and then starts to invade and is recognized clinically as intraductal/predominantly intraductal carcinoma. Among breast cancers of high histologic grade, amplification of the c-*erb*B-2 gene was suggested to occur relatively infrequently in the former pathway, whereas it appeared to occur frequently in the latter pathway. The reason for this difference is still unknown. Long-term intraductal spread might subject cancer cells to a special type of stress, and amplification of the c-*erb*B-2 gene and overexpression of its protein might be involved in overcoming this stress.

Germline mutation of the p53 gene is documented in cancer family syndrome.[33, 34] In the present study, p53 mutations were more frequent in breast carcinomas occurring in premenopausal patients, especially in those of younger age (26 to 30 years old). p53 mutations seemed to play an important role in the development of breast cancer in these young patients. Otherwise, such high incidence of p53 mutations might be explained by the fact that all of these young patients carried Grade 3 tumors, in which p53 mutations are frequent.

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