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Analysis of Oncogenes and Tumor Suppressor Genes in Human Breast Cancer

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Oncogenes (c-*erb*B-2, c-*myc*, and some genes linked to the 11q13 lesion), tumor suppressor genes (retinoblastoma gene, p53) and an antimetastatic gene (*nm*23/nucleoside diphosphate kinase) play important roles in breast cancer progression. Amplification of c-*erb*B-2, c-*myc*, and *int*-2, and expression of RB, p53(mutant), and NDP kinase were determined in 77 primary breast cancer specimens. *nm*23-H1 allelic loss was also studied. c-*erb*B-2 and c-*myc* amplification, loss of RB expression, p53(mutant) expression, and *nm*23-H1 allelic loss were also found in non-invasive carcinoma. *int*-2 amplification was significantly correlated with lymph node status ($P=0.02$) and a significant association was found between p53(mutant) expression and tumor size ($P=0.04$). c-*erb*B-2 amplification was strongly associated with disease-free and overall survival in multivariate analysis ($P=0.002$). All of the c-*erb*B-2 amplified cases and all but one of the *int*-2 amplified cases in node-positive patients had relapsed within 2 years post resection. The cancer cells may acquire new proliferative pathways sequentially as a result of multiple genetic alterations which enable them to bypass the estrogen-dependent proliferation.

Key words: Breast cancer — Oncogene — Tumor suppressor gene — *nm*23/NDP kinase

Tumorigenesis is a complex, multistep process.[1] In human breast cancer development, mammary epithelial cells are transformed, then estrogen may induce proliferation when its receptor signals c-*myc* expression.[2] We have demonstrated that the expression of estrogen receptors in human breast cancer decreases as the tumor invades extraductal tissue and that the level of Ki-67 expression, a presumed marker of cell proliferation, is higher in extraductal invasive areas than in the intraductal component.[3] A number of oncogenes (c-*erb*B-2, c-*myc*, and some genes linked to the 11q13 lesion), tumor suppressor genes (retinoblastoma gene, p53, and other genes detected as allelic loss) and an antimetastatic gene (*nm*23) may play important roles in breast cancer progression. c-*erb*B-2 gene amplification and overexpression appear to be prognostic factors in breast cancer patients[4,5] and this gene is frequently amplified and overexpressed in non-invasive breast tumors.[6,7] Further, c-*myc* gene amplification and rearrangement,[8–10] and *int*-2 gene amplification[11,12] are found in some breast cancer samples. RB[13–15] and p53[16,17] alteration and loss of expression (or mutant p53 expression) are also detected. In addition, the expression of *nm*23/nucleoside diphosphate (NDP) kinase is associated with a good prognosis.[18,19]

In this series, we measured amplification of c-*erb*B-2, c-*myc*, and *int*-2, expression of RB, p53(mutant), and NDP kinase, and *nm*23-H1 allelic loss in fresh-frozen breast cancer specimens obtained from 1983 to 1991. In our analysis of these data and other clinical parameters, we sought insight into the genetics of breast cancer progression.

MATERIALS AND METHODS

Tumors and blood samples Seventy-seven human breast cancer samples were obtained by mastectomy or surgical biopsy at the Nagoya City University Hospital from 1983 to 1991, and stored at −80°C with or without treatment with "Tissue-Tek, OCT Compound" (Miles, Inc., Elkhart, IN). Blood samples were collected from 49 patients whose tissues had been stored.

Estrogen and progesterone receptor analyses Frozen tissue specimens were analyzed for estrogen and progesterone receptors by enzyme immunoassay[20] using ER·EIA "Abbott" and PgR·EIA "Abbott" (Dainabot Inc., Tokyo). Estrogen receptor levels of <10 fmol/mg protein and progesterone receptor levels of <15 fmol/mg protein were considered negative.

DNA extraction and hybridization analyses Genomic DNA from the breast cancer specimens or blood samples was extracted by standard techniques.[21] DNA (10 μg) was digested with appropriate restriction enzymes, separated on 0.8% agarose gels, transferred to nylon membranes (Hybond N$^+$, Amersham, Tokyo), and hy-

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bridized overnight at 42°C with multiprime-[^{32}P]dCTP-labeled (Amersham) probe DNA. After washing at high stringency (final wash 0.1×SSC/0.1% SDS at 60°C for 30 min), autoradiography was performed for 1 to 3 days at −80°C using Kodak XRP-5 film. The membranes were stripped and rehybridized to other probes. Probes obtained from the Japanese Cancer Research Resources Bank (Tokyo) included: c-*erb*B-2 (a 440 bp *Kpn*I-*Xba*I fragment of human c-*erb*B-2 cDNA), c-*myc* (a 1.4 kb *Kla*I-*Eco*RI fragment from human c-*myc* exon 3), *int*-2 (a 0.9 kb *Sac*I fragment from human *int*-2, SS6). Human β-actin cDNA served as a control. A 0.9 kb *Bam*HI fragment of pNM23-H1[22] served as the *nm*23-H1 probe.

RB, p53, and NDP kinase expression analyses Expressions of RB, p53, and NDP kinase in the cancer specimens were detected by immunohistochemical methods as previously described.[3] Briefly, expression of RB or p53 was detected by the avidin-biotinylated peroxidase complex method using the SAB-PO kit (Nichirei Co., Inc., Tokyo) with anti mouse retinoblastoma gene product monoclonal antibody (NCL-RB, Novocastra Laboratories Ltd., Newcastle, UK) or anti-murine p53 monoclonal antibody (Ab-3, Oncogene Science, Inc., NY, USA), which recognizes an epitope on mutant p53. NDP kinase expression was detected by the indirect immunoperoxidase method using a primary polyclonal rabbit anti-rat liver NDP kinase antibody.[23] RB expression was considered positive when nuclear staining was observed in all of the cancer cells. p53 expression was considered positive when more than one cancer cell was stained. NDP kinase expression was scored as either negative-to-weakly or moderately-to-strongly overexpressed, based on the staining intensity in the normal duct epithelium.

Statistical analysis Chi-squared analysis was used to estimate the association between oncogene amplification or protein expression and clinical parameters. Fisher's exact test was added in some cases. The Cox proportional hazard model was used for univariate and multivariate analysis of prognostic values.[24] Disease-free survival was evaluated by the Kaplan and Meier analysis.[25] The differences between survival curves were calculated with the log-rank test.

RESULTS

Estrogen receptor expression in 77 breast cancer tissues There were 45 (58%) estrogen receptor-positive tumors. An inverse association was found between estrogen receptor expression and tumor size ($P=0.02$).

c-*erb*B-2, c-*myc*, *int*-2 amplification and association between other parameters The frequency of amplification seen in the 77 specimens was 25% for c-*erb*B-2, 12% for c-*myc*, and 14% for *int*-2 (Fig. 1, Table I). Rearrangement of c-*myc* was not detected. c-*erb*B-2 and *int*-2 amplification were found in tumors less than 2 cm in size. *int*-2 amplification was significantly correlated with number of positive lymph nodes ($P=0.02$ by chi-squared test, $P=0.01$ between groups 0–3 and >3 by Fisher's exact test). c-*myc* amplification was not found in tumors less than 2 cm in size.

RB, p53(mutant), NDP kinase expression and association between other parameters Expression of RB, p53 (mutant), and NDP kinase was determined as positive in 49%, 25%, and 42% of tumors, respectively (Fig. 2, Table II). In 77 cases analyzed for RB expression, there were 12 (16%) cases with no expression, and 27 (35%) cases with decreased RB expression. Loss of RB expression was found even in tumors of less than 2 cm, but the loss was more frequent in larger tumors. p53(mutant) expression was demonstrated in nuclei except one case. p53(mutant) was not expressed in tumors of less than

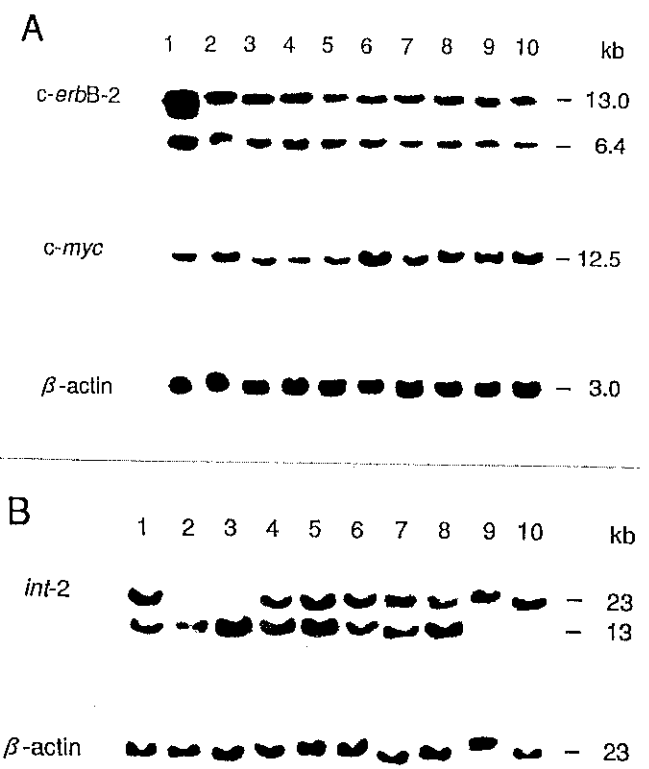

Fig. 1. Southern blot analysis of total genomic DNA extracted from breast cancer specimens digested with *Eco*RI (A) or *Hin*dIII (B) and hybridized with c-*erb*B-2, c-*myc*, or *int*-2 probe. (A) c-*erb*B-2: Lane 1 represents 10 copies, and the other lanes are a single copy. c-*myc*: Lane 6 represents 3 copies, and the other lanes are a single copy. (B) *int*-2: Bands are seen at 23 or 13 kb. Lane 3 and 5 represent 2 copies, and the other lanes are a single copy.

Table I. Association between c-*erb*B-2, c-*myc*, *int*-2 Amplification and Disease Parameters in 77 Breast Cancer Patients

Factors	c-*erb*B-2		c-*myc*		*int*-2	
	Amplified/total (%)	*P*	Amplified/total (%)	*P*	Amplified/total (%)	*P*
Total	19/77 (25)		9/77 (12)		11/77 (14)	
Menopausal status						
Pre/Peri	8/31 (26)		5/31 (16)		4/31 (13)	
Post	11/46 (24)	0.85	4/46 (9)	0.32	7/46 (15)	0.78
Tumor size (cm)						
≦2.0	3/11 (27)		0/11 (0)		2/11 (18)	
2.1–5.0	7/43 (16)	0.12	5/43 (12)	0.34	5/43 (12)	0.75
≧5.1	9/23 (39)		4/23 (17)		4/23 (17)	
Number of positive lymph nodes						
0	8/30 (27)		3/30 (10)		3/30 (10)	
1–3	1/19 (5)	0.06	1/19 (5)	0.39	0/19 (0)	0.02*
>3	10/28 (36)		5/28 (18)		8/28 (29)	
Estrogen receptor status						
Negative	6/32 (19)		5/32 (16)		2/32 (6)	
Positive	13/45 (29)	0.31	4/45 (9)	0.36	9/45 (20)	0.09
Progesterone receptor status						
Negative	7/32 (22)		5/32 (16)		3/32 (9)	
Positive	12/42 (29)	0.51	4/42 (10)	0.43	8/42 (19)	0.25

P values by chi-squared test. * $P<0.05$ is considered significant.

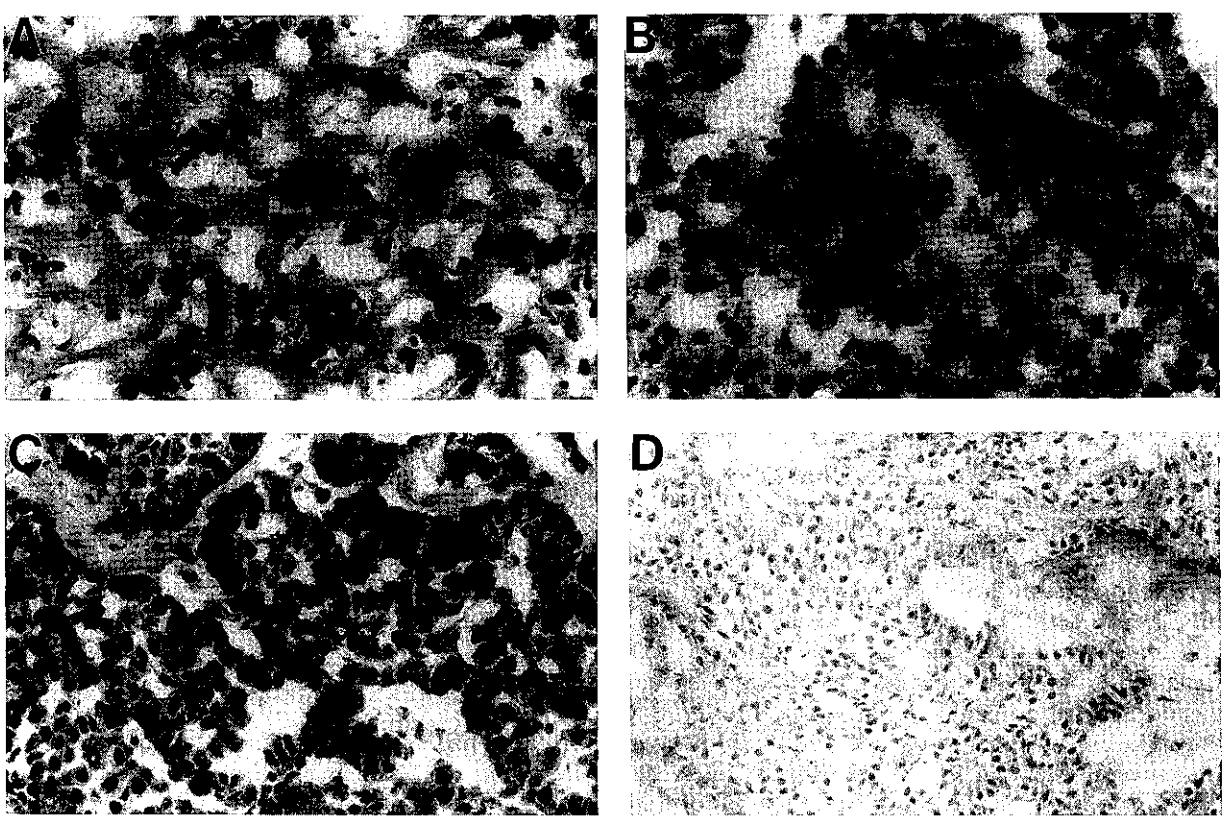

Fig. 2. Immunohistochemical staining of RB (A), p53 (B), and NDP kinase (C) in breast cancer. (A) All cancer cells were stained in nuclei (×200). (B) Cancer cells were stained in nuclei (×200). (C) Both nuclei and cytoplasm are moderately to strongly stained (×200). (D) Negative control on p53 expression. No cancer cells were stained (×200).

Table II. Association between RB, p53, NDP Kinase Expression and Disease Parameters in 77 Breast Cancer Patients

Factors	RB		p53(mutant)		NDP kinase	
	Expressed/total (%)	*P*	Expressed/total (%)	*P*	Expressed/total (%)	*P*
Total	38/77 (49)		19/77 (25)		32/77 (42)	
Menopausal status						
Pre/Peri	14/31 (45)		8/31 (26)		11/31 (36)	
Post	24/46 (52)	0.55	11/46 (24)	0.85	21/46 (46)	0.37
Tumor size (cm)						
≦2.0	8/11 (73)		0/11 (0)		7/11 (64)	
2.1–5.0	20/43 (47)	0.24	10/43 (23)	0.04*	19/43 (44)	0.10
≧5.1	10/23 (44)		9/23 (39)		6/23 (26)	
Number of positive lymph nodes						
0	13/30 (43)		6/30 (30)		14/30 (47)	
1–3	10/19 (53)	0.70	4/19 (21)	0.52	6/19 (32)	0.57
>3	15/28 (54)		9/28 (32)		12/28 (43)	
Estrogen receptor status						
Negative	12/32 (38)		11/32 (34)		8/32 (25)	
Positive	26/45 (58)	0.08	8/45 (18)	0.10	24/45 (53)	0.01*
Progesterone receptor status						
Negative	16/32 (50)		11/32 (34)		12/32 (38)	
Positive	20/42 (48)	0.84	8/42 (19)	0.13	17/42 (41)	0.80

P values by chi-squared test. * $P<0.05$ is considered significant.

2 cm and a significant association between its expression and tumor size was observed ($P=0.04$ by chi-squared test, $P=0.03$ between groups ≤2.0 cm and ≥2.1 cm by Fisher's exact test). NDP kinase expression was significantly correlated with estrogen receptor status ($P=0.01$) and it tended to decrease with increasing tumor size.

***nm*23-H1 allelic loss** Among 49 breast cancer patients, 12 were informative and allelic loss was detected in only one case (Fig. 3). NDP kinase expression was not detected in this case.

Cases with greater than 90% intraductal component Five cases were found. In these cases, c-*erb*B-2 amplification was observed in 3 cases, c-*myc* amplification in 1 case, loss of RB expression in 3 cases, p53(mutant) expression in 2 cases, and decreased NDP kinase expression in 2 cases. *nm*23-H1 allelic loss was found in 1 case. *int*-2 amplification was not detected in any of these cases.

c-*myc* amplified cases without RB expression There were 3 cases. Tumor sizes in these cases were 9 cm, 10 cm, and 11 cm. c-*erb*B-2 amplification was also found in 2 cases. Three cases with both c-*myc* amplification and p53 (mutant) expression were 2.3 cm, 5 cm, and 9 cm in size.

Prognostic analysis In multivariate analysis, c-*erb*B-2 amplification was strongly associated with both disease-free and overall survival ($P=0.002$, Table III). *int*-2 amplification was correlated with disease-free and overall survival in univariate analysis ($P=0.009$), but no correlation was found in multivariate analysis. All of the c-*erb*B-2 amplified cases (n=11) in node-positive patients (n=47) had relapsed within about 2 years post resection (Fig. 4A). All but one node-positive patient with *int*-2 amplification relapsed within 2 years (Fig. 4B). There were 3 cases with distant metastasis at the time of surgery, and 2 of them had tumors in which both the genes were amplified.

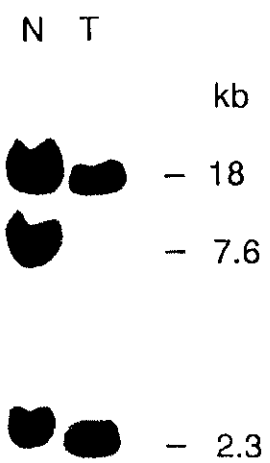

Fig. 3. Allelic deletion of *nm*23-HI in breast cancer. Southern blot analysis of total genomic DNA from normal lymphocyte and breast cancer tissues digested with *Bgl*II and hybridized with a pNM23-H1 probe. An allelic band at 7.6 kilobases was found. N, normal; T, tumor. Total 49 cases; informative 12 cases; alleic loss 1 case.

Table III. Cox Univariate and Multivariate Analyses Showing Correlation of Various Parameters with Disease-free and Overall Survival in Breast Cancer Patients

Factors	Disease-free survival		Overall survival	
	Univariate *P* value	Multivariate *P* value	Univariate *P* value	Multivariate *P* value
Menopausal status	0.18	0.10	0.81	0.67
Tumor size	0.003	0.01	0.002	0.13
Number of positive lymph nodes	<0.001	<0.001	<0.001	0.001
Estrogen receptor status	0.08	0.03	0.21	0.69
c-*erb*B-2 amplification	0.04	0.002	0.008	0.002
c-*myc* amplification	0.22	0.13	0.30	0.01
int-2 amplification	0.009	0.03	0.009	0.74
RB expression	0.53	0.51	0.10	0.03
p53(mutant) expression	0.04	0.35	0.002	0.05
NDP kinase expression	0.38	0.67	0.58	0.34

DISCUSSION

In the early stages of human breast cancer progression following carcinogenesis, the tumor cells proliferate within mammary ducts. They later penetrate the ductal wall, forming invasive cancer nests and finally cause metastasis. Throughout this progression, multiple genetic alterations may occur. In this series, we assayed cancer tissues for oncogenes (c-*erb*B-2, c-*myc*, *int*-2), tumor suppressor genes (RB, p53), and an antimetastatic gene (*nm*23/NDP kinase), to obtain information about breast cancer development.

The c-*erb*B-2 gene product is a growth factor receptor homologous to the epidermal growth factor receptor.[26] The putative ligand of c-*erb*B-2 protein, heregulin,[27] is also homologous to the epidermal growth factor family. Amplification and overexpression of c-*erb*B-2 is associated with poor prognosis in breast cancer patients.[4,5] On the other hand, there have been several reports indicating that the c-*erb*B-2 oncogene is frequently amplified and overexpressed in non-invasive breast tumors.[6,7] Our study has also shown that c-*erb*B-2 amplification may occur in *in situ* carcinomas, the earliest form of breast cancer. In addition, all of the c-*erb*B-2 amplified cases in node-positive patients in our series relapsed within 2 years after resection. This result suggests that breast cancer cells overexpressing c-*erb*B-2 may proliferate rapidly in metastatic foci, such as bone and lung.

As c-*myc* gene encodes a proliferative nuclear DNA-binding protein, its expression may be associated with breast cancer progression. Some reports have indicated that c-*myc* amplification is significantly correlated with tumor size, number of positive lymph nodes, and poor prognosis.[28,29] In this series, c-*myc* amplification could not be detected in tumors of less than 2 cm, but it increased with tumor size. On the other hand, one out of 5 intraductal cancers showed amplification of the c-*myc* gene. Due perhaps to the small study sample, no significant association between c-*myc* amplification and clinical parameters was detected.

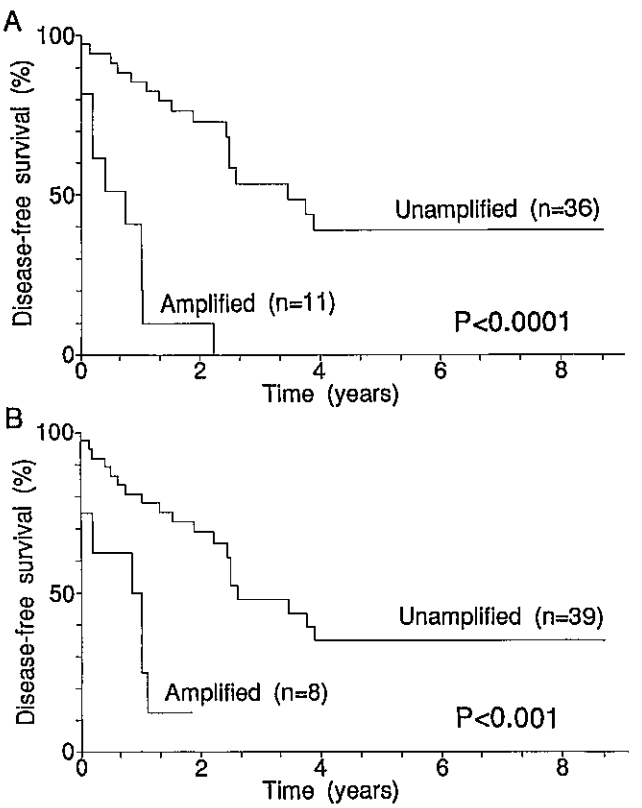

Fig. 4. Disease-free survival in node-positive patients with and without c-*erb*B-2 (A) or *int*-2 (B) amplification.

The *int*-2 gene is often amplified, but not expressed in various cancers. Recent studies found that PRAD1 (linked to the 11q13 lesion) or the cyclin D gene[30, 31] is coamplified and overexpressed in *int*-2 amplified tumors.[32, 33] In our series, all but one node-positive patient with an *int*-2 amplified tumor had relapsed within 2 years after resection. Furthermore, *int*-2 amplification was not found in non-invasive carcinomas. It was more often amplified in cases with severe lymph node involvement. These observation suggest that *int*-2 amplification (cf. overexpression of cyclin D) may be important in malignant transformation of human breast cancer. The amplification and overexpression of cyclin D gene in human breast cancer tissues are to be examined in further experiments.

Although it is not clear that decrease or loss of RB expression detected by immunohistochemistry is caused only by genetic alteration, the retinoblastoma gene protein has been reported to be inactivated by mutations of its gene in breast cancer.[13–15] Though the loss of RB expression was observed even in non-invasive breast cancer, its expression decreased with increasing tumor size in the invasive cases. Furthermore, the cases with amplified c-*myc* gene and no expression of RB protein had tumor sizes greater than 9 cm. Recently, the negative cooperation of c-*myc* and RB proteins in the control of cell proliferation has reported.[34, 35] Our data support the interaction of c-*myc* and RB.

Mutation of the p53 gene has also been reported in breast cancer.[17, 36] Because the half-life of normal p53 is of the order of minutes, it is not usually detectable immunohistochemically in normal untransformed cells. In this series, p53(mutant) expression was not found in tumors of less than 2 cm, and it was significantly correlated with tumor size. However, the expression was detected even in tumors with greater than 90% intraductal components.

The *nm*23 gene was identified as an antimetastatic gene.[37] High expression of its mRNA and the product, NDP kinase, associated with the absence of lymph node metastasis and good prognosis in breast cancer patients.[18, 19, 38] Although our study demonstrated no correlation between NDP kinase expression and lymph node metastasis or disease-free/overall survival of breast cancer patients, its expression was significantly associated with estrogen receptor status and decreased with increasing tumor size. These results may be related that the anti-NDP kinase antibody we used was derived from the α isoform of the rat NDP kinase, which is identical with the human *nm*23-H2 gene product.[39, 40] It is, therefore, suggested that the NDP kinase encoded by the *nm*23-H2 gene may have some role in tumor growth of human breast cancer other than metastasis. Because *nm*23-H1 allelic loss was found in one case with greater than 90% intraductal components, alteration of this gene may occur in an earlier stage of breast cancer progression.

There were 8 (10%) cases with no abnormality in our analysis. In these cases, 6 were node-positive and 1 of them has relapsed. Therefore, genetic alterations not yet described may play a role in breast cancer development in some individuals.

Finally, estrogen receptor expression wanes with increasing tumor size and extraductal invasion. The cancer cells may acquire new proliferative pathways sequentially as a result of multiple genetic alterations which enable them to bypass the estrogen-dependent proliferation.

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