[CANCER RESEARCH 50, 7184–7189, November 15, 1990]

Allelotype of Breast Cancer: Cumulative Allele Losses Promote Tumor Progression in Primary Breast Cancer[1]

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ABSTRACT

Allele loss on a specific chromosome has implied the existence of a tumor suppressor gene such as the p53 gene and the *RB* gene. In order to determine which chromosome(s) carries a tumor suppressor gene(s) that contributes to tumor progression in primary breast cancer, we analyzed the loss of heterozygosity for each autosomal chromosome arm by using 39 restriction fragment length polymorphism markers including 25 variable numbers of tandem repeat probes. In 79 primary breast cancers, we found the frequent loss on the long arm of chromosome 13 (21%), the long arm of chromosome 16 (45%), and the short arm of chromosome 17 (56%). Interestingly, breast cancers in which loss of both chromosomes 13q and 17p was detected showed more malignant histopathological features, and a group of the tumors in which chromosome 16q loss was detected presented with frequent lymph node metastasis. Furthermore, the result of the deletion mapping on chromosome 17p implied the existence of a tumor suppressor gene distal to the p53 gene as well as the p53 gene itself for primary breast cancer. These results suggest that at least 4 tumor suppressor genes exist on chromosomes 13q, 16q, and 17p for primary breast cancer.

INTRODUCTION

The accumulation of genetic alterations contributes to tumor progression including metastatic phenotype and grade of malignancy (1). There have been recent reports of LOH[3] at specific chromosome sites in several types of cancers such as colorectal cancer (5q, 17p, 18q) (2), renal cancer (3p) (3), small-cell lung carcinoma (3p, 13q, 17p) (4–6), bladder cancer (6p, 9q, 11p, 17p) (7, 8), Wilms' tumor (11p) (9), and retinoblastoma (13q) (10, 11). These results imply the existence of a tumor suppressor gene on each chromosome in which a consistent deletion was detected.

In further studies, Vogelstein *et al.* (2) reported that multiple steps of genetic alteration are required for the progression of colorectal cancer and they identified the p53 gene and the *DCC* gene as candidates on chromosomes 17p and 18q, respectively (12, 13). In the case of the p53 gene, point mutations at several different positions or deletions within the retained allele have been detected in several primary tumors and cancer-derived cell lines such as colon, lung, and brain origin (14, 15). The p53 gene thus appears to be one of the common tumor suppressor genes for human cancer (16).

On the other hand, several LOHs on specific chromosomes have been reported for cancers unique to women such as breast cancer (1q, 3p, 11p, 13q, 17p) (17–22), ovarian cancer (6q, 11p, 17p) (23), and cancer of the uterus (3p) (24). In the case of breast cancer, Rodgers *et al.* (25) reported in their cytogenetic studies that the most frequently lost chromosomes were 8, 13, and 16 and the common region of karyotypic change was chromosome 1qter-1q21. Chen *et al.*(17) conducted RFLP analysis of 48 breast carcinomas and suggested that the tumor suppressor gene for breast cancer might be located on chromosome 1q23–32. Furthermore, Devilee *et al.* (18) showed that there were at least 4 different chromosomal regions in which LOH occurred in breast cancer and allele loss of a marker on chromosome 3 (region p14-p21) was found in 7 of 15 informative cases.

In order to study (*a*) how many chromosomes or parts of chromosomes are involved in the development of primary breast cancer, (*b*) whether each LOH is independent or has any association with losses on other chromosomes or with amplification of *erb*B2 (26) or *Int*-2 oncogene (27), and (*c*) whether LOH correlates with histopathological types, we examined 79 pairs of normal tissues and breast cancers for LOH with 39 RFLP probes including 25 VNTR markers (28) covering all the autosomal chromosomes; we also examined *erb*B2 and *Int*-2 oncogenes for evidence of amplification in primary breast cancer.

MATERIALS AND METHODS

Materials. Tumor and normal tissues were obtained from 79 patients with primary breast cancer undergoing mastectomy at the Cancer Institute Hospital, Tokyo, Japan; 69 of them were invasive ductal carcinomas and 10 were classified as special types including 2 mucinous carcinomas and 4 invasive lobular carcinomas. Histopathological classification was based on the typing scheme of the Japanese Breast Cancer Society (29) which is basically the same as the World Health Organization typing scheme for breast tumors.

DNA Extraction from Tissues. All tissue samples were immediately frozen in liquid nitrogen following operation and were ground to very fine powder using a mortar and pestle. The powder was transferred to a 15-ml tube and suspended in 4 ml of 50 mM Tris HCl, pH 7.5-150 mM NaCl-50 mM EDTA with 1% SDS-1 mg/ml proteinase K for 4–16 h at 37°C. Genomic DNA was purified by phenol: chloroform:isoamyl alcohol (25:24:1) extraction and ethanol precipitation. DNAs were spooled onto a glass rod and suspended in 10 mM Tris HCl, pH 7.5-1 mM EDTA, pH 8.5 buffer.

Probes. All probes used in this study are listed in Table 1. The information concerning these probes is described in the Human Gene Mapping 10 (30). c-*erb*B2 (pKX044) (31) and *Int*-2 (SS6) (32) were obtained from the Japanese Cancer Research Resources Bank. p79–2–23 (*D16S7*) was kindly supplied by Dr. Mike Litt.

Hybridization Conditions. After alkali transfer of the DNA in 0.1 N NaOH-0.1 M NaCl, nylon membranes (Pall Biodyne) were neutralized in 2× standard saline citrate (0.15 M NaCl-0.015 M sodium citrate) and fixed by UV cross-linking at 120 mJ with Stratalinker according to the manufacturer's instructions. Prehybridization and hybridization were carried out in 7% polyethylene glycol 8000-10% SDS with 200 μg/ml of human placental DNA or 100 μg/ml of salmon sperm DNA at 65°C overnight. The membranes were hybridized at 65°C for 16–24 h with

Received 5/29/90; accepted 8/14/90.

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[1] The work was supported in part by a grant from Uehara Memorial Foundation.

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[3] The abbreviations used are: LOH, loss of heterozygosity; RFLP, restriction fragment length polymorphism; VNTR, variable number of tandem repeat; SDS, sodium dodecyl sulfate; FAL, fractional allelic loss.

Table 1 *Loss of heterozygosity in human breast cancer*

Chromosome location	Probe	Locus	Enzyme	No. of patients tested	Allelic loss/informative cases (%)
1p	YNZ2[a]	D1S57	*Msp*I	51	4/43 (9)
1q	HHH106	D1S67	*Msp*I	41	2/15 (13)
2p	TBAB5.7[a]	D2S47	*Msp*I	51	0/23 (0)
2q	YNH24[a]	D2S44	*Msp*I	55	5/49 (10)
3p	EFD145	D3S32	*Taq*I	42	3/16 (19)
3q	EFD64.2[a]	D3S46	*Msp*I	43	0/17 (0)
4p	YNZ32[a]	D4S125	*Taq*I	45	1/6 (17)
4q	EFD139[a]		*Msp*I	40	2/34 (6)
5q	MC5.61	D5S84	*Taq*I	32	2/11 (18)
6p	YNZ132[a]	D6S40	*Msp*I	49	5/28 (18)
6q	JCZ30[a]	D6S37	*Taq*I	47	2/23 (9)
7p	RMU7-4	D7S370	*Msp*I	46	1/8 (6)
7q	JCZ67[a]	D7S396	*Msp*I	38	3/20 (15)
8q	MCT128.2[a]	D8S39	*Taq*I	47	1/14 (7)
9p	MCT112	D9S15	*Msp*I	34	1/11 (9)
9q	EKZ19	D9S17	*Taq*I	44	6/35 (17)
10p	TBQ7[a]	D10S28	*Msp*I	41	4/32 (13)
10q	EFD75[a]	D10S25	*Taq*I	42	2/23 (9)
11p	Insulin[a]	INS	*Hae*III	49	3/31 (10)
11q	SS6	INT2	*Taq*I	39	0/12 (0)
12p	THH14	D12S16	*Msp*I	39	2/12 (17)
12q	YNH15	D12S17	*Msp*I	42	1/19 (5)
13q	MHZ47[a]	D13S52	*Msp*I	41	7/33 (21)
14q	CMM101[a]	D14S13	*Msp*I	51	6/47 (13)
15q	YNZ90.1	D15S28	*Bam*HI	41	2/12 (17)
16p	CMM65[a]	D16S84	*Taq*I	48	1/21 (5)
16q	p79-2-23[a]	D16S7	*Taq*I	48	19/42 (45)
17p	YNZ22[a]	D17S30	*Taq*I	46	16/34 (47)
			*Msp*I	40	19/40 (48)
Total (YNZ22)				53	26/50 (52)
	BHP53	P53	*Bam*HI	45	8/25 (32)
	MCT35.1	D17S31	*Msp*I	51	9/28 (32)
	LB17.3[a]		*Msp*I	50	14/38 (37)
Total (chromosome 17p)				59	33/59 (56)
17q	CMM86[a]	D17S74	*Msp*I	32	3/32 (9)
18p	B74	D18S3	*Msp*I	39	1/9 (11)
18q	MCT108.2	D18S24	*Taq*I	44	1/13 (8)
19p	JCZ3.1[a]	D19S20	*Taq*I	48	7/36 (19)
19q	EFD4.2[a]	D19S22	*Taq*I	48	3/19 (16)
20p	CMM6[a]	D20S19	*Taq*I	45	2/37 (5)
21q	MCT15[a]	D21S113	*Msp*I	50	4/26 (15)
22q	EW7.20[a]		*Msp*I	48	1/9 (11)

[a] VNTR markers.

^{32}P-labeled probes (specific activity $>1 \times 10^8$ cpm/μg DNA) labeled using a random primer method (33). After hybridization, membranes were washed twice at 65°C with 0.1 × standard saline citrate-0.1% SDS and exposed for autoradiography to Kodak XAR film at −70°C. The membranes used were stripped in 0.1 N NaOH according to the method of Donis Keller *et al.* (34) and were repeatedly rehybridized.

Statistical Analysis. The χ^2 test and Fisher's exact test were used for statistical analysis of the results.

RESULTS

Multiple Allele Losses in Breast Cancer. We tested all arms of autosomal chromosomes, except the short arms of acrocentric chromosomes, 5p and 8p, with the RFLP markers listed in Table 1. The majority of them were VNTR markers (28) which showed high heterozygosity. The results of Southern blots with markers on chromosomes 16q and 17p are shown in Fig. 1, *A* and *B*. A summary of the frequency of LOH is shown in Fig. 2. No LOH was observed on chromosomes 2p, 3q, or 11q in more than 10 informative cases. Frequent LOHs were observed with RFLP markers on chromosomes 16q and 17p. Allele loss of chromosome 17p was observed in 33 of 59 informative cases (56%), a frequency similar to that reported previously (18, 22). Frequent LOH of chromosome 16q (19 of 42 informative cases, 45%) in breast cancer has not been reported before, although involvement of chromosome 16q has been noted in cytogenetic studies (25). In the present study, losses of chromosomes 1q (13%), 3p (19%), 11p (10%), and 13q (21%) in primary breast cancer were observed less frequently than in previous studies (17–22).

Interestingly, loss of chromosome 16q was observed in 15 of 25 informative cases which showed LOH of chromosome 17p; in contrast, only 4 of 17 informative cases which showed LOH of chromosome 17p lost chromosome 16q ($P = 0.0209$, by Fisher's exact test) (Table 2) and loss of chromosome 13q was found only in the group of tumors which had lost chromosome 17p. This concordant loss of chromosomes 13q and 17p is statistically significant as shown in Table 3 ($P = 0.0118$, by Fisher's exact test). Furthermore, tumors which lost both chromosomes 13q and 17p had more malignant histopathological features (Table 4).

We summarized the FAL value (35) in 57 breast tumors with >5 informative cases by RFLP analysis. The distribution of FAL values is shown in Fig. 3. The median FAL was 0.15 less than that of colorectal cancer (FAL = 0.20, 56 tumors) (35). Allelic losses of >30% in the evaluable chromosome arms were observed in 9 breast tumors. This result is similar to the

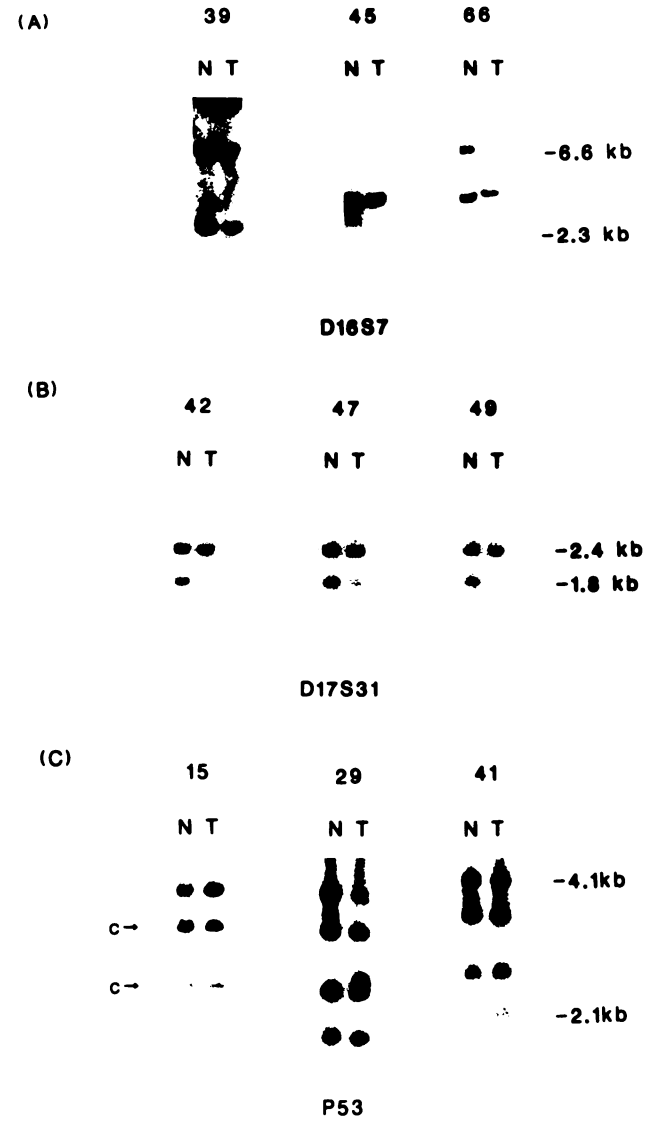

Fig. 1. Southern blot analysis demonstrating LOH of DNAs from tumor (*T*) and normal (*N*) tissues in patients with primary breast cancer. The probes used are MCT35.1 (*D17S31*) (*A*), p79-2-21 (*D16S7*) (*B*), and BHP53 (*P53*) (*C*). *Abscissa*, tumor numbers; *arrows* (*C*), constant bands; kb, kilobases.

Table 2 *Association of allele loss between chromosomes 16q and 17p*[a]

ch17p	LOH of chromosome 16q +	−	Total
LOH			
+	15	10	25
−	4	13	17
Total	19	23	

[a] $P = 0.0209$, by Fisher's exact test.

Table 3 *Association of allele loss between chromosomes 13q and 17p*[a]

ch17p	LOH of chromosome 13q +	−	Total
LOH			
+	7	12	19
−	0	14	14
Total	7	26	

[a] $P = 0.0118$, by Fisher's exact test.

Table 4 *Relation between concordant loss of chromosomes 13q and 17p and histopathological diagnosis*

Chromosomal loss	Histopathological class[a] a1	a2	a3	Other
17p loss (+) 13q loss (−)	4 (33%)	2 (17%)	6 (50%)	0
17p loss (+) 13q loss (+)	0 (0%)	1 (14%)	5 (72%)	1 (14%) (b3)
Total distribution in our samples	14 (18%)	16 (20%)	38 (48%)	11 (14%)

[a] Six (a3) of 12 tumors with 17p loss but no 13q loss and 6 (a3 and b3) of 7 tumors with both 17p and 13q losses were classified to the worst histological types ($P = 0.1441$, by Fisher's exact test). a1, papillotubular carcinoma (well differentiated); a2, solid-tubular carcinoma (moderately-poorly differentiated); a3, scirrhous carcinoma (poorly differentiated); b3, invasive lobular carcinoma (poorly differentiated).

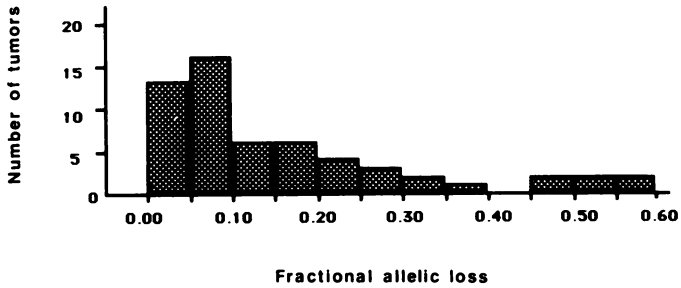

Fig. 3. FAL of primary breast cancers. The FAL in 57 primary breast tumors which showed >5 informative cases by RFLP analysis was defined according to the definition of Vogelstein *et al.* (35).

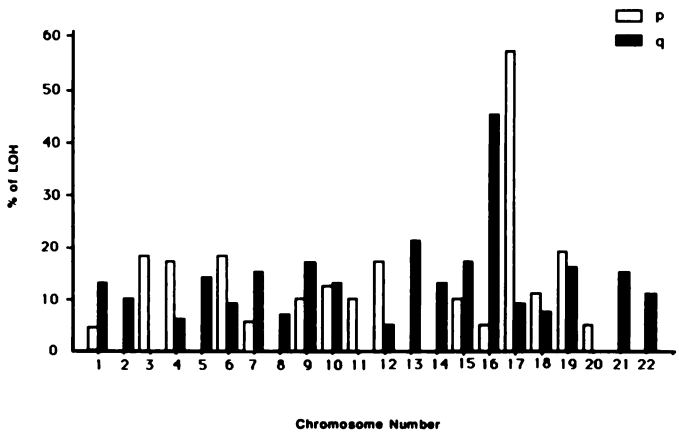

Fig. 2. Frequency of allele losses on individual chromosomal arms. Allele losses were tested by RFLP analyses. The probes used are listed in Table 1. *Open columns*, p arm; *solid columns*, q arm.

distribution of FAL values in colorectal cancers. However, any correlation between FAL values and histopathological features could not be found in this study.

Amplification of *erb*B2 and *Int*-2 Oncogenes in Primary Breast Cancer. We examined *erb*B2 and *Int*-2 oncogenes for amplification since such findings have been reported as useful prognosis values for primary breast cancer (26, 27). Amplification of *erb*B2 and *Int*-2 was detected in 9 of 42 (21%) and 4 of 37 (11%) primary breast cancers, respectively, as shown in Fig. 4. Interestingly, one case showed coamplification of *erb*B2 and *Int*-2 (Fig. 4, tumor 68). This tumor, which was classified as a solid tubular carcinoma, had lymph node metastasis. Because of the small number tested, we could not find any statistical correlation between amplification of *erb*B2 and histopathological data. However, relatively frequent amplification of *erb*B2

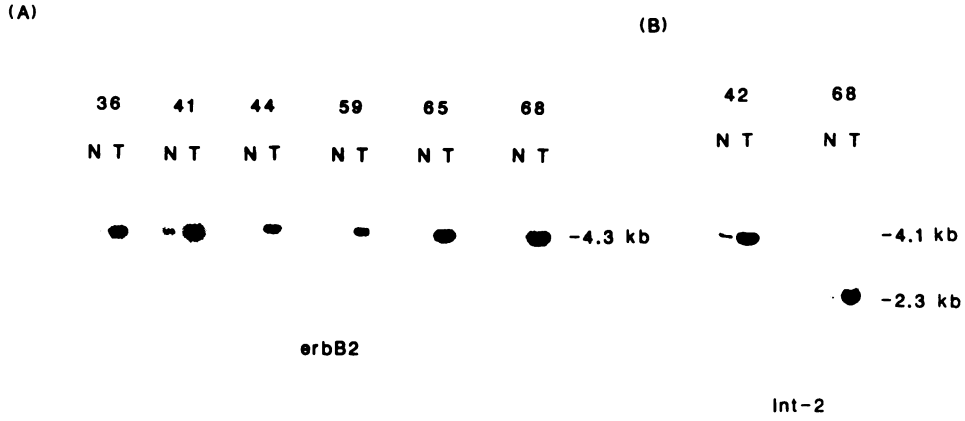

Fig. 4. Amplification of *erb*B2 and *Int*-2 oncogenes in primary breast cancer. All DNAs from each sample (5 μg) were digested with *Taq*I restriction endonuclease. pKX044 which contains genomic DNA of *erb*B2 (25) was used directly as a probe (*A*). SS6 which contains *Int*-2 oncogene (26) was used for the detection of both gene amplification and LOH (*B*). The β-globin gene was used for the detection of nonamplified genes as a control. *Abscissa*, tumor numbers; kb, kilobases; *T*, tumor tissue; *N*, normal tissue.

was observed in breast tumors that showed allele loss on chromosome 17p [7 of 22 (32%)] compared to the group of tumors which did not show such LOH [2 of 20 (10%)] ($P = 0.0881$, by Fisher's exact test).

Deletion Map of Allele Loss on Chromosome 17p. In order to identify a common region of deletion on chromosome 17p in primary breast cancer, we used 4 RFLP markers including MCT35.1 and LB17.3 which map between p53 and YNZ22 by multipoint linkage analysis.[4] To determine LOH at *P53*, we used the cosmid clone BHP53 which contains the polymorphic site of the *Bam*HI restriction endonuclease in the 3′-flanking region of the p53 gene, which is probably one of the tumor suppressor genes of colorectal cancer (12), as a RFLP marker (36). The typical pattern of its LOH is shown in Fig. 1*C*. Eight of 25 informative cases (32%) showed LOH with BHP53, and 26 of 50 cases (52%) revealed LOH at the *D17S30* (YNZ22) locus. LOH at *D17S30* was observed in 10 cases which retained heterozygosity with at least one locus proximal to *D17S30*, including the p53 gene. Furthermore, LOH at both *P53* and *D17S30* was observed in 5 cases. A common region of deletion on chromosome 17p was localized distal to LB17.3 from the results of 26 cases (Fig. 5*A*, tumor 66 in Fig. 6). However, 3 cases showed LOH at *P53* despite retention of heterozygosity at *D17S30* (Fig. 5*B*, tumor 82 in Fig. 6). These results suggest the existence of two different tumor suppressor genes (the p53 gene and a gene distal to LB17.3) for human primary breast cancer.

Correlation between LOH of Chromosome 16q and Lymph Node Metastasis. Frequent allele losses were detected with *D16S7* (p79-2-23) which was localized to chromosome 16q (37). Loss of chromosome 16q frequently coincided with lymph node metastasis [8 of 12 (67%)] compared to the group of tumors which retained chromosome 16q [8 of 20 (40%)] (Table 5). On the other hand, only 13 of 33 tumors (39%) in which 17p deletion was found had lymph node metastasis, and no difference was found in the group showing the retention of chromosome 17p [11 of 26 (42%)] (Table 5). Although the correlation between LOH of chromosome 16q and lymph node metastasis is not statistically significant ($P = 0.1367$, by Fisher's exact test), this weak trend might serve as a prognostic value for the metastatic potential in primary breast cancer.

DISCUSSION

Our results show frequent LOH of chromosome 17p (>50%) in primary breast cancer as reported previously (18, 22). In addition, deletion mapping in tumors suggests the existence of two tumor suppressor genes (P53 as well as a gene in a region distal to LB17.3). The p53 gene could be a common tumor suppressor gene since many mutations of p53 have been found in cancer cell lines or xenografts of primary tumors such as breast, lung, and colorectal cancer (14, 15). However, our results imply the existence of another tumor suppressor gene in the telomeric region of the short arm of chromosome 17 for primary breast cancer. This differs from the case of colorectal cancer (12). A group of breast tumors which showed allele loss of chromosome 13q also revealed LOH of chromosome 17p without exception ($P = 0.0118$, by Fisher's exact test). Six of 7 cases (86%) in which both chromosomes 13q and 17p were lost

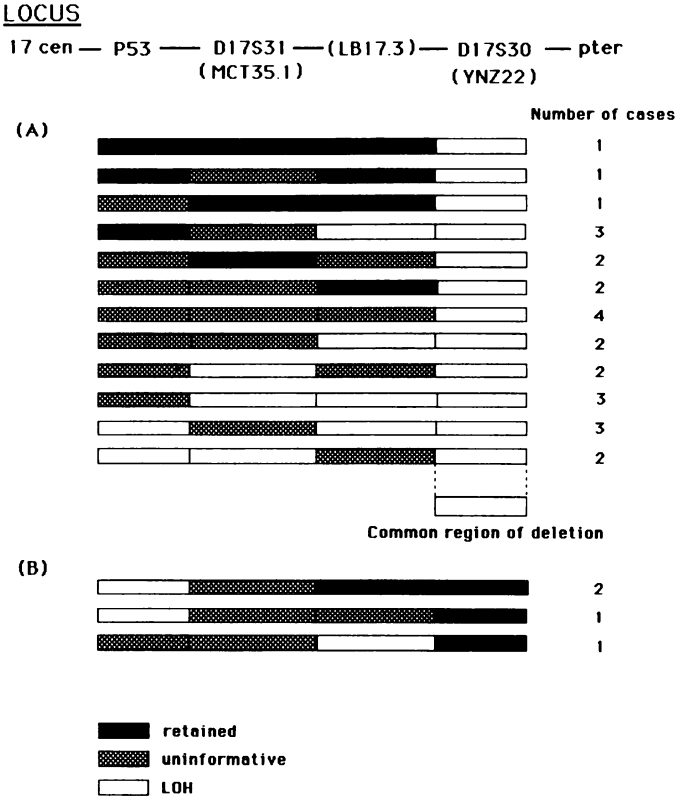

Fig. 5. A schematic representation of the common region of deletion on the short arm of chromosome 17 in primary breast cancer. The order of loci is supported with odds of $>10^5$ except P53 and D17S31. This order is also supported by physical mapping based on Miller-Dieker syndrome patients.[5] *Open columns*, LOH; *hatched columns*, uninformative cases; *solid columns*, no allelic loss.

[4] Y. Nakamura and R. White, unpublished data.

[5] Dr. David Ledbetter, personal communication.

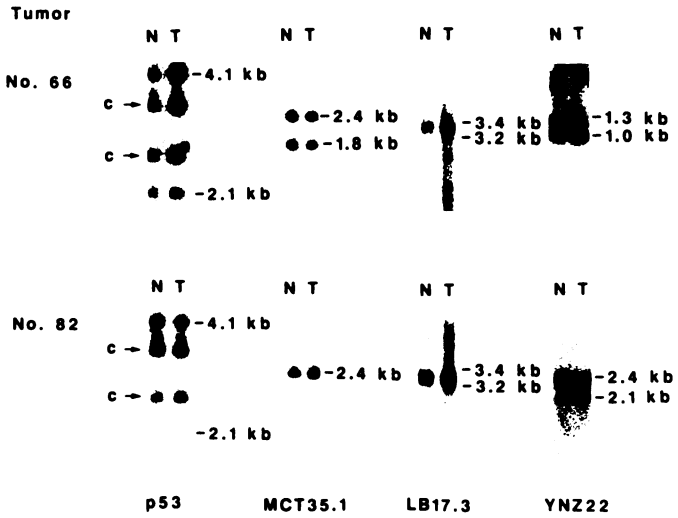

Fig. 6. Southern blot analysis for the tumors demonstrating potential existence of two regions of chromosome 17p. Tumor 66 showed only LOH (1.3-kilobase band) of YNZ22, but both alleles were retained with p53, MCT35.1, and LB17.3. Tumor 82 showed only LOH (4.1-kilobase band) of the p53 gene, but both alleles were retained with YNZ22 and LB17.3. MCT35.1 was not informative in this tumor. *Arrows* (*C*), the constant band.

Table 5 *Relation between lymph node metastasis and loss of chromosomes 16q and 17p*

Frequent lymph node metastasis was observed in a group with loss of chromosome 16q ($P = 0.1367$, by Fisher's exact test).

Lymph node metastasis	LOH of chromosome 16q		LOH of chromosome 17p	
	+	−	+	−
+	8	8	13	11
−	4	12	20	15
Total	12	20	33	26

were classified as scirrhous carcinomas or an invasive lobular carcinoma which showed the highest rate of lymph node metastasis and had the worst prognosis, as previously reported by Page and Anderson (38). Although statistically not significant in this study, allele losses of both chromosomes 13q and 17p may have potential prognostic value at the time of diagnosis of primary breast cancer. Amplification of *erb*B2 and *Int*-2 oncogenes occurred at frequencies similar to those reported previously (26, 27). We detected *erb*B2 and *Int*-2 amplification in 9 of 42 (21%) and 4 of 37 (11%) cancers, respectively (Fig. 4). In our studies, any correlation with *Int*-2 amplification could not be tested because of the small number of cases involved. However, we found a trend of frequent amplification of the *erb*B2 oncogene in tumors which lost chromosome 17p. Frequent LOH on chromosome 16q (45%) was observed in primary breast cancer. Tumors showing loss of chromosome 16q had frequent lymph node metastasis (67%), compared to the group of tumors in which chromosome 16q was not lost (Table 5). Although this is not statistically significant ($P = 0.1367$, by Fisher's exact test), this trend might be of prognostic value for the metastatic potential of breast tumors.

From our results and previous reports, it is clear that accumulation of genetic alterations is required for the transformation of a normal cell to a breast cancer, as in colorectal cancer. Furthermore, our data suggest the sequential genetic alteration during tumor progression in breast cancer. The first step is probably loss (>50%) of chromosome 17p. Inactivation of a growth regulatory gene on chromosome 17p may act at an early stage in primary breast cancer. The second event might be amplification of the *erb*B2 oncogene and/or loss of chromosome 13q, since there was a tendency for frequent amplification of *erb*B2 or frequent loss of chromosome 13q after the loss of chromosome 17p had occurred. The third event, loss of chromosome 16q, might have a significant role in the lymph node metastasis, which is clinically suggested as a prognostic indicator for poor prognosis. In order to confirm associations between specific mutations and clinical diagnosis, further experiments using a large number of primary breast cancers have to be done. Furthermore, because the frequency of allele loss may not be uniform across a chromosomal arm by using one marker per arm, it is necessary to examine LOH with more markers spaced <30 cM on each autosomal chromosome arm.

ACKNOWLEDGMENTS

We thank Drs. Isamu Nishisho, Yoshio Miki, Minoru Fujimori, and Takashi Tokino (Cancer Institute, Tokyo, Japan) for helpful advice, Mike Jones for careful reading of the manuscript, and Hiroko Shimamura for her excellent secretarial assistance. We gratefully acknowledge Dr. Michael Steel and Dr. Bert Vogelstein for comments on the manuscript and for many helpful discussions.

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