Jpn. J. Cancer Res. **92**, 1199–1206, November 2001

Association of Allelic Losses at 3p25.1, 13q12, or 17p13.3 with Poor Prognosis in Breast Cancers with Lymph Node Metastasis

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To identify specific allelic losses that might correlate with postoperative mortality of patients with node-positive breast carcinomas, we examined tumors from a cohort of 263 such patients, who were followed clinically for 5 years postoperatively, for allelic losses among 18 microsatellite markers. Patients whose tumors had lost an allele at 3p25.1, 13q12, or 17p13.3 had significantly higher risks of mortality than those whose tumors retained both alleles at those loci. At 3p25.1, the 5-year mortality rate was 33.8% among patients with losses vs. 16.8% with retention (P=0.0154); at 13q12, 30.3% vs. 13.0% (P=0.0241); and at 17p13.3, 30.4% vs. 16.2% (P=0.0243). Combined losses at 3p25.1 and 17p13.3 increased the predicted postoperative mortality risk by a factor of 4.9 (5-year mortality rate of 38.2% vs. 8.0%, P=0.0006), and combined losses at 3p25.1 and 13q12 raised the predicted postoperative mortality risks by a factor of 2.9 (34.7% vs. 12.7%, P=0.0441). These data indicate that loss of heterozygosity (LOH) at any one or a pair of loci at 3p25.1, 13q12, or 17p13.3 is a significant predictor of postoperative mortality for breast-cancer patients.

Key words: Loss of heterozygosity — Tumor suppressor gene — Prognostic factor — Breast cancer

In an effort to identify chromosomal regions where allelic losses are frequent in breast cancers, we previously examined an average of 200 primary breast cancers for loss of heterozygosity (LOH), using more than 150 polymorphic microsatellite markers derived from throughout the human genome.[1–13] The clinical course of breast cancer varies widely among patients, from modest, non-invasive lesions to aggressive, inflammatory carcinomas. These differences in biological characteristics may be explained by differences in the pattern of alterations among genes that play roles in breast carcinogenesis. Prediction of postoperative prognosis for patients with breast cancer has increased in importance in view of the variety of adjuvant therapies that are now available. However, at present such decisions for an individual patient are still based on conventional prognostic factors such as tumor size, clinical stage, lymph node metastasis, and hormone-receptor status.[14, 15]

We previously described an association of LOH at some chromosomal loci with postoperative prognosis in breast cancers overall.[12, 13] In the present study we looked instead for LOH that might be associated with poor prognosis among aggressive breast cancers specifically, i.e. those that had metastasized to lymph nodes. We examined such tumors from 263 breast-cancer patients for LOH in 18 regions where frequent LOH had been observed in breast cancers in general, using a representative polymorphic marker for each region.

MATERIALS AND METHODS

Patients, specimens and DNA preparation The study population consisted of 263 patients with lymph-node metastasis who underwent surgery for breast cancer between 1989 and 1993 at the Cancer Institute Hospital, Tokyo. Informed consent in the formal style approved by the ethical committee of the Hospital had been obtained from each patient prior to surgery. The majority of the patients received standard or modified radical mastectomy at the Cancer Institute Hospital during the period of 1989 through 1993. All patients were followed clinically for at least 5 years or until decease. A part of the cohort of patients analyzed in the present study overlapped with those analyzed in our previous study. Details of each patient and the clinical data can be obtained by request to the corresponding author, provided that patients agree to public disclosure of additional clinical data. Estrogen receptor (ER) and progesterone receptor (PgR) activity was measured as described previously.[3] All clinical and

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Table I. Clinical Characteristics of 263 Node-positive Breast-cancer Patients

	No. of patients (n=263)
1. Median age (range): 51.1 years (29–79)	
2. t (Tumor stage)[a)]	
t1	54
t2	167
t3	42
3. n (Nodal status)[a)]	
n1α	141
n1β	62
n2	60
4. Pathologic stage[a)]	
stage I	42
stage II	136
stage III	85
stage IV	0
5. Menopausal status	
pre-menopause	130
post-menopause	130
unknown	3
6. Histological type[a)]	
1a (non-invasive)	1
a1 (papillo-tubular)	44
a2 (solid-tubular)	67
a3 (scirrhous)	134
bc (special types)	17
7. Estrogen receptor	
ER (+)	151
ER (−)	112
8. Progesterone receptor	
PgR (+)	171
PgR (−)	92
9. Outcome	
death	55 (20.9%)

a) Clinical recording scheme according to the Japanese Breast Cancer Society (1989).

histopathological data (Table I) were obtained from an electronic database maintained by the Cancer Institute Hospital in a recording format established by the Japanese Breast Cancer Society.[16)] As regards postoperative adjuvant therapy, all patients were treated according to the "Postoperative Clinical Protocol for Breast Cancer" of the Cancer Institute Hospital. In principle, the choice of adjuvant therapy for each patient, whether CMF (cyclophosphamide, methotrexate, fluorouracil) for low-grade metastasis (<10 nodes) or CAF (cyclophosphamide, adriamycin, fluorouracil) for high-grade metastasis (>10 nodes), and/or endocrine therapy for patients with ER-positive status, was strictly determined on the basis of type of surgery, lymph-node involvement, and the presence of local or distant metastases. None of the patients had undergone radiotherapy or chemotherapy prior to surgery. Tumors and samples of non-cancerous breast tissue were excised from each patient, frozen immediately, and stored at −80°C. Genomic DNAs were extracted from the frozen materials as previously described.[17)]

Table II. Chromosomal Regions, Polymorphic Markers, and LOH Frequencies at the 18 Loci Examined in Node-positive Breast Cancers

Chromosomal region	DNA marker	Informative cases/263 cases	LOH (+) cases/ informative cases	LOH frequency (%)
1p36	D1S1612	177	62/177	35
1p34	D1S552	152	40/152	26
1p22	D1S551	178	49/178	28
3p25.1	D3S1286	186	58/186	31
3p14.3	D3S1295	128	50/128	39
6q26–27	D6S503	133	58/133	44
8p22	D8S136	154	84/154	55
9p21–22	D9S157	164	44/164	27
11p15	D11S922	182	56/182	31
11q23–24	D11S1998	168	92/168	55
13q12	D13S171	146	65/146	45
13q14	D13S270	153	51/153	33
16q24.3	D16S413	199	112/199	56
17p13.3	D17S849	185	93/185	50
17p13.1	TP53	182	102/182	56
17q21.1	D17S934	179	63/179	35
18q21.1	D18S474	153	48/153	31
22q13	D22S272	173	66/173	38

Analysis of LOH Procedures for LOH analysis were described elsewhere.[12)] In brief, DNAs from matched normal and cancerous tissues were examined for LOH with respect to the 18 microsatellite markers listed in Table II. Microsatellite sequences were amplified by the polymerase chain reaction (PCR) using 10 ng of genomic DNA, and PCR products were electrophoresed and autoradiographed as described previously. Definition of LOH and distinction from chromosome multiplication were judged according to procedures we have described previously.[12)]

Statistical analysis Postoperative survival was measured from the date of surgery to the date of last follow-up or death. Survival curves were constructed using the Kaplan-Meier method, and the significance of differences in survival rates was tested using the log-rank test as a univariate analysis. Cox's proportional-hazards model for the risk ratio was used to assess the simultaneous contribution of each covariate in the multivariate analysis. Multivariate analysis was carried out with five variables (tumor size, number of positive nodes, LOH at 3p25.1, 13q12, and 17p13.3). P values of <0.05 were considered statistically significant. StatView version 4.5 software (SAS Institute, Inc., San Francisco, CA) was used for those calculations.

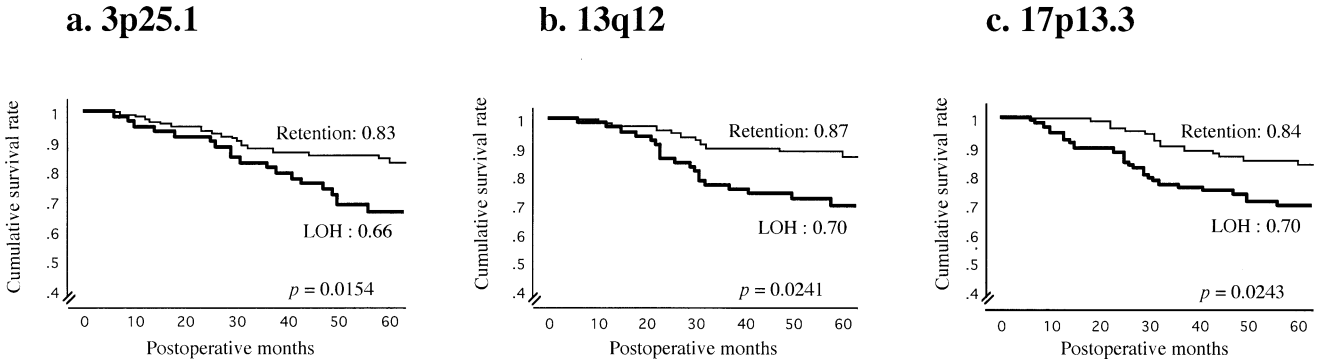

Fig. 1. Kaplan-Meier curves of postoperative overall survival for patients whose tumors retained both alleles (Retention) or had lost one allele (LOH) at each marker locus indicated in each panel.

RESULTS

Table I summarizes conventional clinical data for the cohort of 263 node-positive breast-cancer patients. All surviving patients were followed for at least 5 years. Of the 263 patients, 55 women died within 5 years; the 5-year overall survival rate was 79.1%.

Table II shows the frequency of allelic loss (LOH) at each of the 18 chromosomal regions previously chosen as loci that displayed frequent LOH in breast cancers[12]; LOH ranged from 26 to 56% among the 263 node-positive tumors examined here. D16S413 (at 16q24.3) detected the highest frequency of LOH (112 of the 199 informative tumors, 56.4%). Tumor DNAs in these panels show LOH at D3S1286 on 3p25.1, at D13S171 on 13q12, and at D17S849 on 17p13.3.

Kaplan-Meier analysis of overall survival revealed that postoperative risk of mortality was greater for patients whose tumors showed LOH at 3p25.1, 13q12, or 17p13.3 compared with patients whose tumors retained both alleles (Fig. 1). Table III shows the results of log-rank tests for statistical significance of various parameters in univariate analyses.

Among the 186 patients whose tumors were informative at 3p25.1, 33.8% of those with LOH died within 5 years after surgery, compared with a 16.8% mortality rate among patients whose tumors retained both alleles of the 3p25.1 marker (2.0 times relative risk of mortality; P=0.0154 by log-rank test) (Fig. 1a, Table III). Similarly, Fig. 1b shows the correlation at 13q12, i.e., 30.3% 5-year mortality among patients with LOH and 13.0% among those with retention (2.3 times relative risk of mortality; P=0.0241, Table III). Fig. 1c shows the correlation at 17p13.3, i.e., 30.4% 5-year mortality among patients with LOH and 16.2% among those with retention (1.9 times relative risk of mortality; P=0.0243, Table III). No markers from the other 15 frequently deleted regions showed any correlation of LOH with mortality. When calculated in combination, LOH at both 3p25.1 and 17p13.3 was associated with a risk of mortality 4.8 times higher than for patients who retained all four alleles (5-year mortality rate, 38.2% vs. 8.0%, P=0.0006; Fig. 2a, Table III). Similarly, LOH at both 3p25.1 and 13q12 was associated with a rela-

Table III. Univariate Analysis of Postoperative Mortality According to LOH Status in 263 Breast Cancers

	Region (marker)	LOH status	5-year mortality rate (%)	Log-rank test P value	Relative risk
	3p25.1	retention	16.8	0.0154	2.0
	(D3S1286)	LOH	33.8		
	13q12	retention	13.0	0.0241	2.3
	(D13S171)	LOH	30.3		
	17p13.3	retention	16.2	0.0243	1.9
	(D17S849)	LOH	30.4		
	3p25.1 and	retention	8.0	0.0006	4.8
	17p13.3	LOH	38.2		
	3p25.1 and	retention	12.7	0.0441	2.7
	13q12	LOH	34.7		
Number of positive lymph nodes					
(1–10)	3p25.1	retention	11.6	0.0190	2.3
	(D3S1286)	LOH	27.1		
	13q12	retention	9.4	0.1313	1.9
	(D13S171)	LOH	17.7		
	17p13.3	retention	7.8	0.0173	3.0
	(D17S849)	LOH	23.3		
(Over 10)	3p25.1	retention	39.7	0.049	1.9
	(D3S1286)	LOH	75.0		
	13q12	retention	27.3	0.1175	2.3
	(D13S171)	LOH	61.5		
	17p13.3	retention	47.4	0.1682	1.4
	(D17S849)	LOH	65.9		

tive risk of mortality 2.7 times greater than that for patients who retained all four alleles (5-year mortality rate, 34.7% vs. 12.7%, P=0.0441; Fig. 2b, Table III). Clinical characteristics of each group of patients, classified according to LOH status at each of the three loci, are given in Table IV.

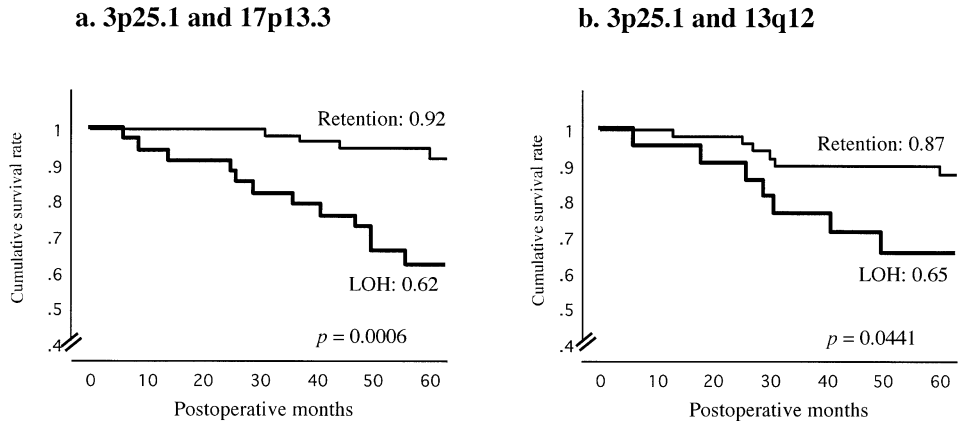

Fig. 2. Kaplan-Meier curves of postoperative overall survival for patients whose tumors retained all four alleles (Retention) or had LOH at both marker loci.

Table IV. Characteristics of Breast Cancer Patients According to LOH Status

	3p25.1		13q12		17p13.3	
	LOH (+) (n=58)	LOH (−) (n=128)	LOH (+) (n=65)	LOH (−) (n=81)	LOH (+) (n=93)	LOH (−) (n=92)
Mean age±SD	50.7±10.7	51.1±10.5	50.7±11.5	50.2±9.6	51.5±11.4	50.9±10.2
Menopausal status						
pre-menopause	27	62	32	42	39	48
post-menopause	31	63	33	38	54	43
unknown	0	3	0	1	0	1
t (Tumor size)[a)]						
t1	9	28	16	15	18	16
t2	38	79	38	52	55	59
t3	11	21	11	14	20	17
n (Nodal status)[a)]						
n1α	28	70	32	49	53	46
n1β	13	32	18	18	15	27
n2	17	26	15	14	25	19
Pathologic stage[a)]						
stage I	8	20	13	11	15	9
stage II	29	68	34	46	43	51
stage III	21	40	18	24	35	32
stage IV	0	0	0	0	0	0
Histological type						
noninvasive	0	1	0	0	0	0
papillotubular	4	24	5	19	10	21
solid tubular	19	29	24	21	31	19
scirrhous	32	63	33	34	47	46
special types	3	11	3	7	5	6

a) Clinical recording scheme according to the Japanese Breast Cancer Society (1989).

Table V. Multivariate Analysis of Five Variable with Respect to Overall Survival among 263 Breast-cancer Patients

Variable	Overall survival		
	P	Relative risk	95%CI
t (Tumor size)	0.0273	1.0	1.0–1.0
Number of positive nodes	<0.0001	1.1	1.0–1.1
3p25.1; LOH	0.0029	2.6	1.4–5.0
13q12; LOH	0.0634	1.9	1.0–4.1
17p13.3; LOH	0.0279	2.1	1.1–4.0

Table V summarizes results of multivariate analyses using the Cox's proportional-hazards regression model. Allelic losses at 3p25.1 and 17p13.3 were significant predictors of earlier postoperative death, as were large tumor size and number of positive lymph nodes. The hazard ratio for LOH at 3p25.1 was 2.6 (95%CI, 1.4–5.0; P=0.0029), and the ratio for LOH at 17p13.3 was 2.1 (95%CI, 1.1–4.0; P=0.0279). LOH at 13q12 showed borderline significance.

We classified the patients into two groups according to grade of lymph-node metastasis, i.e. over 10 or not, and analyzed the prognostic correlation of LOH at 3p25.1, 13q12, or 17p13.3 for each group separately. In the over-10 group, one marker showed remarkable differences in postoperative mortality according to LOH status; the 5-year survival rate was 25.0% among patients with losses vs. 60.3% with retention of both alleles at 3p25.1 (P=0.0499; Fig. 3, Table III). In the other group, two markers showed remarkable differences in postoperative mortality according to LOH status; the 5-year survival rate was 72.9% among patients with losses vs. 88.4% with retention of both alleles at 3p25.1 (P=0.0190; Fig. 4a, Table III); the survival rate was 76.7% with losses vs. 92.2% with retention as to LOH at 13q12 (P=0.0173; Fig. 4b, Table III).

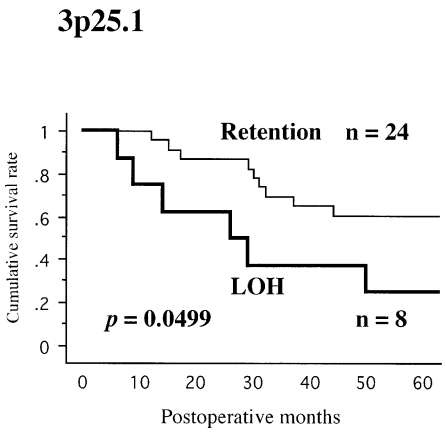

Fig. 3. Kaplan-Meier curves of postoperative overall survival among "high grade (over 10) metastasis" patients whose tumors retained both alleles (Retention) or had lost one allele (LOH).

We then classified the patients according to the ER and PgR status of their tumors.[3] In the ER-positive group, a significant difference in postoperative mortality was noted according to LOH status at 3p25.1; the 5-year mortality rate was 34.6% among patients with losses vs. 12.3% with retention of both alleles at 3p25.1 (P=0.0128; Table VI). In the ER-negative group, a difference in postoperative mortality was found with LOH status at 17p13.3; the 5-year mortality rate was 37.3% among patients with losses vs. 16.7% with retention of alleles at 17p13.3 (P=0.0394; Table VI). No correlation with LOH was found in groups classified by PgR status.

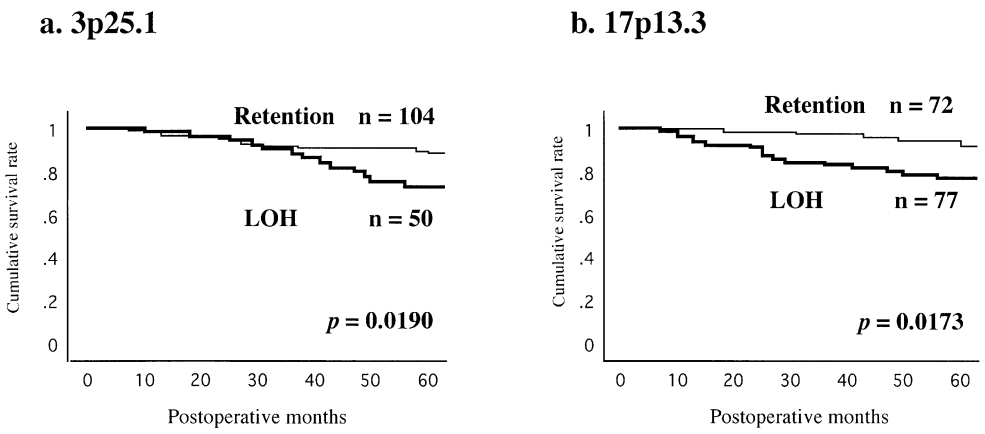

Fig. 4. Kaplan-Meier curves of postoperative overall survival among "low grade metastasis" patients whose tumors retained both alleles (Retention) or had lost one allele (LOH).

Table VI. Univariate Analysis of Postoperative Mortality According to LOH Status for Patients with ER (+) or ER (−) Breast Cancers

ER status	Region (marker)	LOH status	5-year mortality rate (%)	Log-rank test *P* value	Relative risk
Positive	3p25.1	retention	12.3	0.0128	2.8
	(D3S1286)	LOH	34.6		
	13q12	retention	12.2	0.1961	2.0
	(D13S171)	LOH	24.8		
	17p13.3	retention	15.8	0.2865	1.6
	(D17S849)	LOH	24.7		
Negative	3p25.1	retention	24.2	0.3532	1.4
	(D3S1286)	LOH	33.3		
	13q12	retention	14.2	0.0697	2.6
	(D13S171)	LOH	37.4		
	17p13.3	retention	16.7	0.0394	2.2
	(D17S849)	LOH	37.3		

DISCUSSION

In the present study we looked for specific allelic losses that might correlate with poor prognosis among 263 patients with lymph node-positive breast cancers. We found that postoperative risk of mortality was greater for patients whose tumors showed LOH at 3p25.1, 13q12, or 17p13.3 compared with patients whose tumors retained both alleles at these loci.

LOH in the 3p25 region has been described in various types of tumor. The *VHL* gene, a tumor suppressor at 3p25 that is associated with renal-cell carcinoma, might be a target for LOH in breast cancers as well. Other candidate genes in this region include *Rad23* and peroxisome proliferator-associated receptor Gamma (*PPARG*). Rad23 forms a complex with XPC that functions as a nucleotide-excision repair mechanism.[18] *PPARG* is a member of the nuclear-hormone receptor subfamily of transcription factors; mutation within this gene was recently identified in colon cancers.[19] Because LOH at 3p25.1 in particular was a significant prognostic factor in the "n2" group in the present study, the candidate genes mentioned above might play roles in the spreading of tumor cells from metastasized lymph nodes to distant organs.

As to LOH at 13q12, *BRCA2* was mapped there some years ago in families carrying predispositions to breast cancer, and LOH in this region is frequently observed among sporadic primary breast cancers as well.[4, 20] A series of LOH studies in our laboratory has served to emphasize that allelic loss in this region confers an aggressive clinical phenotype on breast cancers that would result in poor survival.

Coles *et al.* revealed that LOH at 17p13.3 was associated with altered expression of p53 mRNA, suggesting that a gene about 20 megabases telomeric to p53 may regulates p53 expression.[21] For example, the *BCPR* gene lies in the 17p13.3 region and is considered as a candidate gene that regulates transcription and expression of p53.

In previous work[12] we examined the relationship between postoperative mortality and LOH at 18 chromosomal regions in a cohort of patients with breast-cancer overall after surgery, and found significant correlations with LOH at 1p34, 13q12, 17p13.3 and 17q21.1. We previously reported that allelic loss in the 1p34–36 region correlated with postoperative recurrence among breast cancers without lymph-node metastasis.[22] We also found a significant prognostic association with LOH at 8p22, specifically in large tumors and in estrogen receptor-negative breast cancers.[23] In a larger cohort of 504 patients, we later noticed a significant association between poor postoperative prognosis and LOH at 3p25.1.[24]

Although we previously reported that allelic loss in the 1p34–36 region correlated with postoperative recurrence of node-negative breast cancers, in the present study allelic loss of 1p was not a significant prognostic factor. These data corroborate the idea that LOH at 1p specifically influences node-negative cancer. In the study reported here, we measured postoperative mortality among patients having metastases to lymph nodes at the time of surgery, and found that in a specific set of tumors with overlapping but distinct LOH status, the latter feature correlated with disease prognosis. Thus, allelic loss at 3p, 13q, and/or 17p is considered to give tumor cells more aggressive character in node-positive breast cancer, suggesting that candidate genes in these regions may inhibit cell growth, vascular invasion, and/or lymphatic permeation from metastatic nodes. Further studies to elucidate such genetic differences will be necessary before we can

fully understand the pathophysiology of breast-cancer progression.

ACKNOWLEDGMENTS

The authors wish to thank Drs. Takaaki Sato, Takuji Iwase, Toyomasa Katagiri, Yousuke Harada, Isao Ito, Kenji Kobayashi, Mieko Matsushima, Aritoshi Iida, Hiroko Saito, Takashi Yokota, Kouichi Bando, Kouichi Fukino, Mayumi Tanaka, Kyoko Shimizu and Yumiko Sakai for their contributions. This work was supported in part by a special grant for Strategic Advanced Research on "Cancer" and "Genome Science" from the Ministry of Education, Science, Sports and Culture of Japan; by a Research Grant for Research from the Ministry of Health and Welfare of Japan; and by a Research for the Future Program Grant of the Japan Society for the Promotion of Science.

(Received June 11, 2001/Revised August 22, 2001/Accepted August 27, 2001)

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