

## Loss of Heterozygosity on Chromosomes 1 and 11 in Sporadic Pheochromocytomas

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Molecular genetic analysis was performed with 20 oncogene probes and 32 polymorphic DNA probes on tumor DNA samples from seven pheochromocytomas; namely, one multiple endocrine neoplasia type 2B, and two familial and four sporadic pheochromocytomas. No amplification or rearrangement of the oncogenes was detected in any of the tumors. However, loss of heterozygosity on chromosome 1p, 11p or 11q was detected in these cases. In addition, a locus related to ETS1 was deleted in two of the sporadic tumors. These results suggest that pheochromocytomas may be genetically heterogeneous, and that inactivation of unknown genes on chromosome 1p, 11p or 11q may contribute to their development.

Key words: Pheochromocytoma — Loss of heterozygosity — Oncogene — Chromosome 11

Pheochromocytoma is a catecholamine-secreting tumor arising from chromaffin cells of the sympathoadrenal system, and it develops in 0.1 to 1.0% of hypertensive patients. Ten percent of the cases are familial and inherited as an autosomal dominant trait either independently or as part of multiple endocrine neoplasia (MEN) type 2A or 2B.<sup>1)</sup>

Recently, two important mechanisms of tumorigenesis have been demonstrated: activation of oncogenes and inactivation of tumor suppressor genes.<sup>2)</sup> Oncogene activation due to point mutation, gene amplification or rearrangement of protooncogenes<sup>3)</sup> has been detected in many malignant tumors. On the other hand, some hereditary tumors have been found to have lost a specific chromosome, such as 13q— in retinoblastoma,<sup>4)</sup> 1p— in neuroblastoma<sup>5)</sup> and 11p— in Wilms' tumor,<sup>6)</sup> indicating inactivation of a specific gene with tumor-suppressing activity.

With progress in molecular genetic analysis, it has become possible to detect mitotic recombination or chromosomal deletion localized in a small region of the chromosome as loss of heterozygosity, even in a solid tumor.<sup>7)</sup> This loss of heterozygosity on a specific chromosome has been found in both hereditary and non-hereditary<sup>8)</sup> tumors, and is thought to be an important mechanism of tumorigenesis.

On analysis of a case of familial pheochromocytoma complicated with congenital aniridia,<sup>9)</sup> we suspected the involvement of the short arm of chromosome 11. Furthermore, we recently reported that two independent pancreatic tumors from a patient with multiple endocrine neoplasia type 1 did not show amplification, rearrangement or point mutational changes of oncogenes, but both had lost heterozygosity on chromosome 11p.<sup>10)</sup> From these findings we supposed that disorganization of some genetic information from chromosome 11 was involved in the development of pheochromocytomas.

To clarify this hypothesis, we examined DNA samples from sporadic, familial and MEN-2B pheochromocytomas for the presence of oncogene abnormalities and loss of heterozygosity.

### MATERIALS AND METHODS

**Patients** Samples of pheochromocytoma were obtained from the seven patients listed in Table I. Patient 1 had MEN type 2B, patients 2 and 3 were familial cases of pheochromocytoma, and patients 4, 5, 6 and 7 were sporadic cases. All tumor tissues were obtained at operation and stored at  $-80^{\circ}\text{C}$  until use. Peripheral leukocytes obtained from each patient were also stored at  $-80^{\circ}\text{C}$ .

**Isolation of DNA** High-molecular-weight DNA was prepared from the tumor tissues and peripheral leukocytes by the method of Blin and Stafford.<sup>11)</sup>

**DNA probes** We used the following 20 oncogene and 32 polymorphic DNA probes<sup>12)</sup> to detect oncogene abnormality or restriction fragment length polymorphism (RFLP) at 41 loci on 18 autosomal chromosomes: FGR (1p36), D1S7 (1p35-p33), MYCL (1p32), AT3 (1q23), APOB (2p24-p23), TGFA (2p13), MYCN (2p24), RAF1 (3p25), ERBA2 (3p22), D3S3 (3p14), FMS (5q33), MYB (6q22-q23), MET (7q31-q32), ERBB1 (7p11-p13), MOS (8q11/q22), MYC (8q24), ASS (9q11-q22), ABL (9q25-q26), RBP3 (10p11-q11), D10S5 (10q21), GLUD (10q23-q24), CYP2E (10), HRAS1 (11p15), INS (11p15), IGF2 (11p15), D11S12 (11p15), PTH (11pter-p15), CALCA (11p15), CAT (11p13), D11S151 (11p13), PGA (11q13), INT2 (11q13), APOA1 (11q23-qter), ETS1 (11q23), KRAS2 (12p12), D13S2 (13q22), FOS (14q21-q31), FES (15q25-q26), D17S4 (17q23-q25), ERBB2 (17q11-q12), D18S3 (18p11), D18S1 (18), C3 (19p13), D19S9 (19q15-q13), D20S6 (20p12), D20S5 (20p12), D21S16 (21q11-q21), IGLC (22q11), D22S9 (22q11), D22S1

Table I. Patients Analyzed

Patient	Age (yr)	Sex	Diagnosis	Site of tumor
1	28	F	MEN type 2B	bilateral adrenal
2	44	F	familial pheochromocytoma (mother)	left adrenal
3	19	F	familial pheochromocytoma (daughter)	left adrenal
4	64	F	sporadic pheochromocytoma	right adrenal
5	69	F	sporadic pheochromocytoma	right adrenal
6	50	F	sporadic pheochromocytoma	left adrenal
7	48	M	sporadic pheochromocytoma	right adrenal

(22q11-qter), SIS (22q12-q13). These probes were radiolabeled with [ $\alpha$ - $^{32}$ P]dCTP by the random hexanucleotide primer method.<sup>13)</sup>

**Southern blot analysis** Aliquots of 5  $\mu$ g of DNA were digested with appropriate restriction endonucleases for

12 h according to the manufacturer's recommendations (Toyobo, Japan). The resulting DNA fragments were separated by electrophoresis in 0.7% or 1.4% agarose gel and transferred to a nitrocellulose membrane by the method of Southern.<sup>14)</sup> DNA on the filter was hybridized at 42°C for 16 h in a mixture of 50% formamide, 6 $\times$  standard saline citrate (SSC), 5 $\times$  Denhardt's solution, 10 mM EDTA, 0.5% sodium dodecyl sulfate, 100  $\mu$ g/ml denatured salmon sperm DNA,  $^{32}$ P-labeled denatured probe DNA and 100 mg/ml dextran sulfate. The filter was then washed three times for 40 min each time with 0.2 $\times$ SSC at 52°C and subjected to autoradiography using Kodak X-ray film (XRP-5 or XAR-5) with a Dupont Cronex intensifying screen at -70°C. When necessary, the membranes were reprobbed after removing the first probe in boiling water.

## RESULTS

**Southern blot analysis of protooncogenes** We analyzed the oncogenes on DNAs obtained from tumor tissues to determine whether any cellular protooncogenes were

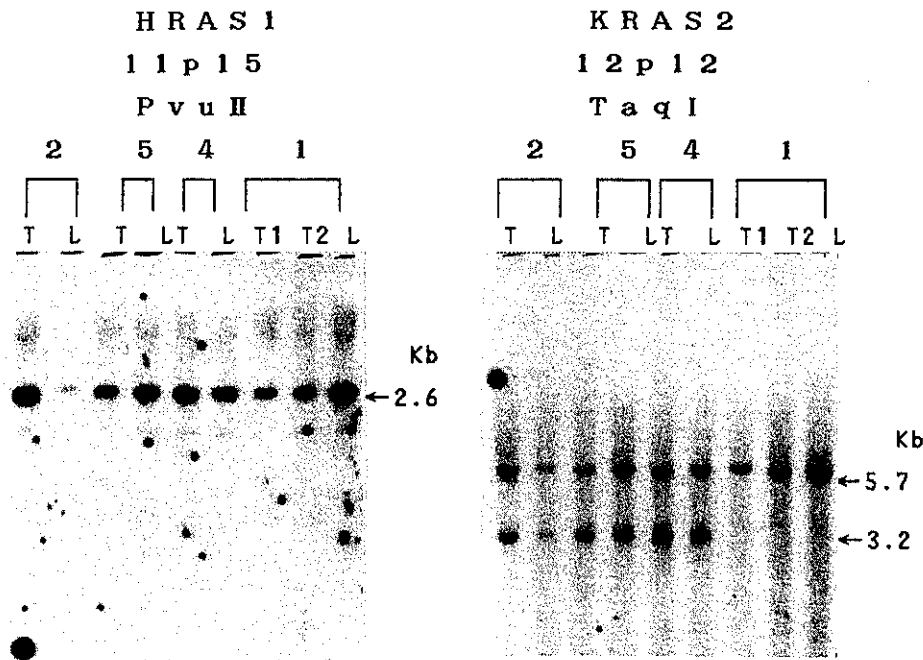


Fig. 1. Southern blot analyses of H-ras and K-ras oncogenes. DNAs of tumors and peripheral leukocytes from patients 1, 2, 4 and 5 were digested with appropriate restriction endonucleases, separated by electrophoresis in 0.7% agarose gel and transferred to nitrocellulose membranes. Each membrane was hybridized with a  $^{32}$ P-labeled DNA probe for the H-ras (4.6 kb *Bam*HI/*Kpn*I fragment of HRAS1) or K-ras (0.64 kb *Eco*RI/*Hind*III fragment of KRAS2) oncogene. Lane L, digest of DNA from peripheral leukocytes. Lane T, digest of DNA from tumor. T1; pheochromocytoma of MEN-2B. T2; thyroid medullary carcinoma of MEN-2B. kb; kilobase pairs.

amplified or rearranged. Fig. 1 shows the results of Southern blot analysis of the H-ras (HRAS1) and K-ras (KRAS2) genes in patients 1, 2, 4 and 5. The DNAs from all these patients showed constitutional homozygosity at HRAS1. DNA from patient 1 also showed constitutional homozygosity at KRAS2, but the others showed heterozygosity. Neither amplification nor rearrangement of the protooncogenes was observed in any case. Further analysis showed that 18 other oncogenes, namely, the protein kinase group (*fgr, abl, fps/fes, erbB1, erbB2, fms, mos, raf, met*), growth factor group (*sis, int 2*) and intranuclear protein group (*c-myc, L-myc, N-myc, myb, fos, erbA2, ets1*), also contained no amplification or rearrangement of protooncogenes in any tumor.

**Loss of 1p, 11p and 11q alleles in pheochromocytomas**

Next, we examined loss of heterozygosity in the tumor DNAs using polymorphic DNA probes of 41 loci on 18 chromosomes other than chromosomes 4, 14, 15, and 16 and the sex chromosome. DNA samples from all the patients showed constitutional homozygosity on MOS (8q11/q22), MYC (8q24), D10S5 (10q21) and HRAS1 (11p15), and so they were not informative at these loci.

As can be seen in Table II, in the case of MEN 2B, a total of 21 informative loci were obtained on chromosomes 1, 3, 5, 6, 7, 9, 10 and 11, but no loss of heterozygosity was observed. In the cases of familial pheochro-

mocytoma, there were 19 informative loci, but in all these heterozygosity was preserved in the tumor DNA.

In the cases of sporadic pheochromocytomas, 22 loci were informative. Loss of heterozygosity was observed in 2 cases, but no common loss of any allele was observed. In case 6, loss of heterozygosity was observed at MYCL (1p35-p33) and D1S7 (1p32) on chromosome 1p (Fig. 2). This tumor did not give the 7.6 kb *HinfI* fragment of D1S7 or the 6.6 kb *EcoRI* fragment of MYCL. The presence of a weak band of the 7.6 kb *HinfI* fragment in D1S7 was due to contamination of the DNA with DNA from peripheral leukocytes or normal tissue cells. Because the AT3 locus (1q23) was constitutionally homozygous, loss of allele on the long arm of chromosome 1 could not be assessed.

On the other hand, the tumor in patient 4 showed loss of heterozygosity at four loci on chromosome 11: the 0.68 kb *PvuII* fragment of INS (11p15), the 6.7 kb *EcoRI* fragment of D11S151 (11p13), the 0.84 kb *BamHI* fragment of INT2 (11q13) and the 2.2 kb *PstI* fragment of APOA1 (11q23-qter) were absent in this tumor (Fig. 3). In this case, loss of heterozygosity should extend from the short arm to the long arm of chromosome 11.

The tumors in patients 4 and 6 did not show loss of heterozygosity on the other chromosomes examined, suggesting that these chromosomal losses were not random events.

Table II. Frequency of Loss of Heterozygosity in Seven Pheochromocytomas

Chromosome	Probes <sup>a)</sup> (n)	Loss of heterozygosity (n)/informative loci (n) <sup>b)</sup>						
		P1	P2	P3	P4	P5	P6	P7
1	3	0/1	0/1		0/3	0/2	2/2	0/2
2	2				0/1			
3	2	0/2	0/3		0/3	0/3		
5	1						0/1	
6	1	0/1			0/1			
7	1	0/1			0/1	0/1		
8	1							
9	1	0/1	0/1		0/1	0/1		
10	4	0/3	0/3	0/1	0/2	0/2		
11	12	0/5	0/6	0/3	4/6	0/5	0/1	
12	1		0/1		0/1	0/1		
13	1	0/1					0/1	0/1
17	1	0/1	0/1					0/1
18	2	0/1				0/1	0/1	0/1
19	2				0/1			
20	2	0/1					0/1	0/1
21	1	0/1	0/1		0/1	0/1	0/1	
22	3	0/2	0/2		0/1	0/2		

a) Number of DNA probes used for analysis on each chromosome.

b) Number of cases of loss of heterozygosity per total number of informative loci. P: patient.

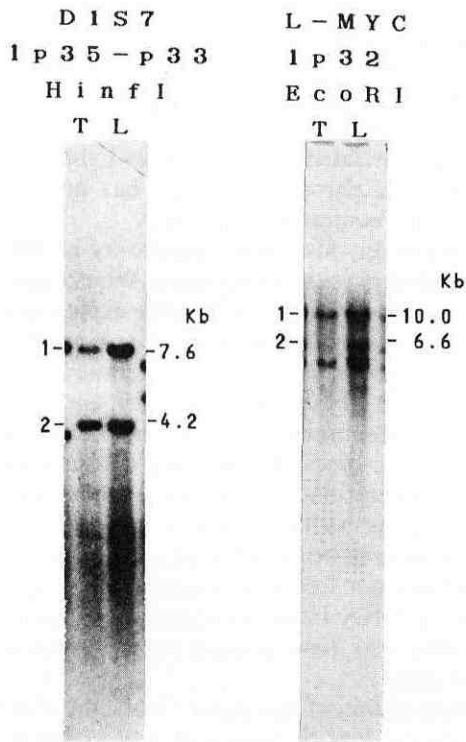


Fig. 2. Loss of heterozygosity at loci on chromosome 1p in the sporadic pheochromocytoma of patient 6. DNAs of the tumor and peripheral leukocytes from patient 6 were digested with appropriate restriction endonucleases, separated by electrophoresis in 0.7% agarose gel and transferred to a nitrocellulose membrane. Each membrane was hybridized with a  $^{32}\text{P}$ -labeled DNA probe for DIS7 or MYCL (1.8 kb *Sma*I/*Eco*RI fragment of MYCL). The numbers 1 and 2 on the left indicate the observed alleles. Lane L, digest of DNA from peripheral leukocyte. Lane T, digest of DNA from tumor. kb; kilobase.

**Chromosomal loss at ETS locus** In the tumor in patient 4, loss of heterozygosity extended from 11p to 11q and extensive chromosomal lesion was suspected on chromosome 11. Therefore, we examined the ETS1 gene located at the terminal of the long arm of chromosome 11. Our probe, a 5.4 kb *Eco*RI fragment of human *c-ets* gene cloned into pKH47 (American Type Culture Collection, USA), revealed 4 bands (8.3, 6.2, 2.3 and 2.0 kb) after *Ss*I digestion. The bands of 2.3 kb and 2.0 kb were due to genetic polymorphism.<sup>12)</sup> The bands of 8.3 kb and 6.2 kb have not been reported previously and their origins are not clear. Southern blot analysis showed that in the tumors from patients 4 and 6, heterozygosity at ETS1 (11q23) was preserved, but the 8.3 kb *Ss*I fragment was

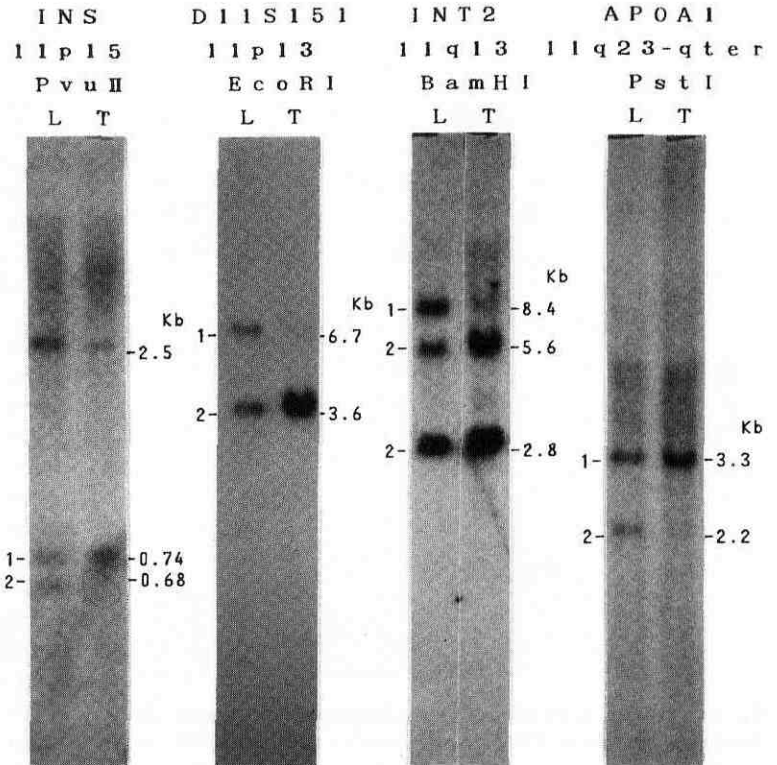


Fig. 3. Loss of heterozygosity at loci on chromosome 11 in the sporadic pheochromocytoma of patient 4. DNAs of the tumor and peripheral leukocytes from patient 4 were digested with appropriate restriction endonucleases, separated by electrophoresis in 0.7% agarose gel (D11S151, INT2, and APOA1) or in 1.4% agarose gel (INS) and transferred to nitrocellulose membranes. The membranes were hybridized to  $^{32}\text{P}$ -labeled DNA probe for INS, D11S151, INT2 and APOA1, respectively. The numbers 1 and 2 on the left indicate the observed alleles. Lane L, digest of DNAs from peripheral leukocyte. Lane T, digest of DNA from tumor.

lost (Fig. 4). Both the leukocytes and the tumors of patients 1, 2, 3, 5 and 7 gave only the band of 6.2 kb.

To characterize these extra bands, we performed Southern blot analysis using this probe on placenta DNAs and leukocyte DNAs obtained from several normal individuals. We found one or both of these bands in all cases. In addition, Mendelian inheritance of these bands was demonstrated in one family (data were not shown).

## DISCUSSION

Until recently, there has been no clue to the mystery of tumor development, malignant change or inheritance of pheochromocytomas. In MEN type 2, some chromosomal abnormalities have been proposed to be located on 6p,<sup>15)</sup> 16q,<sup>16)</sup> 20p12<sup>17)</sup> or 22p11<sup>18)</sup> from the results of

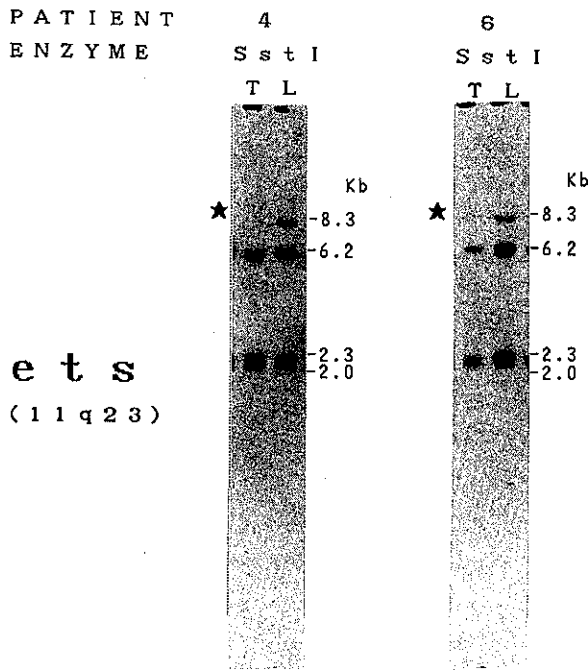


Fig. 4. Loss of ETS-related DNA fragment in two sporadic pheochromocytomas. DNAs of the tumors and peripheral leukocytes from patients 4 and 6 were digested with *Sst*I, separated by electrophoresis in 0.7% agarose gel and transferred to nitrocellulose membranes. The membranes were hybridized to <sup>32</sup>P-labeled DNA probe for ETS1 (*c-ets*). The asterisk (\*) on the left indicates the observed loss of a fragment of ETS. Lane L, digest of DNA from peripheral leukocytes. Lane T, digest of DNA from the tumor.

cytogenetic or linkage analysis. Recently, using DNA markers, Simpson *et al.*<sup>19)</sup> and Mathew *et al.*<sup>20)</sup> demonstrated close linkage between RBP3 on chromosome 10p11-q11 and the MEN type 2A locus, and Takai *et al.*<sup>21)</sup> confirmed the close linkage between RBP3 and the MEN type 2A locus in Japanese MEN type 2 families. But there have been no molecular genetic studies on the mechanism of development of sporadic pheochromocytoma.

In this work we analyzed the DNAs from seven pheochromocytomas using oncogene and polymorphic probes. No amplification or rearrangement of the 20 oncogenes examined was observed in any of these tumors, but two sporadic cases (Nos. 4 and 6) showed loss of heterozygosity. Case 6 showed loss of heterozygosity on chromosome 1p as observed by Mathew *et al.*<sup>22)</sup> in MEN type 2. Case 4 showed loss of heterozygosity at *INS*, *D11S151*, *APOA1* and *INT2*, indicating that the chromosomal change extended from the short arm to the long arm of chromosome 11. These two cases both

showed loss of an *Sst*I fragment detected by *c-ets*. Our preliminary characterization suggested that this fragment might be a new RFLP of *c-ets*. But as there are many reports on *ets*-related sequences on different human chromosomes,<sup>23-25)</sup> another possibility is that this fragment may be an *ets*-related sequence cross-hybridizing with our probe. This chromosomal loss has not been reported before and requires further study.

We did not detect any loss of heterozygosity in MEN type 2B and familial pheochromocytoma. We examined only 7 tumors, but there are several possible explanations for our result. First, pheochromocytoma may be a genetically heterogeneous disease and the abnormalities responsible for MEN type 2A, MEN type 2B, and familial and sporadic pheochromocytomas may be located separately on chromosomes. Second, the chromosomal lesions in these tumors may be too small to detect by Southern blot analysis with currently available probes. Third, these tumors may originate from polyclonal cells, and these cells may not lose alleles uniformly. Fourth, the contaminating DNA from peripheral leukocytes or normal tissue cells may have masked loss of heterozygosity in tumor cells.

During the preparation of this paper, Tsutsumi *et al.*<sup>26)</sup> also reported similar loss of heterozygosity at *HRAS1* and *INS* on chromosome 11 and *MYCL* on chromosome 1 in three of five cases of sporadic pheochromocytoma. Fong *et al.*<sup>27)</sup> observed loss of heterozygosity on 1p or 11p in embryologically related tumors derived from neural crest cells, such as melanoma, medullary thyroid carcinoma and pheochromocytoma, and suggested the existence of a common mechanism of tumor development. We also suppose that chromosomes 1 and 11 have some tumor suppressor genes and that inactivation of these genes due to chromosomal loss leads to tumor development. This chromosomal loss may be a secondary event in tumor development, but the possibility of loss of tumor suppressor genes is partly supported by the findings that introduction of a normal human chromosome 11<sup>28)</sup> or chromosome 1<sup>29)</sup> into tumor cell lines by cell fusion resulted in suppression of the tumorigenic phenotype of the cells. Extensive characterization of this activity on chromosome 1 and 11 is required before any definite conclusion can be reached on this point.

In summary, our data suggest that pheochromocytomas may be genetically heterogeneous and that at least loss of some sequence on chromosome 1 or 11 may be involved in their development.

#### ACKNOWLEDGMENTS

We thank Drs. T. Sekiya, T. Awata, A. S. Bleaudet, S. C. Bock, J. Breslow, C. D. Bridges, W. Cavenee, B. G. Forget, R. R. Frant, M. Jansen, R. G. Korneluk, H. M. Kronenberg, P.

Leder, J.-L. Mandel, J. Minna, B. D. Nelkin, M. Obinata, G. J. B. van Ommen, D. J. Show, G. F. Sounders, G. D. Stewart, R. A. Weinberg, B. N. White, R. White, and J. Yokota for polymorphic DNA probes. DNA probes were also provided by Amersham, the Japanese Cancer Research Resources Bank and the American Type Culture Collection. This work was sup-

ported by a Grant-in-Aid for Research on Intractable Diseases from the Ministry of Health and Welfare, and a Grant-in-Aid for Scientific Research (No. 63770853) from the Ministry of Education, Science and Culture of Japan.

(Received December 20, 1989/Accepted March 28, 1990)

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