Jpn. J. Cancer Res. 84, 402–408, April 1993

Deletion Mapping of Chromosome 1p and 22q in Pheochromocytoma

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To identify the localization of tumor suppressor genes, 22 pheochromocytomas (9 hereditary and 13 sporadic) were examined for loss of heterozygosity (LOH) on the short arm of chromosome 1 and on the long arm of chromosome 22 by using 11 polymorphic DNA markers on each chromosome arm. LOH on 1p was observed in 12 of 22 informative cases (55%) and on 22q in 8 of 20 informative cases (40%). There was no significant difference in the frequency of LOH on 1p or 22q between hereditary and sporadic cases. We could localize the commonly deleted regions as distal to D1S73 and proximal to D1S63 on 1p and distal to D22S24 and proximal to D22S1 on 22q. In addition, the relationship between LOH on 1p and 22q was studied in 20 pheochromocytomas which were informative for probes on both chromosome arms. Of eight tumors that showed LOH on 22q, allelic loss on 1p was also detected in seven. Thus, LOH on 22q was correlated significantly with LOH on 1p (P=0.0249; Fisher's exact test). These results suggest that inactivation of multiple tumor suppressor genes may be required for development and progression of hereditary and non-hereditary pheochromocytoma.

Key words: Pheochromocytoma — Deletion map — Loss of heterozygosity — Multiple endocrine neoplasia type 2

Pheochromocytomas, which occur in 0.1–1% of hypertensives,[1] originate in the chromaffin cells of the sympathoadrenal system. Ten percent of the cases are inherited as an autosomal dominant trait, either independently or as part of multiple endocrine neoplasia type 2 (MEN 2). Linkage analysis has mapped the gene predisposing to MEN 2 to the pericentromeric region of chromosome 10.[2-4] Because of early onset, multifocal occurrence, and precedence of adrenal chromaffin cell hyperplasia in hereditary cases,[5,6] it seems reasonable to explain the development of pheochromocytoma in terms of the two-mutational model postulated by Knudson *et al.*[7] In hereditary retinoblastoma, one allele of the retinoblastoma gene is mutated germinally, and tumor development is caused by subsequent somatic inactivation of the remaining allele. Thus, inactivation of both copies of the predisposing gene is sufficient for tumorigenesis of retinoblastoma. In this instance, molecular genetic analysis often identifies somatic inactivation of one allele as LOH.[8] On the other hand, although deletion or LOH at the pericentromeric region of chromosome 10, where the predisposing gene for MEN 2 resides, is rarely found in pheochromocytoma,[9-11] LOH has been found on chromosomes 1p, 3p, 17p, and 22q.[11-15] These findings suggest that tumorigenesis of pheochromocytoma cannot be explained solely by the two-mutational theory and that inactivation of additional tumor suppressor gene(s) may be required for development of pheochromocytoma, as in the case of colorectal cancers.[16]

In our present study, we examined 22 hereditary and sporadic pheochromocytomas for LOH on chromosomes 1p and 22q. On each chromosome arm, the commonly deleted region could be narrowed down by using 11 polymorphic DNA markers on 1p and 11 markers on 22q. The relationship between LOH on 1p and on 22q was also studied.

MATERIALS AND METHODS

Tumor and blood samples In each case, tumor tissue and peripheral blood were obtained in the operating room at the time of surgery. The tissue was immediately frozen and stored at −80°C until DNA extraction. Peripheral leukocytes were separated from obtained whole blood at the earliest opportunity. High-molecular-weight DNA was then extracted from the tumor tissue and peripheral leukocytes as described elsewhere.[17]

DNA analysis Each DNA sample (5 μg) was completely digested with appropriate restriction enzymes and the resultant DNA fragments were separated by agarose gel (0.6–0.8%) electrophoresis. The DNA was then transferred to a nylon membrane by a modification of the procedure of Southern.[18] DNA probes were labeled with [α-^{32}P]dCTP by random primer extension.[19] The membranes were hybridized overnight with radioactive probes at 65°C in 7% polyethylene glycol, 10% sodium dodecyl

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sulfate (SDS) with 0.1 mg/ml denatured herring sperm (or human placenta) DNA. The membranes were washed once at room temperature in 2×standard saline citrate (SSC) for 10 min and twice at 65°C in 0.1×SSC, 0.1% SDS for 10 min and then exposed to Kodak XAR 5 films at −80°C overnight.

The intensity of hybridization signals was measured by densitometry (Personal Scanning IMAGER PD110, Molecular Dynamics, Sunnyvale, CA). Allelic loss was recorded when the ratio of alleles in the tumor decreased to less than 50% of the ratio in the corresponding leukocyte DNA.

DNA probes We used 11 polymorphic probes on chromosome 1p and two polymorphic probes on chromosome 1q; D1S80 (pMCT118, 1p36-35),[20] D1S76 (pCMM12, 1p36-34),[21] *PND* (pJA110, 1p36),[22] *FGR* (pB8, 1p36.2-36.1),[23] D1S63 (pCMM8.1, 1p35-32),[24] D1S57 (pYNZ2, 1p35-32),[21] *MYCL* (L-*myc*, 1p32),[25] D1S64 (pEFZ13, 1p22.1-13),[26] D1S73 (pEFD53.2, 1p21-cen),[27] *NGFB* (N8C6, 1p13),[28] *NRAS* (pMCR3, 1p13),[29] D1S67 (pHHH106, 1q21),[30] D1S81 (pTHH33, 1q32-44).[21] The physical and linkage maps of 1p were constructed previously.[31–33]

We used 11 polymorphic probes on chromosome 22q; D22S24 (W21G, 22q11.1-11.2),[34] D22S9 (p22/34, 22q-11.1-11.2),[35] *IGLC* (p1A5, 22q11.1-11.2),[36] *IGLV* (V4-A, 22q11.1-11.2),[37] D22S10 (22C1-18, 22q11.1-11.2),[38] *BCR* (pbcr, 22q11),[39] D22S1 (pMS-18, 22q11.2-12),[40] D22S32 (pEFZ31, 22q12-13),[41] D22S29 (W22D, 22q),[34] D22S102 (KI-436, 22q12-13),[42] D22S22 (W110D, 22q).[34] The physical and linkage maps of 22q were constructed previously.[34, 43, 44]

RESULTS

LOH on chromosome 1p Restriction fragment length polymorphism (RFLP) analysis was performed for 22 matched blood/tumor pairs of pheochromocytomas (9 tumors from MEN 2A patients, 13 sporadic) at 11 loci on chromosome 1p. Representative examples of the LOH on 1p are shown in Fig. 1. All samples investigated were informative for at least two of the 11 markers (Fig. 2). LOH was observed in 12 of 22 pheochromocytomas (55%), five of which were from MEN 2A patients and seven sporadic. Chromosome 1p was partly deleted in eight of 12 pheochromocytomas that showed LOH on the chromosome arm. In cases 50 and 57, LOH was observed at the *MYCL* locus, while heterozygosity was retained at D1S73. Both alleles at D1S73 were retained in case 37 which showed LOH at D1S57. Moreover, heterozygosity was retained at D1S63 in cases 28 and 61 in which LOH was observed at the loci proximal to D1S63. These results enabled us to localize the commonly deleted region as distal to D1S73 and proximal to D1S63.

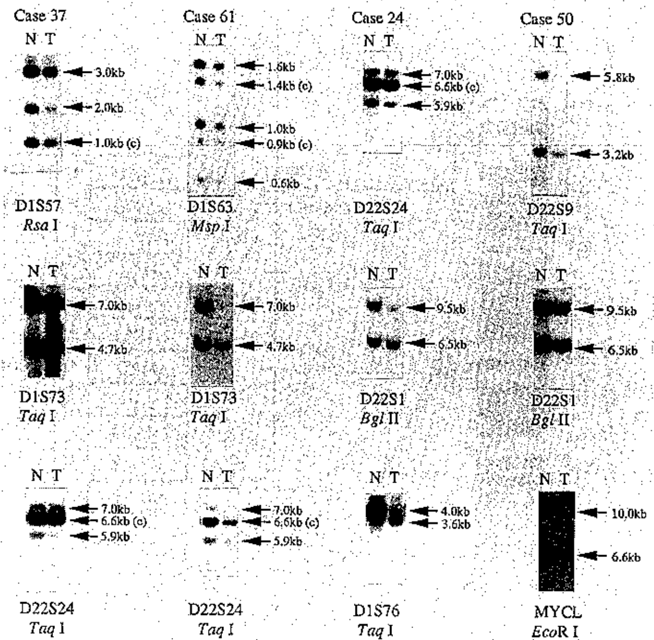

Fig. 1. Results of restriction fragment length polymorphism studies. Each panel shows a comparison of N (DNA from peripheral leukocytes), and T (DNA from pheochromocytoma tumor tissue). Each sample was digested with the indicated restriction enzyme and hybridized with probes at the designated locus. Alleles are indicated by arrows and numbers on the right refer to band sizes. (c), constant bands; kb, kilobases.

LOH on chromosome 22q Twenty of 22 pheochromocytomas were informative for at least two of the 11 probes tested (Fig. 3). Eight pheochromocytomas (4 from MEN 2A, 4 sporadic) showed LOH on the chromosome arm (40%). Representative RFLP studies on 22q are shown in Fig. 1. Interstitial deletion on 22q was observed in two pheochromocytomas (cases 50 and 66). In case 50, heterozygosity was lost at the D22S9, *IGLC* and D22S10 loci, while it was retained at the D22S24 and D22S1 loci. LOH was also observed at the *IGLC* locus in case 66. These results indicate that the commonly deleted region in pheochromocytomas is enclosed by the D22S1 locus distally and proximally by D22S24.

Relationship between LOH on 1p and on 22q Twenty of 22 pheochromocytomas were informative for probes on both 1p and 22q. As summarized in Table I, seven tumors showed LOH on both chromosome arms, while eight did not show any loss on either chromosome arm. Seven out of eight pheochromocytomas that showed LOH on 22q had also lost alleles on 1p. On the other hand, seven of 11 tumors with LOH on 1p had lost alleles on 22q (64%). LOH on 22q thus correlated significantly with LOH on 1p (P=0.0249; Fisher's exact test). In addition, we com-

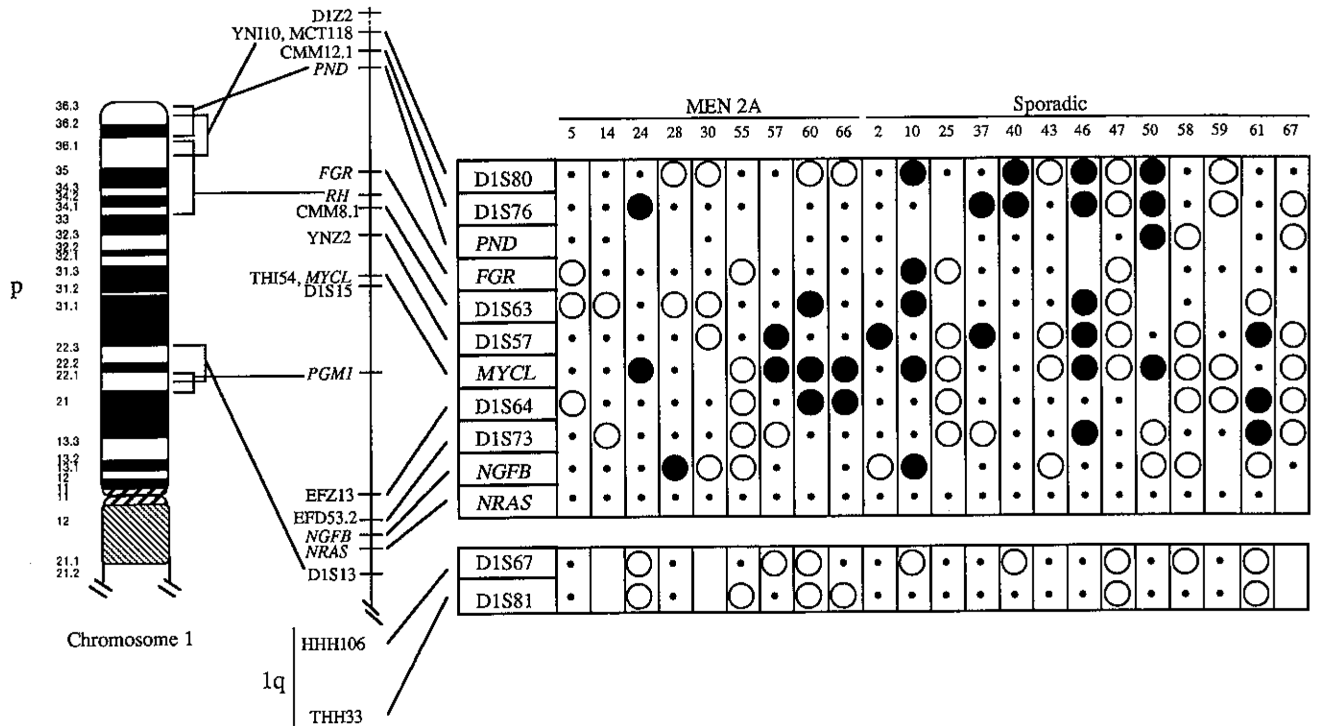

Fig. 2. Analysis for LOH at various chromosome 1 loci in 22 pheochromocytomas. Relative location of probes on chromosome 1 are listed on the left. The physical and linkage maps of 1p were constructed previously.[31-33] Informative loci are indicated by circles (●, loss of heterozygosity; ○, retention of heterozygosity), and noninformative loci (homozygous loci) by black dots (•). A blank indicates that the analysis was not performed. The case number is shown at the top.

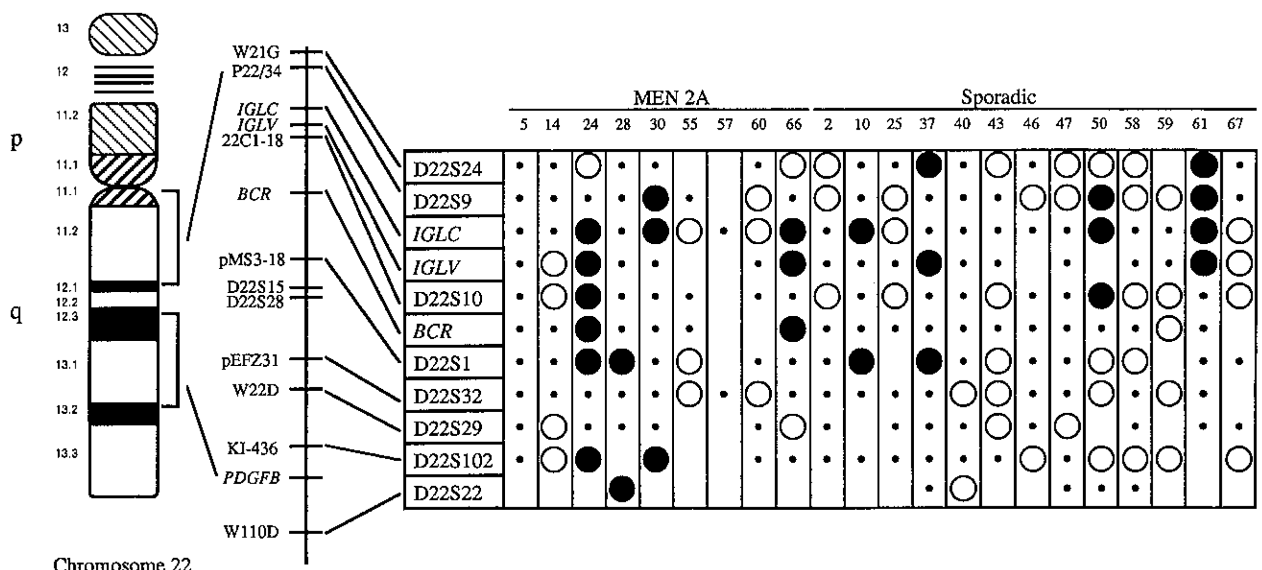

Fig. 3. Analysis for LOH at 11 chromosome 22q loci in 22 pheochromocytomas. Relative location of probes on chromosome 22q are listed on the left. The physical and linkage maps of 22q were constructed previously.[34, 43, 44] Informative loci are indicated by circles (●, loss of heterozygosity; ○, retention of heterozygosity), and noninformative loci (homozygous loci) by black dots (•). A blank indicates that the analysis was not performed. The case number is shown at the top. Case 28 was previously reported.[15]

Table I. Correlation of Allelic Loss on Chromosomes 1p and 22q

Allelic loss on 1p	Allelic loss on 22q		Total
	+	−	
+	7	4	11
−	1	8	9
Total	8	12	20

P=0.0249; Fisher's exact test.

pared clinicopathological characteristics (sex, age, stage, and tumor size) of patients with and without allelic losses on 1p or 22q. No significant correlations were noted between these characteristics and LOH on 1p or 22q.

DISCUSSION

Recent studies have revealed that tumor development is brought about by accumulation of multiple genetic changes. In colorectal tumors, the finding that several chromosomal regions are often deleted in the tumor suggests that inactivation of multiple tumor suppressor genes is required for progression to full malignancy.[16] In fact, several candidate tumor suppressor genes, such as *TP53*,[45] *DCC*,[46] *MCC*,[47] and *APC*[48,49] have been identified in the commonly deleted regions in these tumors. Moreover, RFLP analysis of breast cancers and lung cancers have indicated the existence of multiple tumor suppressor genes.[50–53] Thus, examination of LOH at specific loci is important for identification of tumor suppressor genes.

In our RFLP study using 11 polymorphic DNA markers on chromosome 1p, we have demonstrated a high frequency of LOH (55%) in pheochromocytomas. Previous reports have identified corresponding ratios ranging from 42 to 67%.[11,14,54] Recently, Moley *et al.*[54] indicated that the frequency of LOH in patients with the MEN 2A and 2B syndromes (9 of 9: 100%) was significantly different from that in patients with sporadic disease (2 of 7: 29%). Our study, however, detected no significant difference in the frequency of LOH on 1p between MEN 2A (5 of 9: 56%) and sporadic (7 of 13: 54%) pheochromocytomas. Our results suggest that, irrespective of the genetic background of the patients, genes on chromosome 1p are involved in the tumorigenesis of pheochromocytoma.

We could localize the commonly deleted region on 1p as distal to D1S73 and proximal to D1S63. Although the region defined in this study overlaps with that identified in the previous report[54] in the case of pheochromocytoma, the consistently deleted region (1p36) in neuroblastoma,[55] which has the same neuroectodermal origin as pheochromocytoma, is not included in this region. Consequently, it is reasonable to assume that a potential suppressor locus involved in pheochromocytoma may be different from that involved in neuroblastoma. More detailed analysis is required to determine conclusively whether more than one suppressor locus on 1p is involved in development of these two types of tumors.

We found LOH on 22q in eight of 20 informative pheochromocytomas. The frequency of LOH (40%) identified in this study is slightly lower than that reported by us (53%).[15] Although only three tumors showed interstitial deletion on 22q, the commonly deleted region could be localized as distal to D22S24 and proximal to D22S1. Previous reports have placed the neurofibromatosis type 2 (NF2) locus between D22S1 and D22S28[56] and the tumor suppressor gene associated with meningioma distal to D22S15[57] or between D22S1 and D22S15.[58] The commonly deleted region on 22q in pheochromocytoma defined in this study is proximal to the loci of NF2 and meningioma. Our results thus suggest that an additional suppressor locus may be involved in pheochromocytoma.

It is noteworthy that seven of eight pheochromocytomas that showed LOH on 22q had also lost alleles on 1p, showing a significant correlation between LOH on 22q and on 1p. Taken together with the finding that LOH on 1p (55%) was more frequent than on 22q (40%) in pheochromocytoma, the significant correlation between these two genetic events raises the possibility that the tumor suppressor genes on 1p may be involved in the relatively early stages of tumorigenesis of pheochromocytoma and subsequent inactivation of the tumor suppressor gene on 22q may be associated with further tumor development and progression.

In this study, we demonstrated that alleles on 1p and 22q were frequently lost in pheochromocytoma. These findings strongly suggest that multiple genetic alterations are required for tumorigenesis of hereditary and nonhereditary pheochromocytoma. At least two tumor suppressor loci, one on 1p and the other on 22q, may be involved in pheochromocytoma, although the exact role of the genes remains unknown. Further investigation is required to narrow down the localization of these suppressor genes and to clarify the correlation between LOH on 1p and on 22q.

ACKNOWLEDGMENTS

We thank Drs. Y. Ito, M. Kiso, A. Miyauchi and many other doctors for providing tissue samples. We would like to thank the following for providing the polymorphic clones: Dr. Y. Nakamura for pMCT118, pCMM12, pYNZ2, pEFZ13, pEFD-53.2, pMCR3, pHHH106, pTHH33 and pEFZ31; Dr. G. A. Rouleau for 22C1-18, W21G, W22D and W110D; Dr. R.

White for pMS3-18; Dr. P. Leder for p1A5; Dr. J-C. Kaplan for V4A; Dr. B. N. White for p22/34. N8C6, pJA110, pB8, KI-436 and pbcr were obtained from the ATCC. L-*myc* and pCMM8.1 were obtained from the JCRB. We thank K. Karakawa and T. Nakamura for helpful discussions. This work was supported by Grants-in-Aid for Cancer Research from the Ministry of Education, Science and Culture of Japan.

(Received November 4, 1992/Accepted January 12, 1993)

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