Jpn. J. Cancer Res. 86, 929–933, October 1995

Low Incidence of a Nucleotide Sequence Alteration of the Neurofibromatosis 2 Gene in Human Breast Cancers

Sadanori Yaegashi,[1,2] Richard Sachse,[1,3] Noriaki Ohuchi,[2] Shozo Mori[2] and Takao Sekiya[1,4]

[1]*Oncogene Division, National Cancer Center Research Institute, 5-1-1 Tsukiji, Chuo-ku, Tokyo 104 and* [2]*Second Department of Surgery, Tohoku University School of Medicine, 1-1 Seiryo-machi, Aoba-ku, Sendai 980*

We investigated aberrations of the neurofibromatosis type 2 (*NF2*) gene in breast tumors of 60 patients by single-strand conformation polymorphism analysis of polymerase chain reaction products followed by nucleotide sequencing. We detected a tumor-specific single-base substitution in codon 398 in exon 12 of the gene, resulting in an alteration of a single amino acid, in DNA from a breast cancer sample. The results indicated possible but infrequent involvement of mutations of the tumor suppressor *NF2* gene in human breast cancers.

Key words: *NF2* gene — Human breast cancer — Mutation — SSCP analysis — Polymorphism

Neurofibromatosis type 2 (NF2) is a genetic disease inherited in an autosomal dominant fashion and characterized by bilateral vestibular schwannomas and the development of other nervous system tumors, including meningiomas of the cranial nerves and the spinal nerve roots.[1,2] The *NF2* gene on chromosome 22 has been isolated.[3,4] Sequencing the *NF2* gene helped to reveal mutations of the gene in NF2 patients, since single-base substitutions and other mutations were detected by several polymerase chain reaction (PCR)-based methods including single-strand conformation polymorphism (SSCP) analysis.[3–7] Mutations of the gene have been found in NF2-related[3,4,8,9] and sporadic[8–16] tumors, such as schwannomas and meningiomas. These mutations and concomitant loss of heterozygosity (LOH) at loci flanking the *NF2* gene on the long arm of chromosome 22 in many of these tumors[8,10,12] suggest that *NF2* is a tumor suppressor gene.

As the *NF2* gene is expressed in various human tissues,[3,4] aberrations of the gene might be involved in the carcinogenesis of human cells not only in nervous systems, but also in many other tissues. In fact, mutations of the *NF2* gene have been detected in melanomas[11] and colon cancers,[17] although these tumor types are not found in *NF2* patients.

Frequent LOH at loci on chromosome 22q (10 to 40% of tumors) has been found in breast cancers[18,19] and Bianchi *et al.* have detected a frameshift mutation and a missense mutation of the *NF2* gene in one case of the same breast cancer.[11] On the other hand, Arakawa *et al.*[17] and Kanai *et al.*[20] reported no mutations of the *NF2* gene in 55 and 68 breast cancers, respectively. In this study, to evaluate the possibility of the involvement of abnormalities of the *NF2* gene in tumorigenesis of the breast, aberrations of the *NF2* gene in tumor DNA from 60 patients with breast diseases were examined by PCR-SSCP analysis.

MATERIALS AND METHODS

Preparation of DNA from breast tumors Fresh specimens of primary breast cancers, adjacent metastatic lymph nodes, metastatic liver or subcutaneous tumors, phyllodes tumors and non-malignant breast tumors were resected at the Second Department of Surgery, Tohoku University School of Medicine (Sendai) from 1986 to 1992 and stored at −70°C. Genomic DNA was extracted by using the proteinase K-phenol-chloroform method.[21]

PCR-SSCP analysis Regions containing coding exons, except exon 16, of the *NF2* gene[9] were amplified into 16 DNA fragments by PCR. The names and the lengths of amplified DNA, primers used for the PCR, and the regions concerning which information is provided by this analysis are indicated in Table I. These data were based on the published nucleotide sequences for the *NF2* gene.[4,9] DNA samples (0.1 μg) were amplified by PCR in a mixture (5 μl) as described.[22] The annealing temperature for primer pairs was 54 or 55°C, according to the GC content of the primers (Table I). The 5′-ends of primers were labeled by means of the polynucleotide kinase reaction with [γ-^{32}P]ATP as described.[23] The PCR mixture was diluted with 45 μl of deionized water. One μl of this mixture was heated with 1 μl of formamide dye (95% formamide: 20 m*M* EDTA: 0.05% xylene cyanol: 0.05% bromophenol blue), then 1 μl of the preparation was subjected to electrophoresis in 5%

[3] Present address: Medizinische Klinik 1, Krankenhausstr. 12, 91054 Erlangen, Germany.

[4] To whom all correspondence should be sent.

Table I. Primers Used to Amplify the *NF2* Gene

Fragment			Primer			Region analyzed		
Name	Length (bp)	Exon (codon)	Name	Nucleotide sequence	Annealing temperature (°C)	5′-Flanking (bp)	Codon	3′-Flanking (bp)
				5′ 3′				
A	182	1 (1–38)	1C	CTAAAGGGCTCAGAGTGCAG	55	39	1–35	0
			1D	GCCGGTTACCTCGCAATTGA				
B	171	2 (39–80)	2A	CTTCCCCATTGGTTTGTTATT	54	4	39–80	0
			2B	TCATCGAGTTCTAGCCCAAC				
C	165	3 (81–121)	3A	TGCAATTCTGCAGGTACTGGA	54	0	83–121	9
			3B	GAACTGGGGGGTAGCCTTGA				
D	136	4 (122–149)	4A	TATCATGTCTCCCTTGTTGC	54	9	122–149	0
			4B	CATTTTTCTTCTTTGAGCCTAC				
E	123	5 (150–172)	5A	AATCGCCTGCTCTCCCTTTCT	54	9	150–172	0
			5B	TGAAAAGGGAATTTAATCTCTTAC				
F	107	6 (173–200)	6C	TTTTGGTAGGTAATAAATCTGTATC	55	0	178–198	0
			6B	TGAATGGGCCTCACCTGGCT				
G	128	7 (201–225)	7A	ACAGTGTCTTCCGTTCTCCC	54	5	200–225	6
			7B	AAGGAGCTCAGAGAGGTTTCA				
H	168	8 (226–270)	8A	ATCCACAGAATAAAAAGGGCAC	54	0	230–270	4
			8B	CATCTGCAGTACACACATGTC				
I	118	9 (271–295)	9A	TTCTGCTTCATTCTTCCAGTTTA	55	0	272–295	17
			9B	ATGAAAACCAGGATCTCAACTTA				
J	171	10 (296–333)	10A	TAACCTTTTAGTCTGCTTCTGTG	55	7	296–333	4
			10B	CATCAGTTAAAACAAGGTTGTGC				
K	175	11 (334–374)	11A	CAATGACTGTTTTTCTTCACCC	55	7	334–374	3
			11B	AACCCCAGCCCCTCAGAAAT				
L	257	12 (375–447)	12A	CACTTCAGCTAAGAGCACTGTGCC	55	6	375–443	0
			12B	CCCCCTCACCTCCTCTCTGAC				
M	139	13 (448–482)	13A	AGCTGACATCTCATCCTTTCCTT	55	4	447–478	0
			13B	GCTCACCGGGTACGTGGGCT				
N	169	14 (483–525)	14A	AATCCGAAATTTCTCATTAACAGC	55	0	483–524	0
			14B	GCACAGGGGGCTACATACTTT				
O	220	15 (526–579)	15A	GATGCATGATACCCTCTTGCC	55	5	525–579	7
			15B	CAGCAAAATACAAGAAAGAGACCC				
P	177	17 (580–595)	17A	CCTCTCAGCTTCTTCTCTGCTTT	55	7	580–595	72[a)]
			17B	AGCTCCCTATGGATGGCTCTCTT				

a) Nucleotide sequence in non-coding region of exon 17.

non-denaturing polyacrylamide gel containing 45 m*M* Tris-borate (pH 8.3) and 4 m*M* EDTA. Two gels were used, one containing 5% glycerol and the other without glycerol. Electrophoresis proceeded at 30 W for 1 to 4.5 h with fan cooling. The gel was dried and exposed to X ray film at −80°C for 6 to 18 h with an intensifying screen.

Direct DNA sequencing DNA fragments with abnormal mobility shifts in SSCP analysis were eluted from gel pieces and amplified by 30 PCR cycles using the same primers.[24] The amplified DNA fragments were denatured and annealed with a 5′-labeled primer for sequencing. Chain elongation and termination[25] were performed with a dsDNA Cycle Sequencing kit (GIBCO BRL) and the products were analyzed in 5% HydroLink gels containing 7 *M* urea.

RESULTS

We analyzed the *NF2* gene by PCR-SSCP, in the DNAs of 73 surgical specimens from 52 patients with breast cancer, 2 with phyllodes tumor and 6 with non-malignant breast tumors. Among the 52 patients with breast cancer, metastatic tumors were also obtained from the adjacent lymph nodes of 7 patients, from the liver of 2 and from the subcutaneous tissue of one. In one patient with a phyllodes tumor, the tumor locally recurred within 3 months of primary tumor resection. The recurrent tumor was also examined. Non-malignant tumors examined included 4 fibroadenomas, a mammary dysplasia and a mastopathy.

Coding exons, except exon 16,[9] and adjacent sequences of introns of the *NF2* gene indicated in Table I

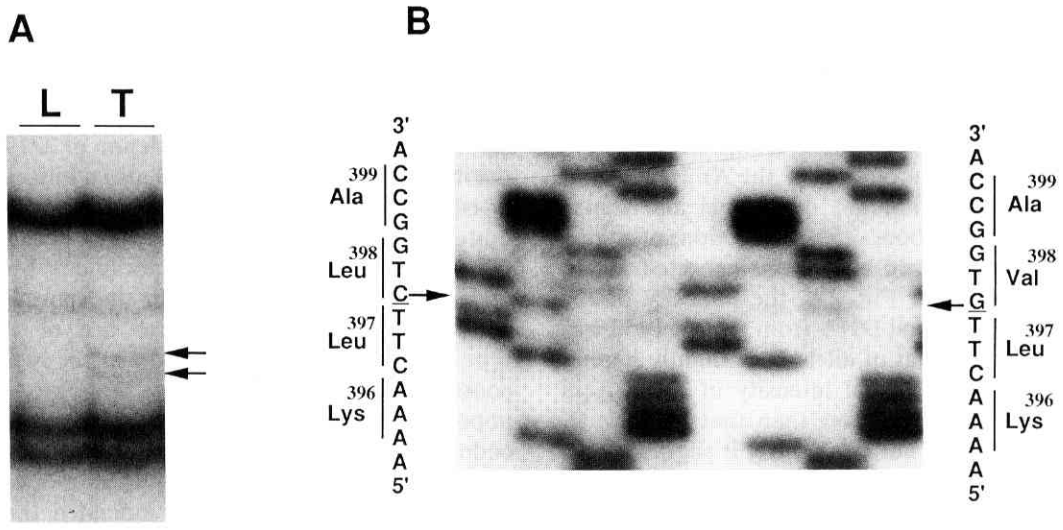

Fig. 1. SSCP and nucleotide sequence analyses of fragment L containing exon 12 of the *NF2* gene. (A) DNA fragment L amplified from genomic DNAs from breast cancer cells (T) and lymphocytes (L) from patient Br52 were analyzed by SSCP. Two isoconformers of a shifted DNA fragment are indicated by arrows. (B) DNA fragment L from cancer DNA showing mobility shifts in bands of isoconformers indicated by arrows in (A) were eluted from the gel and sequenced. The sequencing ladders obtained from abnormal fragments (T) were compared with that of normal lymphocyte DNA (L). The two fragments indicated by arrows in (A) showed the same sequencing ladder.

were amplified into 16 DNA fragments and analyzed by SSCP. Although the signal was faint, a tumor-specific mobility shift in fragment L from the breast cancer DNA of patient Br52 was reproducibly observed (Fig. 1A). A set of two bands observed was due to isoconformers from the same fragment. A similar SSCP analysis of the same DNA sample for the *p53* gene showed a shifted band in the DNA fragment carrying exon 5 of the gene and indicated that the DNA sample contained about the same amount of normal and tumor DNAs (data not shown). Therefore, the faint signal observed in Fig. 1A might not be due to small population of the tumor DNA in the sample. PCR-SSCP analysis sometimes shows unexpectedly weak signals of a DNA fragment from a polymorphic allele. The result observed in an allele of the *NF2* gene from patient Br2 (allele B in Fig. 2B) is one such example. Thus, the faint signal of the shifted band in tumor DNA in patient Br52 might be due to this type of feature. However, the possibility remains that only a fraction of the cancer cells with *p53* abnormality has the mutated *NF2* gene. Determination of the nucleotide sequence of the shifted DNA fragments eluted from the gel confirmed the presence of the target fragment of the *NF2* gene and revealed a C-to-G transversion in codon 398 in exon 12, resulting in an amino acid substitution from leucine to valine (Fig. 1B). No tumor-specific mobility shifts of DNA fragments were evident in other breast tumors.

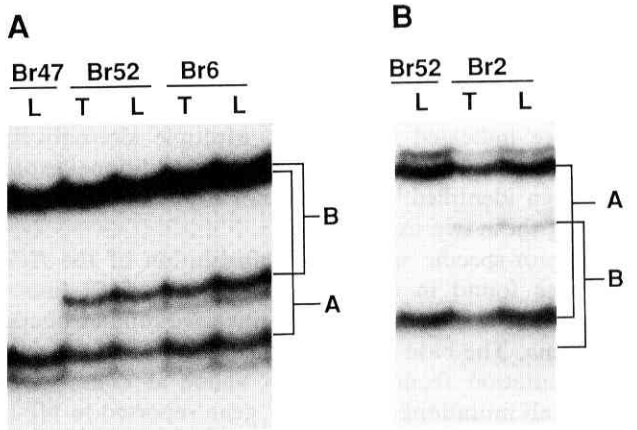

Fig. 2. Polymorphic changes of the nucleotide sequence of the *NF2* gene. Mobility shifts of fragment K (A) and fragment A (B) due to polymorphisms were detected by SSCP analysis. L and T indicate DNA from lymphocytes and tumors, respectively. The shifted bands are indicated by B in (A) and (B).

During the analysis, there were mobility shifts of DNA fragments due to polymorphisms in fragment K from DNA of patients Br6 and Br52 (allele B in Fig. 2A) and in fragment A from the DNA of patient Br2 (allele B in Fig. 2B). Nucleotide sequencing of the shifted fragment K revealed a single-base change from C to T in the third

base of codon 371 (asparagine, AAC to AAT). The shifted fragment A with the faint signal described above contained the polymorphic base change of G to A 18 base pairs upstream from the first ATG codon.

As described above, the tumor DNA sample from patient Br52 showing a mutation of the *NF2* gene contained about the same amount of normal and tumor DNAs and therefore about 25% reduction of signal intensity of one of the two alleles was expected, if the sample showed LOH. In Fig. 2A, the intensity of the signal for allele A in normal DNA from patient Br52 was essentially the same as that of allele B or slightly higher. On the other hand, the signal intensity of allele A in tumor DNA seemed to be lower than that of allele B. Although the result was not conclusive, it suggested the presence of LOH at the *NF2* locus in the tumor DNA from patient Br52.

DISCUSSION

Here, we analyzed the regions of the tumor suppressor *NF2* gene containing the coding exons and their flanking intron sequences[9] in DNAs from human breast tumors. When the primers used in this study were designed, available nucleotide sequences of introns of the *NF2* gene were limited. Therefore, mutations at the 5′-end of exons 3, 6, 8, 9 and 14 and in adjacent introns, and in introns adjacent to the 3′-end of exons 1, 2, 4, 5, 6, 12, 13 and 14 and at the 3′-end of exons 1, 6, 12, 13 and 14 could not be detected. The regions providing information in this study are indicated in Table I. Multiple alternatively spliced transcripts of the gene[15] and two additional exons have been identified.[17] We did not examine the region carrying these two exons.

A tumor-specific single-base substitution of the *NF2* gene was found in the DNA from one of 52 breast cancers that was diagnosed as an invasive tubular ductal carcinoma. The base substitution should result in a missense mutation from leucine to valine at codon 398. Almost all mutations of the *NF2* gene reported in NF2-related cancers[3-9, 11, 12] and in sporadic cancers[4, 8-14, 16, 17] are nonsense and frameshift mutations that result in truncation of the NF2 protein. In breast cancers, only one case with mutations of the *NF2* gene has been reported so far. The breast cancer was also diagnosed as a poorly differentiated ductal carcinoma and showed a frameshift mutation due to a deletion of 221 base pairs in the middle of the transcript and also a missense mutation of isoleucine to phenylalanine at codon 273.[11] Missense mutations of the *NF2* gene have also been found in an NF2-related schwannoma,[9] sporadic schwannomas[8, 9] and a sporadic melanoma.[11] In the sporadic colorectal carcinomas, the two mutations are of the missense type.[17] These have been observed in the regions that are conserved among species and are therefore thought to be important for biological functions of the protein. The codon in which we observed a missense mutation was also in the region conserved in human moesin and mouse schwannomin.[3, 26] Our results indicated that mutations of the tumor suppressor *NF2* gene might be involved in some human breast cancers, although the frequency of such mutations was low. Because of the low frequency of the *NF2* mutation, frequent allelic loss at loci on chromosome 22q reported in breast cancers[18, 19] might suggest the presence of another tumor suppressor gene as proposed in human meningiomas.[27]

Besides the tumor-specific base substitution, we detected polymorphic base changes, C to T in the third base of codon 371 in 2 of 60 individuals and G to A 18 base pairs upstream from the first ATG codon in one of 60 individuals.

ACKNOWLEDGMENTS

This work was supported by a Grant-in-Aid from the Ministry of Health and Welfare for the 2nd Term Comprehensive 10-Year Strategy for Cancer Control, Japan, and a Grant for Research on Aging and Health from the Ministry of Health and Welfare. R.S. was a recipient of a Research Fellowship under the Program for Invitation of Foreign Scientists to Japanese Institutes from the Foundation for Promotion of Cancer Research of Japan.

(Received May 22, 1995/Accepted July 20, 1995)

REFERENCES

1) Martuza, R. L. and Eldridge, R. Neurofibromatosis 2. *N. Engl. J. Med.*, **318**, 684–688 (1988).
2) Evans, D. G., Huson, S. M., Donnai, D., Neary, W., Blair, V., Teare, D., Newton, V., Strachan, T., Ramsden, R. and Harris, R. A. A genetic study of type 2 neurofibromatosis in the United Kingdom. I. Prevalence, mutation rate, fitness, and confirmation of maternal transmission effect on severity. *J. Med. Genet.*, **29**, 841–846 (1992).
3) Trofatter, J. A., MacCollin, M. M., Rutter, J. L., Murrell, J. R., Duyao, M. P., Parry, D. M., Eldridge, R., Kley, N., Menon, A. G., Pulaski, K., Haase, V. H., Ambrose, C. M., Munroe, D., Bove, C., Haines, J. L., Martuza, R. L., MacDonald, M. E., Seizinger, B. R., Short, M. P., Buckler, A. J. and Gusella, J. F. A novel moesin-, ezrin-, radixin-like gene is a candidate for the neurofibromatosis 2 tumor suppressor. *Cell*, **72**, 791–800 (1993).

4) Rouleau, G. A., Merel, P., Lutchman, M., Sanson, M., Zucman, J., Marineau, C., Hoang-Xuan, K., Demczuk, S., Desmaze, C., Plougastel, B., Pulst, S. M., Lenoir, G., Bijlsma, E., Fashold, R., Dumanski, J., de Jong, P., Parry, D., Eldrige, R., Aurias, A., Delattre, O. and Thomas, G. Alteration in a new gene encoding a putative membrane-organizing protein causes neuro-fibromatosis type 2. *Nature,* **363,** 515–521 (1993).
5) Bourn, D., Carter, S. A., Mason, S., Gareth, D., Evans, R. and Strachan, T. Germline mutations in the neurofibromatosis type 2 tumour suppressor gene. *Hum. Mol. Genet.,* **3,** 813–816 (1994).
6) Bourn, D., Carter, S. A., Evans, D. G. R., Goodship, J., Coakham, H. and Strachan, T. A mutation in the neurofibromatosis type 2 tumor-suppressor gene, giving rise to widely different clinical phenotypes in two unrelated individuals. *Am. J. Hum. Genet.,* **55,** 69–73 (1994).
7) MacCollin, M., Ramesh, V., Jacoby, L. B., Louis, D. N., Rubio, M.-P., Pulaski, K., Trofatter, J. A., Short, M. P., Bove, C., Eldridge, R., Parry, D. M. and Gusella, J. F. Mutational analysis of patients with neurofibromatosis 2. *Am. J. Hum. Genet.,* **55,** 314–320 (1994).
8) Irving, R. M., Moffat, D. A., Hardy, D. G., Barton, D. E., Xuereb, J. H. and Maher, E. R. Somatic *NF2* gene mutations in familial and non-familial vestibular schwannomas. *Hum. Mol. Genet.,* **3,** 347–350 (1994).
9) Jacoby, L. B., MacCollin, M., Louis, D. N., Mohney, T., Rubio, M.-P., Pulaski, K., Trofatter, J. A., Kley, N., Seizinger, B., Ramesh, V. and Gusella, J. F. Exon scanning for mutation of the *NF2* gene in schwannomas. *Hum. Mol. Genet.,* **3,** 413–419 (1994).
10) Ruttledge, M. H., Sarrazin, J., Rangaratnam, S., Phelan, C. M., Twist, E., Merel, P., Delattre, O., Thomas, G., Nordenskjöld, M., Collins, V. P., Dumanski, J. P. and Rouleau, G. A. Evidence for the complete inactivation of the NF2 gene in the majority of sporadic meningiomas. *Nature Genet.,* **6,** 180–184 (1994).
11) Bianchi, A. B., Hara, T., Ramesh, V., Gao, J., Klein-Szanto, A. J. P., Morin, F., Menon, A. G., Trofatter, J. A., Gusella, J. F., Seizinger, B. R. and Kley, N. Mutations in transcript isoforms of the neurofibromatosis 2 gene in multiple human tumour types. *Nature Genet.,* **6,** 185–192 (1994).
12) Twist, E. C., Ruttledge, M. H., Rousseau, M., Sanson, M., Papi, L., Merel, P., Delattre, O., Thomas, G. and Ruleau, G. A. The neurofibromatosis type 2 gene is inactivated in schwannomas. *Hum. Mol. Genet.,* **3,** 147–151 (1994).
13) Rubio, M.-P., Correa, K. M., Ramesh, V., MacCollin, M. M., Jacoby, L. B., von Deimling, A., Gusella, J. F. and Louis, D. N. Analysis of the neurofibromatosis 2 gene in human ependymomas and astrocytomas. *Cancer Res.,* **54,** 45–47 (1994).
14) Deprez, R. H. L., Bianchi, A. B., Groen, N. A., Seizinger, B. R., Hagemeijer, A., van Drunen, E., Bootsma, D., Koper, J. W., Avezaat, C. J. J., Kley, N. and Zwarthoff, E. C. Frequent *NF2* gene transcript mutations in sporadic meningiomas and vestibular schwannomas. *Am. J. Hum. Genet.,* **54,** 1022–1029 (1994).
15) Pykett, M. J., Murphy, M., Harnish, P. R. and George, D. L. The neurofibromatosis 2 (NF2) tumor suppressor gene encodes multiple alternatively spliced transcripts. *Hum. Mol. Genet.,* **3,** 559–564 (1994).
16) Sainz, J., Huynh, D. P., Figueroa, K., Ragge, N. K., Baser, M. E. and Pulst, S.-M. Mutations of the neurofibromatosis type 2 gene and lack of the gene product in vestibular schwannomas. *Hum. Mol. Genet.,* **3,** 885–891 (1994).
17) Arakawa, H., Hayashi, N., Nagase, H., Ogawa, M. and Nakamura, Y. Alternative splicing of the *NF2* gene and its mutation analysis of breast and colorectal cancers. *Hum. Mol. Genet.,* **3,** 565–568 (1994).
18) Sato, T., Tanigami, A., Yamakawa, K., Akiyama, F., Kasumi, F., Sakamoto, G. and Nakamura, Y. Allelotype of breast cancer: cumulative allele losses promote tumor progression in primary breast cancer. *Cancer Res.,* **50,** 7184–7189 (1990).
19) Chen, L.-C., Kurisu, W., Ljung, B.-M., Goldman, E. S., Moore, D., II and Smith, H. S. Heterogeneity for allelic loss in human breast cancer. *J. Natl. Cancer Inst.,* **84,** 506–510 (1992).
20) Kanai, Y., Tsuda, H., Oda, T., Sakamoto, M. and Hirohashi, S. Analysis of the neurofibromatosis 2 gene in human breast and hepatocellular carcinomas. *Jpn. J. Clin. Oncol.,* **25,** 1–4 (1995).
21) Blin, N. and Stafford, D. M. A general method for isolation of high-molecular-weight DNA from eukaryotes. *Nucleic Acids Res.,* **3,** 2303–2308 (1976).
22) Saiki, R. K., Gelfand, D. H., Stoffel, S., Scharf, S. J., Higuchi, R., Horn, G. T., Mullis, K. B. and Erlich, H. A. Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. *Science,* **239,** 487–491 (1988).
23) Hayashi, K., Orita, M., Suzuki, Y. and Sekiya, T. Use of labeled primers in polymerase chain reaction (LP-PCR) for a rapid detection of the product. *Nucleic Acids Res.,* **17,** 3605 (1989).
24) Suzuki, Y., Sekiya, T. and Hayashi, K. Allele-specific polymerase chain reaction: a method for amplification and sequence determination of a single component among a mixture of sequence variants. *Anal. Biochem.,* **192,** 82–84 (1991).
25) Murray, V. Improved double-stranded DNA sequencing using the linear polymerase chain reaction. *Nucleic Acids Res.,* **17,** 8889 (1989).
26) Claudio, J. O., Marineau, C. and Rouleau, G. A. The mouse homologue of the neurofibromatosis type 2 gene is highly conserved. *Hum. Mol. Genet.,* **3,** 185–190 (1994).
27) Akagi, K., Kurahashi, H., Arita, N., Hayakawa, T., Monden, M., Mori, T., Takai, S. and Nishisho, I. Deletion mapping of the long arm of chromosome 22 in human meningiomas. *Int. J. Cancer,* **60,** 178–182 (1995).