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LOH on Chromosome 11q, but not SDHD and Men1 Mutations Was Frequently Detectable in Chinese Patients with Pheochromocytoma and Paraganglioma

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Recently, the succinate dehydrogenase subunit D (*SDHD*) gene has been reported as one of the major susceptibility genes for pheochromocytoma (PCC) and paraganglioma (PGL). In addition, loss of heterozygosity (LOH) on chromosome 11, mainly in 11q23 and 11q13, is observed frequently in PGL. Based on the fact that mutation frequency of the *SDHD* gene is less than that of allelic loss at chromosome11q, where the *SDHD* gene is located, this region may contain other candidate tumor-suppressor genes involved in pathogenesis of PCC/PGL. The tumor-suppressor gene *Men1* located in 11q13 is responsible for multiple endocrine neoplasia type 1 (Men1). However, the involvement of the *Men1* gene in tumorigenesis of sporadic PCC/PGL is yet to be determined. To understand the roles of the two tumor-suppressor genes and LOH on chromosome 11q in Chinese patients with sporadic PCC or PGL, we performed mutation detection of the *SDHD* and *Men1* genes in tumors from 35 Chinese patients with PCC/PGL; we also did LOH analysis at chromosome 11q for 25 patients out of the 35. No mutation was found in all of 35 patients. However, LOH was detected at one or more loci in 11 of the 25 (44%) tumor samples. The highest frequency of LOH occurred at D11S2006 (41%). Our results suggested that mutation in *SDHD* or *Men1* gene was not found in Chinese patients with sporadic PCC/PGL. However the loss of chromosome 11q might be critical in development of PCC or PGL.

Key Words: Pheochromocytoma; tumor-suppressor gene; mutation; loss of heterozygosity.

Introduction

Pheochromocytoma (PCC) is a type of catecholamine-producing tumor that usually arises from the adrenal medulla. If it arises from extraadrenal chromaffin tissue, it is called paraganglioma (PGL) or extraadrenal pheochromocytoma. The genetic mechanisms underlying the tumorigenesis of sporadic PCC are poorly understood. Recently, the main susceptibility gene for familial paraganglioma syndrome has been characterized and shown to encode succinate dehydrogenase subunit D (*SDHD*), which is part of the complex II in the mitochondrial respiratory chain and plays an important role in both the tricarboxylic acid cycle and the aerobic respiratory chain *(1,2)*. In addition, loss of heterozygosity (LOH) on chromosome 11, mainly in 11q23 (PGL1) and 11q13 (PGL2), has been observed in PGL recently *(3)*. *SDHD* gene is located in the PGL1 region, and previous work indicates that it is the putative PGL1 gene *(1)*. However, the tumor-suppressor gene(s) in the PGL2 region has not been identified. It is conceivable that more tumor-suppressor genes may locate on chromosome11q, such that allelic deletion of those genes may enhance tumor formation.

SDHD mutations have been identified widely in patients with familial or sporadic PCC/PGL *(4–9)*. However, some recent studies have shown that *SDHD* may not participate in sporadic PCC/PGL pathogenesis *(10,11)*. Loss of heterozygosity (LOH) at Chr.11q23, where the *SDHD* gene is located, has been detected frequently in PCC/PGL *(4,10, 12)*. Comparing with the mutation rates of *SDHD* gene, the

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frequency of 11q LOH is higher. It indicates that the existence of other unidentified tumor-suppressor genes except *SDHD* are located in Chr.11q. These tumor-suppressor genes will be lost when LOH happens in Chr.11q. So, it is possible that the additional tumor-suppressor genes take part in the tumorigenesis in PCC/PGL.

In the 11q13 region, there is another known tumor-suppressor gene, multiple endocrine neoplasia type 1 (*Men1*) gene. However, the involvement of the *Men1* gene in tumorigenesis of sporadic PCC/PGL is yet to be determined. It has also been hypothesized that the combined loss of wild-type *Men1* and another tumor-suppressor gene in Chr.11q is essential for tumorigenesis of endocrine tumors *(13)*. The frequency of either *SDHD* or *Men1* mutation in sporadic endocrine tumors does not correspond to the relative high frequency of LOH at chromosome 11q. To understand the roles of these two tumor-suppressor genes and LOH of chromosome 11q in Chinese patients having sporadic PCC or PGL, we performed a mutation screening of both *SDHD* and *Men1* genes in tumors from 35 Chinese patients with PCC/PGL, but LOH analysis at chromosome 11q was carried out for 25 patients out of the 35. In addition, we detected mutation of a potential candidate tumor-suppressor gene named Family with sequence similarity 111, member B (*FAM111B,* Gene ID: 374393) in tumors. The gene submitted to Genebank is predicted to code cancer-associated nucleoprotein.

Results

Clinical Findings

Among the participating patients with tumor, there were 23 females with a mean age of 45 yr (range 17–63 yr), and 12 male patients with a mean age 43 yr (range 17–74 yr). Approximately 74% (26/35) of the patients had suffered from pheochromocytoma trilogy (headache, palpation, and sweating). Twenty-five of them were diagnosed as sporadic PCC (1 malignant) and 10 of them were diagnosed as sporadic PGL (two malignant), based on no family history and other signs of Men1, MEN2, VHL, and NF1.

The tumors in PCC and PGL were unilateral or isolated. According to the pathological characteristics, 3 of these 35 cases presented malignant morphologic behaviors, like capsule invasion and/or vessel tumor embolus or metastasis. There was no recurrence in all of cases except one whose brain metastasis had been observed 18 mo after operation. Clinical data, including blood pressure, urinary epinephrine (E), norepinephrine (NE), dopamine (DA), plasma metanephrine (MN), and normetanephrine (NMN), are shown in Table 1.

Mutation Detection

Mutation analyses of the *SDHD*, *Men1*, and *FAM111B* genes were performed in 35 tumor samples, but there was no mutation detected in the complete coding regions of these genes.

Table 1
Clinical Data of Patients with PCC/PGL

Item	Means	SD	Normal range
Age (yr)	44.2	14.4	
Gender (male/female)	12/23		
SBp (mmHg)	173.9	58.7	
DBp (mmHg)	102.2	43.8	
MN (pg/mL)	1267.4	2065.6	14.0–90.0
NMN (pg/mL)	2566.8	2444.6	19.0–121.0
E (μg/24h)	201.3	290.4	<22.0
NE (μg/24h)	728.5	786.5	7–65
DA (μg/24h)	423.4	1223	75–440

NE, urinary norepinephrine (pg/mL); DA, urinary dopamine (pg/mL); E, urinary epinephrine (pg/mL); NMN, plasma normetanephrine (nmol/L); MN, plasma metanephrine (nmol/L)

LOH Analysis

LOH analysis at Chr.11q was performed in the 25 patients with 9 microsatellite markers covering chromosome 11q, spanning a region over 70 cM. We designed marker primers every 5–10 cM with the following order: pter-D11S1985-D11S2006-D11S2371-D11S2002-D11S2000-D11S1986-D11S1998-D11S4464-D11S4463-qter. To refine the LOH region, an additional six markers located between D11S2006 and D11S2371 were included later, with the following order: D11S4191-D11S4076-D11S4205-D11S1889-D11S4113-D11S1975 (primer information can be gain at www.gdb.org). The *Men1* gene is located between D11S2006 and D11S2371, *FAM111B* between D11S1985 and D11S2006*, SDHD* between D11S1986 and D11S1998. The distance from telomere to every marker is shown in Table 2. The average informative rate of all these markers was 76%. Allelic deletion was detected at one or more loci in 11 of the 25 (44%) samples. Three of the 11 identified samples only had one locus deletion, and the other 8 had multilocus allelic deletions (8/25, 32%), out of which 7 samples almost had the whole long arm deleted (Table 2). We observed the highest LOH frequency (41%, 7/17) occurring at D11S2006 (Table 3). Five of 7 samples with the marker deletion had almost the whole long arm deleted. The boundaries for LOH regions in 5/7 patients were labeled as STR marker D11S1985 and D11S4463, which are close to centromere and telomere on chromosome 11q, respectively. Two other samples showed partial deletion. The partial deletion cases allowed us to refine the allelic loss regions of the marker *D11S2006* (Fig. 1). The boundaries for LOH regions in 2/7 patients were labeled as STR marker D11S 1985 and D11S4191, which spans 1.45 Mb (Table 2). The frequency of LOH at Chr.11q was similar in PCC and PGL patients (9/19 PCC vs 2/6 PGL). The clinical characteristics, such as age, blood pressure, and laboratory tests, which included 24-h urinary epinephrine (E), norepinephrine (NE) and dopamine (DA), plasma metanephrine (MN), and nor-

Table 2
A. Pattern of LOH on 11q Detected in 25 Samples

Tumor type	Code	D11S1985 58.3 Mb	D11S2006 59.5 Mb	D11S2371 73.2 Mb	D11S2002 79.6 Mb	D11S2000 105.1 Mb	D11S1986 110.7 Mb	D11S1998 117.2 Mb	D11S4464 123.1 Mb	D11S4463 130.4 Mb
PCC	1	●	●	●	—	●	●	●	●	●
	2	●	●	●	●	*Nº*	●	●	●	●
	3	●	●	●	●	—	●	●	●	●
	4	●	●	●	●	—	●	●	●	●
	5	—	—	●	●	●	●	●	●	●
	6	○	○	○	●	●	●	●	●	●
	7	○	●	○	○	*Nº*	○	○	○	○
	8	○	●	—	—	○	○	○	○	—
	9	●	○	○	○	—	○	○	○	—
	10	—	○	○	○	—	—	—	○	○
	11	○	○	—	—	○	○	○	—	○
	12	○	—	○	○	○	—	—	○	○
	13	○	○	○	—	○	○	—	○	—
	14	○	○	○	○	○	○	○	○	—
	15	○	○	—	○	○	○	○	○	○
	16	○	○	○	—	○	○	○	○	○
	17	○	—	○	○	*Nº*	○	—	○	○
	18	○	—	○	○	○	○	○	—	○
	19	—	○	—	○	—	○	○	○	—
PGL	1	—	●	—	●	●	●	—	●	●
	2	●	*Nº*	●	○	○	○	●	●	○
	3	○	—	—	○	—	○	—	○	○
	4	○	—	○	○	○	○	○	○	○
	5	○	—	○	—	○	○	○	○	○
	6	○	○	—	○	○	○	○	—	○

B. LOH Refine Analyses

Code	D11S1985 58.3 Mb	D11S2006 59.47 Mb	D11S4191 59.75 Mb	D11S4076 61.1 Mb	D11S4205 62.9 Mb	D11S1889 63.1 Mb	D11S4113 68.5 Mb	D11S1975 70.0 Mb	D11S2371 73.2 Mb
7	○	●	○	○	—	○	○	○	○
8	○	●	●	○	○	—	○	○	—

●, Loss of heterozygosity (ratio > 1.5); ○, retention of heterozygosity; —, no informative (homozygosity); *Nº*, No result; FAM111B: 58.6 Mb; Men1: 64.3 Mb; SDHD: 111.5 Mb. The *Men1* gene is located between D11S2006 and D11S2371, FAM111B between D11S1985 and D11S2006, *SDHD* between D11S1986 and D11S1998. The physical position of every marker is listed in the table.

Table 3
LOH Frequency Analysis in 25 Samples

Marker	Mb from pter	No. of informative cases (%)	LOH (%)
D11S1985	58.3 Mb	21 (84%)	6 (28.6%)
D11S2006	59.5 Mb	17 (68%)	7 (41.2%)
D11S2371	73.2 Mb	18 (72%)	6 (33.3%)
D11S2002	79.6 Mb	18 (72%)	6 (33.3%)
D11S2000	105.1 Mb	15 (60%)	4 (26.7%)
D11S1986	110.7 Mb	22 (88%)	7 (31.8%)
D11S1998	117.2 Mb	19 (76%)	7 (36.8%)
D11S4464	123.1 Mb	22 (88%)	8 (36.4%)
D11S4463	130.4 Mb	19 (76%)	7 (36.8%)

metane-phrine (NMN), tumor location and size scanning by CT, were analyzed, but neither the clinical characteristics nor the result of the lab test was found to have no correction with allelic deletion.

Discussion

Mutations in the *SDHD* gene or LOH on chromosome 11 recently have been demonstrated to play roles in the pathogenesis of PCC/PGL *(1,3,4–10,12)*. It is hypothesized that one or more tumor-suppressor genes on chromosome 11q may be responsible for the development of PCC or PGL. The *SDHD* gene is located in the 11q23 region, and there is a known tumor-suppressor gene *Men1*, which is located in 11q13, near the *SDHD* gene. The *Men1* gene contains nine exons, coding for a tumor-suppressor protein, menin. The majority of *Men1* tumors have LOH of chromosome 11q13 and a few have been shown to harbor somatic mutations consistent with the Knudson two-hit hypothesis *(14)*. *Men1* mutations occur not only in *MEN1* (both familial and sporadic), but also in sporadic endocrine tumors *(15)*. However, the involvement of *Men1* gene in the development of sporadic PCC/PGL is yet to be determined. In this

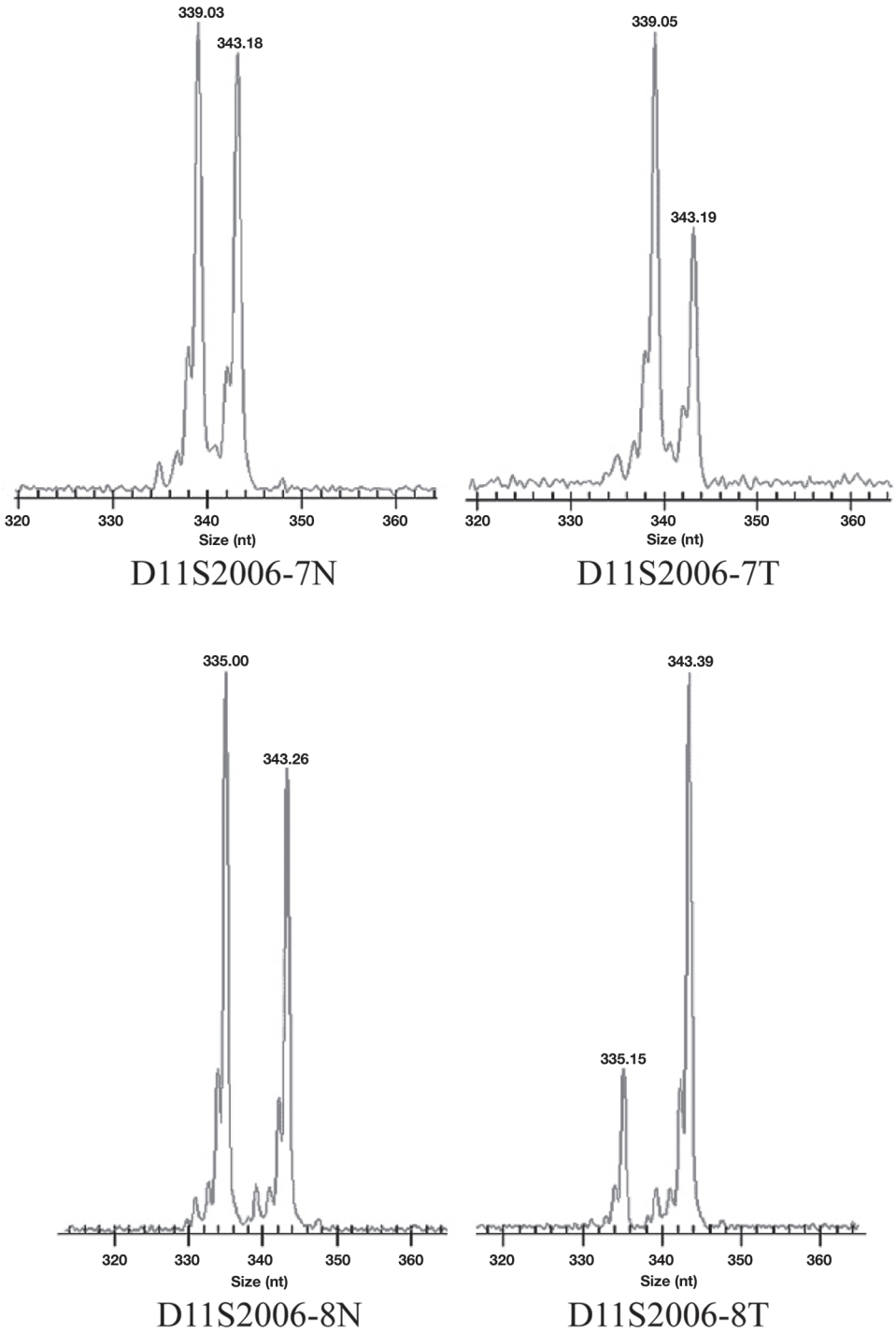

Fig. 1. The LOH results of D11S2006 in tumor 7 and 8, the left image comes from normal blood tissue and the right from tumor.

study, there was no mutation in *Men1* gene detected in tumor of the 35 Chinese patients having PCC/PGL. Edstrom et al. reveal no mutation in *Men1* gene in their 30 subjects of PCC/PGL either; this suggests that the gene was rarely involved in the formation of sporadic PCC/PGL *(16)*. We detected mutation of *SDHD* gene in tumors of 35 patients. Our result demonstrated that mutation in *SDHD* gene was not found in this sample of Chinese patients with sporadic PCC/PGL.

Recent findings of comparative genomic hybridization (CGH) and LOH studies provide new points to investigate the genetic mechanisms of tumors. Studies of genome-wide scanning for copy number changes by CGH in PCC/PGL report that losses are more common than gains. This indicates that the inactivation of tumor-suppressor gene plays a critical role in tumorigenesis *(16)*. The CGH studies on PCC and PGL have shown frequent loss of chromosome 11 *(16–18)*. LOH on chromosomes 11q has also been reported

in PCC and PGL *(3,4,12,18)*. The results of these studies suggest the involvement of one or several tumor-suppressor genes, located within 11q, in the development of PCC or PGL. In our study, we performed LOH analysis at Chr.11q in the 25 patients with PCC/PGL, and the LOH was found in 11 (44%) of them. The multilocus allelic deletions were identified in eight (32%) of them, seven patients had almost the whole long arm deleted. The highest frequency of LOH occurred at D11S2006 (41%), after refining with additional six microsatellite markers in the region, the deletion region was indicated to be about 1.5 Mb size from *D11S1985* to *D11S4191*. One potential candidate tumor-suppressor gene, *FAM111B* (Gene ID: 374393) which is submitted to Genebank as a cancer-associated nucleoprotein coding gene, is within this region according to NCBI database (http://www.ncbi.nlm.nih.gov). It has been characterized in hepatocellular carcinoma and codes 734 amino acids. The information about the function of the *FAM111B* is unknown. The gene locates near D11S2006 locus, where the highest LOH occurred, was thought to be one potential candidate tumor-suppressor gene. We carried out genetic analysis of four exons of the *FAM111B* gene, including its splicing and coding regions. It is the first report where the FAM111B is related with the development of PCC and PLG. However, no mutation was found in our study of sporadic PCC/PGL. Although no function mutation of the gene was found in our samples, other candidate genes located in this region might be related to the development of the tumor.

Our research failed to find any mutations in *SDHD*, *Men1,* or *FAM111B* gene. However, it was found that 11/25 tumor samples displayed LOH in a vast region covering Chr.11q. It is suggested that the loss of chromosome 11q could be an important event in the development of PCC or PGL. The further research is necessary to find the unidentified tumor-suppressor genes responsible for the development of tumors in addition to *SDHD* and *Men1* in this region.

Materials and Methods

Clinical Studies

The clinical evaluation including physical examination and personal and family history was performed and reviewed by at least two physicians in our clinical center. The clinical screening program including urinary epinephrine (E), norepinephrine (NE), dopamine (DA), plasma metanephrine (MN), and normetanephrine (NMN), and CT scanning was used for characterizing the tumors. Patients were first hospitalized for diagnosis at the Clinical Center for Endocrine and Metabolic Diseases in Shanghai RuiJin Hospital, and then transferred to the Department of Urology for resection of tumors. The diagnosis of all these 35 patients was ascertained by pathological laboratory findings. Distant metastases or infiltration of surrounding tissue were diagnosed as malignant. Follow-up study was performed after the operation.

Patients and DNA Sample

Thirty-five unrelated patients with PCC/PGL were hospitalized and operated from January 1, 2003 to September 30, 2005. Their tumor tissue samples were obtained, but only 25 peripheral blood samples were retained. The tumor samples were cut into pieces in an area of 1 cm^2 when resected and immediately frozen in liquid nitrogen. The peripheral blood samples were also collected and frozen at −70°C. DNA from tumor tissue and leukocytes was extracted by using a commercial kit (Qiagen) according to the manufacturer's instruction. This study was approved by hospital ethical board committee, and all patients had given the informed consent in accordance with institutional guidelines and national regulations that have their origin in the Declaration of Helsinki.

Mutation Detection

The analysis was carried out by tumor tissue genomic DNA amplification, with PCR and direct sequencing, of all four exons and the exon–intron boundary regions of *SDHD*, all nine exons and the exon-intron boundary regions of *Men1*, and all four exons and the exon–intron boundary regions of *FAM111B*. Intronic primers used for PCR were designed based on the published *SDHD, Men1,* and *FAM111B* genomic sequence (GenBank accession number: NT_033899, NT_033903, NT_033903) (shown in supplementary information, Table 4). PCR was performed in a volume of 50 μL containing 0.25 U LA-Taq (TaKaRa, Japan), 5 μL 10X buffer, 0.25 mm dNTP, 100 ng genomic DNA, and 1 μ*M* forward and reverse primer. Amplification was performed by 30 cycles of denaturation at 94°C for 1 min, annealing at 55–62°C for 30 s (shown in supplementary information, Table 4), and extension at 72°C for 1 min. The reaction was carried out in PTC-225 DNA Engine Tetrad (MJ Research, Waltham, MA). The PCR products were purified by a commercial kit (Qiagen), and then they were directly sequenced by ABI PRISM 3700 Genetic Analyzer (Applied Biosystems, Foster City, CA).

LOH Analysis

DNA from leukocytes and tumor tissues of the 25 patients (19 PCC and 6 PGL) was used for LOH analysis, with nine microsatellite markers covering chromosome 11q. The markers were designated as follows: D11S1985, D11S2006, D11S2371, D11S2002, D11S2000, D11S1986, D11S1998, D11S4464, and D11S4463. To refine the LOH region, additional markers, D11S4191, D11S4076, D11S4205, D11S1889, D11S4113, and D11S1975, were included. DNA fragments were labeled with fluorescent dye by using the M13 universal tailed primer method. PCR products were analyzed using Beckman-Coulter CEQ 8800 sequencer (Beckman Coulter, Fullerton, CA). Data collection and analysis were performed with Fragment Analysis Module (Beckman Coulter). LOH was defined as either a total loss or a reduction of 70% or more as compared to the consti-

Table 4
Primer Sequences Used for PCR, Annealing Temperature, and Amplicon Size

Gene	Amplicons	Forward (5'-3')	Reverse (5'-3')	Annealing temperature (°C)	Amplicon size (bp)
SDHD	Exon1	AGTAAACTGCGCCTTCTGC	CCTTCGGGTAAACATCTGG	58	550
	Exon2	ATAGGATTTGGCGATTGA	CTAGAGCCCAGAAAGCAG	58	440
	Exon3	CCTGGACCACTAACTTACAT	CAATCAACTTCTCCCTCAT	55	593
	Exon4	TGGAGTGGCAAATGGAGA	AAGCAGAGGCAAAGAGGC	61	550
Men1	Exon1a	TCCCTCCCCCGGCTTGCCTT	ACGTTGGTAGGGATGACGCG	62	220
	Exon1b	TGCTGGGCTTCGTGGAGCAT	GAGACACCCCCTTCTCGAGG	62	220
	Exon1c	GCCCGCTTCACCGCCCAGAT	GGAGGGTTTTGAAGAAGTGG	59	230
	Exon2	TCATTACCTCCCCCTTCCAC	AGGCTGGGGGGAGGGAACAA	59	254
	Exon3	AGGGTGGGCCATCATGAGAC	TAGCCCAGTCCTGCCCCATT	62	207
	Exon4,5	CATAACTCTCTCCTTCGGCT	TCTGCACCCTCCTTAGATGC	58	260
	Exon6	GGATCCTCTGCCTCACCTCC	GCAGGCCCTAGTAGGGGGAT	63	189
	Exon7	GTCGCAACCGCAATGTGC	AAATACCTTCAGTCCCGTCCAA	62	840
	Exon8	AGAGACTGATCTGTGCCCTC	AGACCTCTGTGCAGCTGTCC	62	227
	Exon9a	GGGTGGACACTTTCTGCTTC	CACGGGCTTGTCAGACTTTT	62	600
	Exon9b	GCCAGCACTGGACAAGGGCC	CAGCAGCTCCTTCATGCCCT	62	205
	Exon9c	GGGTCCAGTGCTCACTTTCC	CAAGCGGTCCGAAGTCCCCA	62	218
FAM111B	Exon1	CCCTTCAATCACTGTCTGG	AGGTCCTTCTGCCTACTGC	61	356
	Exon2	ATGTCTTTGGCCTGTGAC	AATTAGCCCTAGTTCTGC	61	596
	Exon3	GCCATAGGGCTTACCTCATA	TTGCCTGTCTACACTTTCCA	61	548
	Exon4	GTTGCGGTATTTCCACTC	CTCCCATTCCGTAGGTTG	61	2995
		CCAGTTATTCTAGCCACATT			
		GGCATAAGAAAGTGTAGCAG			
		TATTTATGCCTTGAAGGGTG			
		ATCTGGGTAGGCGGTATG			
		TCTGCCCTACTCCTGACA			
		CTTACCCACTGTGCCTCC			

tutional DNA, in which the allele peak ratio between germline DNA peak and tumor DNA peak was more than 1.5. To confirm the LOH results, a second PCR reaction was done for those markers showing LOH, and LOH was considered only when the result was replicated. The LOH frequency of a locus was equal to the ratio between allelic loss and informative cases.

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