DNA Fragmentation Factor 45 (*DFF45*) Gene at 1p36.2 Is Homozygously Deleted and Encodes Variant Transcripts in Neuroblastoma Cell Line[1]

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Abstract

Recently, loss of heterozygosity (LOH) studies suggest that more than two tumor suppressor genes lie on the short arm of chromosome 1 (1p) in neuroblastoma (NB). To identify candidate tumor suppressor genes in NB, we searched for homozygous deletions in 20 NB cell lines using a high-density STS map spanning chromosome 1p36, a common LOH region in NB. We found that the 45-kDa subunit of the DNA fragmentation factor (*DFF45*) gene was homozygously deleted in an NB cell line, NB-1. DFF45 is the chaperon of DFF40, and both molecules are necessary for caspase 3 to induce apoptosis. DFF35, a splicing variant of DFF45, is an inhibitor of DFF40. We examined 20 NB cell lines for expression and mutation of *DFF45* gene by reverse transcription (RT)–polymerase chain reaction (PCR) and RT-PCR–single-strand conformation polymorphism. Some novel variant transcripts of the *DFF45* gene were found in NB cell lines, but not in normal adrenal gland and peripheral blood. These variants may not serve as chaperons of DFF40, but as inhibitors like DFF35, thus disrupting the balance between DFF45 and DFF40. No mutations of the *DFF45* gene were found in any NB cell line, suggesting that the *DFF45* is not a tumor suppressor gene for NB. However, homozygous deletion of the *DFF45* gene in the NB-1 cell line may imply the presence of unknown tumor suppressor genes in this region. *Neoplasia* (2001) **3,** 165–169.

Keywords: *DFF45* gene, *DFF40* gene, neuroblastoma, 1p36, homozygous deletion.

Introduction

Neuroblastoma (NB) is derived from neural crest and is the most common extracranial malignant solid tumor in childhood. Cytogenetic studies have suggested that the deletion of the short arm of chromosome 1 (1p) occurs frequently in NB, and is associated with a poor prognosis [1–3]. Loss of heterozygosity (LOH) studies have defined the commonly deleted region as 1p36 between D1S244 and D1S243 in NB [4,5]. More than two tumor suppressor genes are predicted to lie on this region [6,7]. Several genes on 1p36 have been analyzed as possible candidates for tumor suppressor genes of NB. These include the cyclin-dependent kinase (CDK) homologue *CDC2L1* [8]; the zinc finger–containing transcription factors *PAX7* [9], *ID3* [10], and *E2F2* [11]; the tumor necrosis factor receptor 2 (*TNFR2*) [12]; transcription factor *HKR3* [5]; and the *p73* gene [13,14]. However, no tumor suppressor gene has yet to be identified in NB. The homozygously deleted region on 1p36 would be a key to identifying the tumor suppressor genes for NB. For this purpose, we performed a high-density PCR screening [15] around D1S244 in a panel of human NB cell lines as described [16]. We found the *DNA fragmentation factor 45* (*DFF45*) gene to be homozygously deleted in one NB cell line, NB-1 [17] (http://cellbank.nihs.go.jp). The *DFF45* gene maps to chromosome band 1p36.2–36.3 between D1S244 and D1S1460 [18]. The counterpart of DFF45 in mice is called inhibitor of caspase activation DNase (ICAD) [19]. DFF45/ICAD is the substrate of caspase 3, and dimerizes with DFF40/CAD. Cleavage of DFF45/ICAD by caspase 3 releases DFF40/CAD, which degrades chromosome DNA into nucleosomal fragments, the hallmark of apoptosis [19–21]. Recently, DFF35, a new isoform of DFF45, was identified. Functional studies have shown that DFF35 can bind DFF40 very strongly and inhibit its nuclease activity, cannot assist in the synthesis of active DFF40 [22]. An abnormality in this pathway may disrupt apoptosis through DNA fragmentation. We here identified homozygous deletion of the *DFF45* gene, and examined the expression and mutation of the gene in NB cell lines.

Materials and Methods

Cell Lines and Primary Tumors

Twenty NB cell lines were investigated, namely, SCMC-N2, SCMC-N3, NH12, TGW, NB-1, NB-16, NB-19, NB-69, GOTO, SK-N-SH, CHP-134, LAN-1, LAN-2, SJNB-1 to

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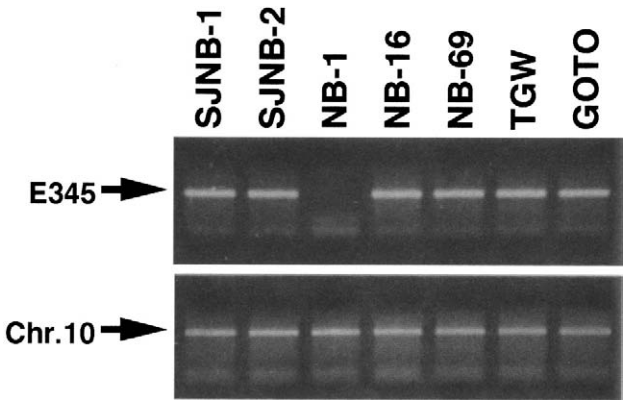

Figure 1. *Detection of homozygous deletion of the DFF45 gene by PCR. E345 is the EST marker mapped to 1p36.2, the area that contained the DFF45 gene. There was no PCR product in the NB-1 cell line when E345 was used; however, there was when the marker D10S2273 for chromosome 10 was used, indicating that there was a homozygous deletion of the DFF45 gene.*

SJNB-5, SJNB-7, and SJNB-8. SCMC-N2 and SCMC-N3 were established by us [23], and SJNB-1 to SJNB-5, SJNB-7, and SJNB-8 were a generous gift from Dr. A.T. Look. The other cell lines including NB-1 [17] were obtained from Japanese Collection of Research Bioresources (http://cellbank.nihs.go.jp). All the cell lines were cultured in RPMI-1640 medium supplemented with 9% fetal bovine serum [14].

Normal Peripheral Blood and Total RNA from Adrenal Gland

Normal peripheral blood was obtained from four adults in our laboratory with their informed consent. Total RNA from the adrenal gland was obtained from Clontech (Palo Alto, CA).

Total RNA Isolation and DNA Extraction

Total RNA was extracted from the cell lines and tissues using the acid guanidine thiocyanate–phenol–chloroform method [24]. High-molecular-weight DNA was extracted from 20 cell lines by proteinase K digestion and phenol/chloroform extraction [14,25].

Screening the Region Homozygously Deleted at 1p36 using PCR

Sixty sets of STS markers or EST markers around D1S244 were selected from 99′ Genemap (http://www.ncbi.nlm.nih.gov/genome/guide/) or designed by ourselves based on the data from our 1p36 contig map (http://www.ncc.go.jp/research/1p-genome/) [15]. Each NB cell line was used for PCR with different primer sets. The reaction mixture was as follows: 50 ng of template DNA, 1×PCR reaction buffer, 2 μl of 2 mM dNTP, 20 pmol of each primer, and 1.25 units of Gold Taq polymerase (Perkin-Elmer, NJ) in a final volume of 25 μl. PCR was performed with GeneAmp PCR system-9700 (Perkin Elmer, Norwalk, CT): denaturing at 94°C for 12 minutes followed by a 35-cycle amplification (94°C for 30 seconds, the respective annealing temperature for 30 seconds, and 72°C for 30 seconds) and 7 minutes extension at 72°C. The PCR products were subjected to electrophoresis on 3% agarose gel, stained in ethidium bromide, and photographed under UV light.

Table 1. Primers used in this study.

DFF S1	TCGAAGGTGCGCAGGTCCCACCTTG
DFF A1	CCCTGCCCCGCTGTCTGTTTCATC
DFF S2	TGGAGGTACAGCTTGGATTTC
DFF A2	CAGTGCAGTAAGGATGTGGCTCGC
DFF S3	GACACGGGTATCAGCAGAGAGACC
DFF A3	CAAATGATGAGGCTGAGGGGTGTCTA

RT-PCR Analysis of the DFF45 Gene

Randomly primed cDNA was made from total RNA, reverse transcribed with a cDNA synthesis kit as previously described [23]. A set of primers that covered the entire coding region of *DFF45* was designed (Table 1). RT-PCR was performed with GeneAmp PCR system-9700 (Perkin-Elmer, Norwalk, CT): denaturing at 94°C for 12 minutes followed by a 35-cycle amplification (94°C for 30 seconds, 62°C for 30 seconds, and 72°C for 30 seconds) and 7 minutes extension at 72°C. The reaction mixture contained 0.8 μM of each primer, 100 μM of each dNTP, 1×PCR buffer, and 1.25 units of Taq polymerase (Perkin-Elmer, NJ). The PCR products were run on a 3% agarose gel, stained in ethidium bromide, and photographed under UV light. The PCR products were subcloned into TA cloning vector (Invitrogen, San Diego, CA). Thirty white colonies were selected from each transformation for colony PCR with the same primers used in original PCR reaction. Colonies with different insertion were grown up for plasmid purification. Those plasmid DNA were subjected to direct sequencing [26].

PCR–SSCP and Direct Sequencing

The mutation analysis was carried out within the coding region of the *DFF45* gene. The primers used for this study are shown in Table 1. PCR amplification was performed with GeneAmp PCR system-9700 (Perkin-Elmer, Norwalk, CT): denaturing at 94°C followed by a 35-cycle amplification (94°C for 30 seconds, the appropriate annealing temperature for 30 seconds, and 72°C for 30 seconds) and 7 minutes extension at 72°C. The reaction mixture contained 50 ng of genomic DNA or 1 μl of cDNA, 0.8 μM of each primer, 100 μM of each dNTP, 1×PCR buffer, and 0.25 units of Taq polymerase in a final volume of 5 μl. Following the

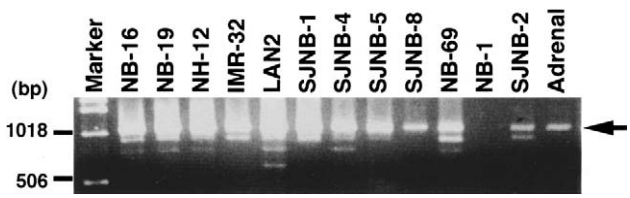

Figure 2. *Expression of the DFF45 gene in NB cell lines. The arrow indicates the predicted product of the DFF45 gene. The NB cell lines contained different-sized products in addition to the predicted product, whereas the adrenal gland revealed only the predicted product.*

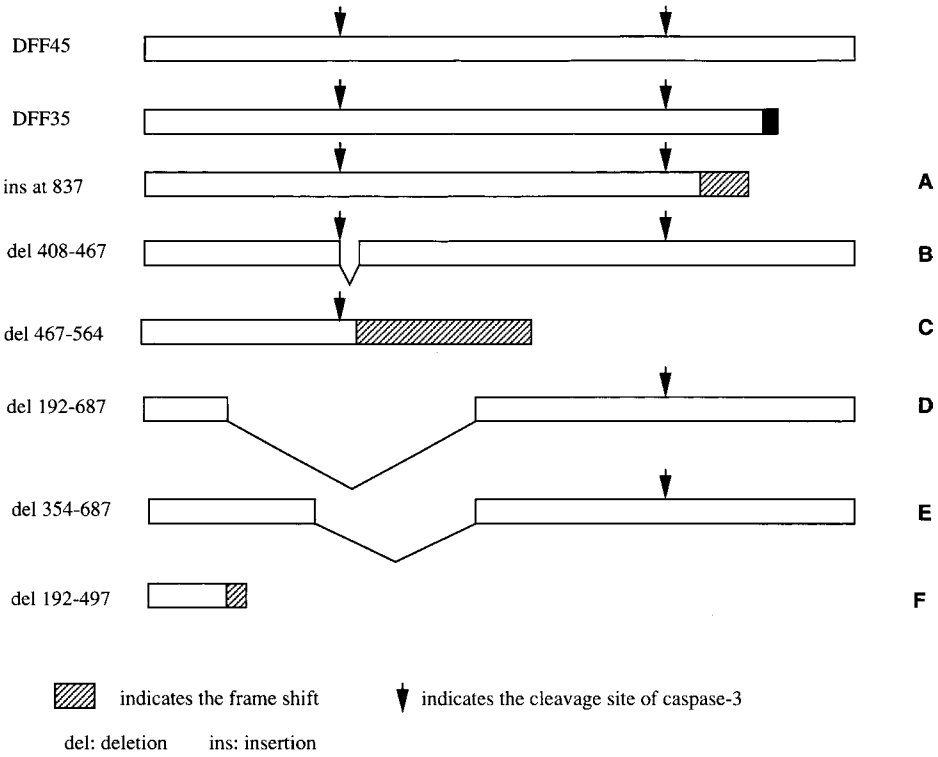

Figure 3. *Schematic presentation of the predicted protein of the variant products of the DFF45 gene in NB cell lines. The arrow indicates the caspase 3 cleavage site. The shaded boxes indicate the region without homology to DFF45 because of a frame shift after the deletion or insertion.*

amplification, 45 μl of a formamide denature dye mixture (95% formamide, 20 mM EDTA, 0.05% xylene cyanol, and 0.05% bromophenol blue) was added to the PCR mixture, then denatured at 80°C for 5 minutes. Two microliters of denatured PCR product was subjected to electrophoresis in a nondenaturing polyacrylamide gel with 5% glycerol at 40 W for 2–4 hours, keeping the temperature of the gel at 25°C. The gel was dried and exposed to X-ray film. If an abnormal band was identified, direct sequencing was carried out [26].

Southern Blotting

Ten micrograms of DNA was digested with *Bam*HI, electrophoresed through 1.0% agarose gel, transferred to charged nylon filters, hybridized, and exposed to X-ray film as described previously [14]. Two cDNA probes covering the coding region of the *DFF45* gene were used.

Results

The DFF45 Gene Is Homozygously Deleted in the NB Cell Line NB-1

Using 60 sets of primers around D1S244, we performed genomic PCR analysis in 20 NB cell lines. All NB cell lines except NB-1 revealed the same sized product as normal placenta DNA (Sigma, Japan). In the NB-1 cell line, we could not obtain the PCR product using an EST marker (T89469) derived from the *DFF45* gene, suggesting that NB-1 had a homozygous deletion of the *DFF45* gene (Figure 1). This result was confirmed by Southern blotting with two cDNA probes that covered the coding region of *DFF45*. The further study confirmed that the homozygous deletion flanked 480 kb [16]. Similar result was also reported by other researchers [27]. Neither rearrangements nor homozygous deletions were found in any other NB cell line. The NB-1 cell line revealed no *DFF45* band although it revealed the germline band of the *p73* gene in the same filter in our previous study [14].

Variant Splicing of the DFF45 Gene in NB Cell Lines

We performed expression analysis on NB cell lines and normal tissue by RT-PCR using primers covering the coding region of the *DFF45* gene. In addition to the predicted sized transcript (wild-type *DFF45* transcript), we found several different-sized transcripts in NB cell lines, but not in normal controls (peripheral lymphocytes and adrenal gland) (Figure 2). *DFF35*, one of the transcripts of the *DFF45* gene, was not detectable with the primers we used because the 3′ UTR of *DFF45* and *DFF35* are different due to their splicing. Thus, all the transcripts except the wild type of *DFF45* were found to be new variant transcripts of the *DFF45* gene. The RT-PCR products from each NB cell line were subcloned and followed by sequencing directly. Their predicted proteins are shown in Figure 3.

Mutational Analysis of the DFF45 Gene

The RT-PCR–SSCP analysis of the *DFF45* gene revealed no abnormal bands and the directly sequencing

did not show any nucleotide changes in 20 NB cell lines, suggesting that *DFF45* gene was not mutated in these 20 NB cell lines.

Discussion

One commonly deleted region in NB was located at chromosome band 1p36 between D1S244 and D1S243 [4,5]. To search for the homozygously deleted region, high-density PCR screening was performed using BAC-based STS [15]. We found that the *DFF45* gene that mapped to this region was homozygously deleted in one of 20 NB cell lines, NB-1. Varieties of variant transcripts of *DFF45* gene were found in most of the NB cell lines, but not in the normal adrenal gland or normal peripheral blood. No mutations were found in any NB cell line in this study.

DFF45/ICAD, a chaperon of DFF40/CAD, dimerizes to DFF40/CAD and inhibits the activity of DFF40/CAD. However, their dimerization is necessary to activate DFF40/CAD through protein unfolding [19–21] (Figure 3). Normally, the *DFF45/ICAD* gene encodes two kinds of protein, DFF45/ICAD-L and DFF35/ICAD-S, due to alternative splicing [19,20,28]. It has been confirmed that an intact DFF45 is needed to function as chaperon and to assist in the synthesis of active DFF40. The amino acid residues 101–180 of DFF35/45 mediate its binding to DFF40. The residues 23–100, which are homologous between DFF35/45 and DFF40, may function to inhibit the activity of DFF40 [22]. The balance between DFF35 and DFF45 may strictly control the cell death machinery [22]. An imbalance in the expression of DFF45, DFF35, and DFF40 could otherwise have a catastrophic effect on the cell by disrupting chromatin condensation and degradation of DNA [20–22]. A recent study suggested that DFF40 was not a tumor suppressor gene for NB [29]. In the present study, we found that the *DFF45* gene was homozygously deleted in the cell line NB-1. Moreover, *DFF45* physically mapped to 1p36.2 [16], a candidate region harboring tumor suppressor genes for NB. It is suggested that the *DFF45* gene is a candidate tumor suppressor gene for NB.

To examine the *DFF45* gene further in NB, expression and mutation analyses were carried out. We found six variant transcripts (A–F) of *DFF45* in NB cell lines (Figure 3). The variant A had an insertion resulting in a truncated protein that contained the amino acid residues 23–100 functioning to bind and inhibit the activity of DFF40. Thus, variant A may function as an inhibitor, not a chaperon, of DFF40. Variants D, E, and F may not have an effect on DFF40 according to a recent study [22]. For the variants B and C, it is difficult to predict whether they can inhibit DFF40 or not. At this point, the variant transcripts, including variant A, are assumed to interfere with the balance between DFF35/45 and DFF40. Overinhibition of DFF40 may result in disruption of apoptosis through DNA fragmentation in some NB cell lines. Two types of apoptotic cell death, one with and one without DNA fragmentation, were reported in NB [30]. Therefore, there might be an abnormality in the pathway of apoptosis through DNA fragmentation in some forms of NBs. Further studies on the function of DFF45 and its variants in NB are needed.

In conclusion, the *DFF45* gene was homozygously deleted at 1p36.2 in one NB cell line, and variant transcripts were expressed in NB cell lines. Although the *DFF45* gene may not be a tumor suppressor gene for NB, homozygous deletion of *DFF45* in the NB-1 cell line suggested that unknown tumor suppressor gene(s) in NB is located in the vicinity of the *DFF45* gene.

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