Differential Expression of NELL2 and A Splice Variant of Fzd6 in Differentiated BE(2)-C Neuroblastoma Cells

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Abstract:

Human neuroblastoma, the most common extracranial solid cancer in early childhood, arises in neural crest derived elements. Both tumors and cell lines established from neuroblastoma show heterogeneous features. Three cell types could be identified based on their morphological appearance and biochemical properties: N-type neuroblastic cells, I-type stem cells, and S-type Schwann cells. The cause of neuroblastoma is not clear, though it is suggested to be the accidental cell growth that occurs during normal development. Fzd6 encodes the receptor for extracellular signaling molecules Wnt, and it is suggested to be a negative regulator of canonical Wnt signaling. NELL2 encodes a secreted glycoprotein that contains six EGF repeats, and may be active in a neuron-specific signaling pathway. The highly regulated spatial and temporal expression of NELL2 is closely related to the neuronal differentiation. In this study we investigated the mRNA expression of Fzd6 and neural epidermal growth factor-like like 2 (NELL2) in differentiated BE(2)-C neuroblastoma cells. Lower expression of NELL2 was found in BUdR treated cells; a splice variant of Fzd6 was detected, and the expression of the two Fzd6 isoforms was investigated. The large isoform was predominatly expressed in the three samples and no significant change in the ratio of large/small isforms was found in

the three samples. These results indicate that NELL2 may be involved in neuroblastoma signaling and differentiation. Fzd6 may not be a critical regulator of neuroblastoma cell differentiation, although the function of small isoform is unknown yet.

Key words: neuroblastoma, Fzd6, NELL2

Introduction:

Neuroblastoma, the most common extracranial tumor of childhood, is precursor of sympathetic ganglia derived from malignant tumors originating from neural crest (1). It accounts for approximately 10% of all pediatric cancers (2), and patients less than 1 year old or with lower stage usually have better outcome than older patients or those with advanced stage diseases (3).

Cellular heterogeneity is the hallmark of both tumors and derived cell lines (6). Histopathological examinations have identified three major cell types: N- (neuroblastic), S- (Schwann), and I-type (intermediate) neuroblastoma cells (6). The most common cells are N-type cells, which have small and rounded cell bodies with neuritic processes. They attach poorly to the substrate and usually form aggregates in culture. S-type cells, which resemble nonneuronal precursor cells, have large and flattened cell bodies. They display a glial-like morphology, and show contact inhibition of cell growth. I-type cells have an intermediate morphology between N- and S-type cells, and are suggested to be the neuroblastoma stem cells (4, 5). These cells can be differentiated into N-type cells with Retinoic Acid (RA) treatment and into S-type cells with 5-bromo-2′-deoxyuridine (BUdR) treatment (5).

Wnt signaling plays a critical role in the control of cellular proliferation, differentiation, and apoptosis (7, 8), and it has been implicated in carcinogenesis (9). The Frizzled gene family encode for the seven-transmembrane receptors for Wnts, the extracellular signaling glycoproteins (10). The varied interactions between Wnts and Fzds could activate distinct downstream pathways, which can be summarized into the canonical and the non-canonical Wnt signaling cascades that exhibit antagonistic interactions (20, 21).

In the canonical pathway, the binding of Wnts with Fzd receptors activates the Dishevelled-1 (Dvl-1) proteins, which lead to the stabilization of β-catenin. The Stabilized β-catenin then translocates into the nucleus where it forms a transcription complex with lymphoid enhancer factor-1 (LEF)/T-cell factor (TCF) transcription factors. The target genes activated by the transcription complex include oncogenes such as c-MYC and cyclin D1, which trigger cell proliferation, oncogenic transformation, and inhibition of apoptosis (11-14). Fzd6 protein has been suggested as a negative regulator of the canonical Wnt signaling cascade. It could activate an antagonistic cascade that interfere with the binding of TCF·β-catenin transcription complex to DNA, thereby inhibiting the transcription of Wnt target genes (15). In neuroblastoma, the disturbance of Fzd6, a tumor suppressor gene, might be critical for tumorigenesis, and it may play a role in the differentiation of I-type neuroblastoma cells.

Neural epidermal growth factor-like like 2 (NELL2) is a secreted glycoprotein that contains six EGF-like repeats (16). It was found to be highly expressed in neuroblastoma cells and little in glioblastoma cells (18). Studies of it have shown that expression of NELL2 is highly regulated spatially and temporally, with its predominant expression in

neuronal cell lineage (17, 18). Like other EGF-like proteins such as Delta and Jagged, Nell2 proteins are suggested to function as ligands of the Notch signaling (17). Notch signaling controls cell fate including stem cell renewal and differentiation, and its deregulation is closely related to cancer (19). These results suggest that Nell2 proteins may act as important signal molecules in neuroblastoma cells.

In this study, we investigated the expression of Fzd6 and NELL2 in three phenotypically different neuroblastoma cells (Table 1). Additionally, differential expression between two Fzd6 isoforms was also examined by RT-PCR amplification. The results may contribute to future studies on the cause of tumorigenicity of neuroblastoma.

Table 1. Neuroblastoma cell samples studied in this project.

Cells	Chemical treatment	Phenotype
BE(2)-C	—	I
BE(2)-C	RA	N
BE(2)-C	BUdR	S

Materials and methods

Cell samples

Untreated and RA or BUdR treated BE(2)-C cells were kindly provided by Barbara Spengler and Dr. Robert Ross. The BE(2)-C cells were cultured in a mixture of 45% Eagle's Minimum Essential Medium with non-essential amino acids, 45% Ham's Nutrient Mix F12 (Invitrogen, Carlsbad, CA) and 10% fetal bovine serum without antibiotics. The differentiated cells were maintained in the same culture as above except that RA or BUdR was added to make a final concentration of 10^{-8} M in the mixture.

Primers

Primers were designed based on the reported human NELL2 mRNA sequence (GenBank Accession No. NM_006159) to amplify a region spanning exons 5 through 9, with the forward primer in exon 5 and the reverse primer in exon 9. Two sets of primers for human Fzd6 mRNA sequence (GenBank Accession No. NM_003506) were designed. Fzd6 primer set 1 was designed to amplify a region spanning exons 4 and 5, and primer set 2 was designed to amplify a region spanning exons 4 and 6. As a loading control for this study, GAPDH was used. The primers for GAPDH were designed to produce a PCR amplified cDNA of 228bp. Information regarding the primers is provided in the Table 2.

Table 2. Primers used in RT-PCR.

Primer		Sequence (5' → 3')	Position*	Intron in between the primers	Predicted size from RT-PCR product
FZD6	Forward1	GCTACTTTGTACTCTTGCCAC	Exon4	2.8kb	386bp
	Reverse1	CTTCCAACCCAGAAGACAGC	Exon5		
FZD6	Forward2	TGACCTGGATGCTTCTCG	Exon4	4.0kb	488bp
	Reverse2	CCTGTAGTACTCTTCGAC	Exon6		
NELL2	Forward	TGATGACAAGTGGCACAAGC	Exon5	3.4kb	362bp
	Reverse	TACAGCCGTCTATCCAGGAC	Exon9		
GAPDH	Forward	AACGGATTTGGTCGTATTGG	Exon2,3	1.9kb	228bp
	Reverse	TTTGGAGGGATCTCGCTCCT	Exon4,5		

*FZD6 mRNA, GenBank Accession No. NM_003506; NELL2 mRNA GenBank Accession No. NM_006159; GAPDH mRNA GenBank Accession No. NM_002046.

Total RNA Extraction

Total RNA from the three neuroblastoma cell samples was isolated using RNeasy Plus Mini Kit following manufacturer's protocol with minor modifications. The pelleted cells were disrupted by adding 600 µl of Buffer RLT Plus and vortexed to obtain a homogenous lysate. The homogenized

lysate was transferred to a gDNA Eliminator spin column placed in a 2 ml collection tube, and was centrifuged for 30s at 13,000 rpm. The column was discarded, and the flow-through was saved. 600ul of 70% ethanol was added to the flow-through and mixed by pipetting. One aliquot of the sample was transferred to an RNeasy spin column placed in a 2 ml collection tube, and was centrifuged for 30s at 13,000 rpm. The flow-through was discarded. The remained aliquot of smaple was transferred to the same RNeasy column and centrifuged for 30s at 13,000 rpm. The flow-through was discarded. 700μl of Buffer RW1 was added to the RNeasy spin column and centrifuged at 13,000 rpm for 30 s. 500μl of Buffer RPE was then added and centrifuged at 13,000 rpm for 30s, and this step was repeated but centrifuged for 2min. The RNeasy spin column was placed in a new 2ml collection tube and centrifuged at 13,000 rpm for 1min. Then the spin column was placed in a new 1.5ml collection tube, and was eluted with 30μl deionized water. Extracted RNA was stored at -80℃.

RT-PCR

RT-PCR was performed on the total RNA samples prepared from the three neuroblastoma cell samples using the Qiagen OneStep RT-PCR kit. Twenty nanograms of RNA was amplified in 25μl RT-PCRs (5μl 5×RT buffer, 1μl 10mM dNTPs, 0.5μl 10pmol/μl forward primer, 0.5μl 10pmol/μl reverse primer, 1μl enzyme mix, 4μl 5ng/μl RNA and 13μl ddH$_2$O). One-step RT-PCR was performed according to the following protocol: one cycle of 50℃ for 30min and 95℃ for 15min, 94℃ for 30s, 56℃ for 30s, and 72℃ for 30s, and a final extension of 72℃ for 10min followed by a final hold at 4°C. For the Fzd6 primers, amplification was performed for 33, 35 and 37 cycles. For the NELL2 primers, amplification was performed for 35, 37 and 38 cycles. To control for the amount of RNA present in the samples, RT-PCR amplification of GAPDH was performed on all RNA samples for 28 cycles. 5μl of loading dye was added to each RT-PCR product. 8μl of each product was then added to a 1.2% agarose gel, and electrophoresis was performed at 130V. Band intensities were quantified using SigmaGel Scanner.

Sequencing

Purified DNA from RT-PCR product or agarose gel was diluted and quantified using spectrophotometer. 80ng of the purified DNA was mixed with 4ul of 10 pmol/ul primer and ddH$_2$O to 12ul. The samples were then sent out for sequencing to Genewiz.
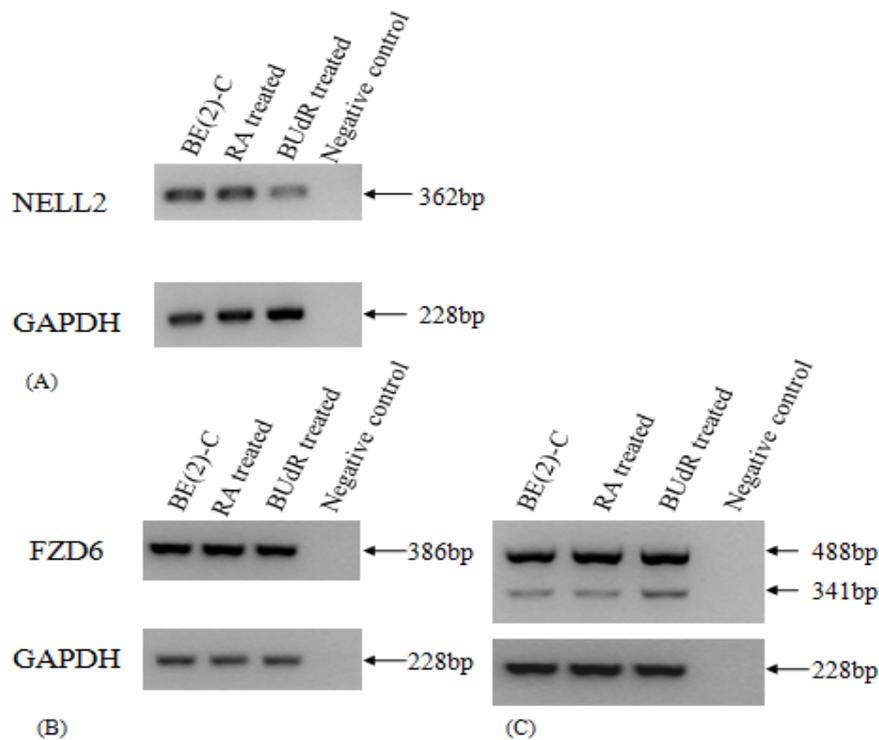
Results

Figure 1. Expression of NELL2 and Fzd6 in differentiated BE(2)-C neuroblastoma cells. (A) RT-PCR examining NELL2 was performed on RNA isolated from untreated, RA treated and BUdR treated BE(2)-C cells. Primers are located in exon5 and exon9. (B) RT-PCR using Fzd6 primer set 1 was performed on RNA isolated from untreated, RA treated and BUdR treated BE(2)-C cells. Primers are located in exon4 and exon5. (C) RT-PCR of Fzd6 using primer set 2. Primers are located in exon4 and exon6. All the amplified products were fractionated on a 1.2% agarose gel. RT-PCR amplification of GAPDH mRNA was used to monitor the amount of RNA present in the samples.

To study the expression of NELL2 and Fzd6 in I-, N-, and S-type cells, RT-PCR of mRNA isolated from these three samples was performed, using specific primers for each gene. RT-PCR amplification of GAPDH was used to monitor the amount of RNA in samples. PCR products were then analyzed on 1.2% agarose gel (Figure 1). NELL2 mRNA was detected in all three samples. BUdR treated cells showed the lowest expression level of NELL2 with relatively higher level of GAPDH, indicating a higher initial amount of total RNA. The difference between untreated and RA treated cells was slight. The differential expression of Fzd6 was examined using two sets of primers. Fzd6 expression was relatively uniform across the samples regardless of which primer pair was used. Notably, by using primer set2 (in Fzd6 exon4 and exon6) a smaller PCR product was amplified in all three samples, which was unexpected. In order to confirm the sequence identity, PCR products were purified and sequenced by Genewiz.

A BLAST search using the sequencing result of 362 bases was performed against the NCBI database. 100 % homology between 180 bases of PCR-product and NELL2 mRNA (NM_006159) confirmed that the expected region of NELL2 was amplified in the PCR reactions (Figure 2-A). The BLAST comparison of the 386bp sequence derived from Fzd6 primer set 1 and 488bp sequence from Fzd6 primer set2 showed 100% homology to the Fzd6 mRNA (NM_003506). (Figure 2-B, C).

The BLAST comparison of the 341bp sequence derived from Fzd6 primer set 2 revealed homology to two separated parts of the Fzd6 mRNA, with 149bp unaligned in between the two parts of Fzd6 mRNA (NM_003506) (Figure 2-D).

This result indicates that the 341bp fragment is the RT-PCR product of a splice variant of Fzd6. Sequence comparison of 341bp sequence with Fzd6 mRNA confirms that the splice variant is due to the removal of exon 5. The excision of exon 5 results in the coding frameshift. If translated, the small Fzd6 splice variant may encode a truncated protein without the seventh transmembrane domain and the C-terminal function region (Figure 3).

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PCR product    1      CTCCACAGACTTGCCTCTAGGCACAACATTTTGGCTAGGACAGAGAAATAATGCGCATGG    60
                      ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
NELL2 mRNA    633     CTCCACAGACTTGCCTCTAGGCACAACATTTTGGCTAGGACAGAGAAATAATGCGCATGG    692

PCR product   61      ATATTTTAAGGGTATAATGCAAGATGTCCAATTACTTGTCATGCCCCAGGGATTTATTGC    120
                      ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
NELL2 mRNA    693     ATATTTTAAGGGTATAATGCAAGATGTCCAATTACTTGTCATGCCCCAGGGATTTATTGC    752

PCR product   121     TCAGTGCCCAGATCTTAATCGCACCTGTCCAACTTGCAATGACTTCCATGGACTTGTGCA    180
                      ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
NELL2 mRNA    753     TCAGTGCCCAGATCTTAATCGCACCTGTCCAACTTGCAATGACTTCCATGGACTTGTGCA    812

   (A)

PCR product    1      TCGGATGTTACGTCTATGAGCAAGTGAACAGGATTACCTGGGAGATAACTTGGGTCTCTG    60
                      ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Fzd6 mRNA    1571     TCGGATGTTACGTCTATGAGCAAGTGAACAGGATTACCTGGGAGATAACTTGGGTCTCTG    1630

PCR product   61      ATCATTGTCGTCAGTACCATATCCCATGTCCTTATCAGGCAAAAGCAAAAGCTCGACCAG    120
                      ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Fzd6 mRNA    1631     ATCATTGTCGTCAGTACCATATCCCATGTCCTTATCAGGCAAAAGCAAAAGCTCGACCAG    1690

PCR product   121     AATTGGCTTTATTTATGATAAAATACCTGATGACATTAATTGTTGGCATCTCTGCTGTCT    180
                      ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Fzd6 mRNA    1691     AATTGGCTTTATTTATGATAAAATACCTGATGACATTAATTGTTGGCATCTCTGCTGTCT    1750

   (B)

Upper band     1      ACCATATCCCATGTCCTTATCAGGCAAAAGCAAAAGCTCGACCAGAATTGGCTTTATTTA    60
                      ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
FZD6 mRNA    1646     ACCATATCCCATGTCCTTATCAGGCAAAAGCAAAAGCTCGACCAGAATTGGCTTTATTTA    1705

Upper band    61      TGATAAAATACCTGATGACATTAATTGTTGGCATCTCTGCTGTCTTCTGGGTTGGAAGCA    120
                      ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
FZD6 mRNA    1706     TGATAAAATACCTGATGACATTAATTGTTGGCATCTCTGCTGTCTTCTGGGTTGGAAGCA    1765

Upper band    121     AAAAGACATGCACAGAATGGGCTGGGTTTTTTAAACGAAATCGCAAGAGAGATCCAATCA    180
                      ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
FZD6 mRNA    1766     AAAAGACATGCACAGAATGGGCTGGGTTTTTTAAACGAAATCGCAAGAGAGATCCAATCA    1825

   (C)

Lower band     1      CTAAAGAAATTTATGATTCGAATTGGAGTCTTCAGCGGCTTGTATCTTGTGCCATTAGTG    60
                      ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
FZD6 mRNA    1504     CTAAAGAAATTTATGATTCGAATTGGAGTCTTCAGCGGCTTGTATCTTGTGCCATTAGTG    1563

Lower band    61      ACACTTCTCGGATGTTACGTCTATGAGCAAGTGAACAGGATTACCTGGGAGATAAACTTG    120
                      |||||||||||||||||||||||||||||||||||||||||||||||||||||||| ||||
FZD6 mRNA    1564     ACACTTCTCGGATGTTACGTCTATGAGCAAGTGAACAGGATTACCTGGGAGATAA-CTTG    1622

Lower band    121     GGTCTCTGATCATTGTCGTCAGTACCATATCCCATGTCCTTATCAG    166
                      ||||||||||||||||||||||||||||||||||||||||||||||
FZD6 mRNA    1623     GGTCTCTGATCATTGTCGTCAGTACCATATCCCATGTCCTTATCAG    1668


Lower band    167     TCCAATCAGTGAAAGTCGAAGAGTACTACAGG    198
                      ||||||||||||||||||||||||||||||||
FZD6 mRNA    1818     TCCAATCAGTGAAAGTCGAAGAGTACTACAGG    1849

   (D)
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**Figure 2.** Alignments of PCR products with reported sequences in NCBI.

(A) Alignment of PCR product using NELL2 primers with a previously reported sequence (NM_006159). (B) Alignment of PCR product using Fzd6 primer set1 with a previously reported sequence (NM_003506). (C) Alignment of large PCR product using Fzd6 primer set 2 with a previously reported sequence (NM_003506). (D) RT-PCR, using Fzd6 primer set 2 identifies a splice variant of FZD6 mRNA. Alignment of small PCR product using Fzd6 primer set 2 with a previously reported sequence (NM_003506).

(A)
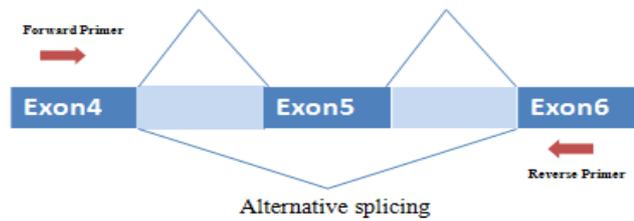
Alleles

```
        I   P   C   P   Y   Q   A   K   A   K   A   R   P   E   L   A   L   F   M   I   K   Y   L
Large   ATCCCATGTCCTTATCAGGCAAAAGCAAAAGCTCGACCAGAATTGGCTTTATTTATGATAAAATACCTG

Small   ATCCCATGTCCTTATCAG----------------------------------------------------
        I   P   C   P   Y   Q
```
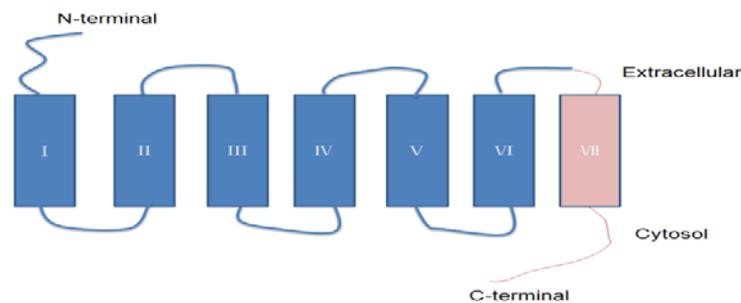
```
        M   T   L   I   V   G   I   S   A   V   F   W   V   G   S   K   K   T   C   T   E   W   A
Large   ATGACATTAATTGTTGGCATCTCTGCTGTCTTCTGGGTTGGAAGCAAAAAGACATGCACAGAATGGGCT

Small   ---------------------------------------------------------------------
```

```
        G   F   F   K   R   N   R   K   R   D   P   I   S   E   S   R   R   V
Large   GGGTTTTTTAAACGAAATCGCAAGAGAGATCCAATCAGTGAAAGTCGAAGAGTA

Small   ---------------------------TCCAATCAGTGAAAGTCGAAGAGTA
                                    S   N   Q   *
```

(B)

(C)

Figure 3. Excision of exon5 in the splice variant. (A) Splicing of the Fzd6 transcript. The large Fzd6 isoform is due to removal of introns 4 and 5 and in retention of exon5. The small Fzd6 isoform comes from the removal of introns 4 and 5 and exon 5. The primers are indicated as arrows. (B) Alignment of large and small Fzd6 cDNA and amino acid sequences. An alignment of a portion of exons 4-6 of large Fzd6 cDNA with that of small Fzd6 cDNA, demonstrating that the exclusion of exon5 results in a frameshift. The seventh transmembrane domain of Fzd6 protein is indicated in the large allele in red color. (C) A cartoon showing the effect of alternative splicing on protein level. The seventh transmembrane domain and the C-terminal are removed.

Discussion

The detected expression of NELL2 in BE(2)-C neuroblastoma cell line and its differentiated cells confirms the previous report that NELL2 is highly expressed in neuroblastoma cells (18). Additionally, it has been proposed that the spatial and temporal expression of NELL2 is highly regulated, with its predominant expression in neuronal cell lineage (17, 18). Not surprisingly, BUdR treated cells showed the lowest level of NELL2 expression in that BUdR could induce I-type cells to differentiate into Schwann cells. The expression level of NELL2 in I-type BE(2)-C cells and RA treated cells was about the same, suggesting its role in stem cell renewal and differentiation and neuroblastoma cells signaling. However, it is still not clear that whether the secreted NELL2 proteins function in the same way as other EGF-containing Notch ligands including Delta and Jagged or it could activate other signaling pathways.

There appears to be no expression difference of large Fzd6 isoform using two primer sets in three cell samples. According to Tamar Golan et al. (2004), Fzd6 works as a negative regulator of canonical Wnt signaling, and transmits an antagonistic signaling cascade. The present study indicates that Fzd6 might not be a critical player in neuroblastoma cell differentiation and progression of neuroblastoma. However, the possibility that the deregulation of Fzd6 may contribute to the tumorigenicity cannot be excluded. Moreover, the Fzd6 protein expression studies would facilitate the interpretation of Fzd6 in neuroblastoma.

By using primer set 2 for Fzd6, the differential expression of the two Fzd6 isoforms in all three samples were also examined, and no significant difference was found (date not shown). A BLAST search using the small Fzd6 sequence against NCBI nucleotide collection database was performed, and the sequence matches a previously clone cDNA isolated from human brain (BC036106, data not shown). No other reports on this Fzd6 splice variant were found. Whether this Fzd6 isoform is tissue specific is not clear. Neither is its function. Whether the alternative splicing form of Fzd6 could be translated to protein requires further studies. Notably, if translated, the protein will be truncated in its seventh transmembrane domain and the C-terminal putative

function region. In what way can such a truncated protein function is unclear. One possibility is that this protein would still be transported into membrane, and would only bind to ligands but cannot activate downstream signaling. In this way, it can be described as a competitive negative regulator of the Wnt signaling. Another possibility is that it would be a soluble intracellular protein that functions in some signaling pathway. It has been reported that a splicing variant of Frizzled-4 encodes a soluble protein, which functions as a regulator of the Wnt Signaling Pathway (22). Future studies on other tissue samples and research using such methods as Western blot, immunohistochemistry and functional assay would be necessary to determine the role of the splice variant of Fzd6.

Acknowledgements:

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