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LABORATORY INVESTIGATION - HUMAN/ANIMAL TISSUE

Expression profile of frizzled receptors in human medulloblastomas

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Abstract Secreted WNT proteins signal through ten receptors of the frizzled (FZD) family. Because of the relevance of the WNT/β-catenin (CTNNB1) signaling pathway in medulloblastomas (MBs), we investigated the expression of all ten members of the *FZD* gene family (FZD1-10) in 17 human MBs, four MB cell lines and in normal human cerebellum, using real-time PCR. We found that *FZD2* transcript was over-expressed in all MBs and MB cell lines. Western blot analysis confirmed the expression of FZD2 at the protein level. Moreover, the levels of *FZD2* transcript were found to correlate with those of *ASPM* transcript, a marker of mitosis essential for mitotic spindle function. Accordingly, *ASPM* mRNA was expressed at a very low level in the adult, post-mitotic, human cerebellum, at higher levels in fetal cerebellum and at highest levels in MB tissues and cell lines. Unlike FZD2, the other FZDs were overexpressed (e.g., *FZD1*, *FZD3* and *FZD8*) or underexpressed (e.g., *FZD7*, *FZD9* and *FZD10*) in a case-restricted manner. Interestingly, we did not find any nuclear immuno-reactivity to CTNNB1 in four MBs over-expressing both FZD2 and other FZD receptors, confirming the lack of nuclear CTNNB1 staining in the presence of increased FZD expression, as in other tumor types. Overall, our results indicate that altered expression of FZD2 might be associated with a proliferative status, thus playing a role in the biology of human MBs, and possibly of cerebellar progenitors from which these malignancies arise.

Keywords ASPM · β-catenin · CTNNB1 · Frizzled · FZD2 · Medulloblastoma

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Introduction

WNT signaling pathways have been implicated in several biological processes, including cell proliferation, determination of cell fate, and generation of cell polarity [1, 2]. In humans, there are 19 identified WNT-secreted glycoproteins (i.e., WNT1, 2, 2B/13, 3, 3A, 4, 5A, 5B, 6, 7A, 7B, 8A, 8B, 9A/14, 9B/15, 10A, 10B/12, 11, and 16) that function as ligands to govern these processes. These WNT ligands signal through ten receptors of the frizzled (FZD) family, FZD1-10, and two co-receptors, LRP5 and LRP6. FZD receptors transduce the WNT signals via at least three intracellular pathways, that are distinct as 'canonical' and 'non-canonical'.

The canonical WNT pathway is termed WNT/CTNNB1 pathway, as its central molecule, the transcription factor β-catenin (CTNNB1), primarily regulates cell proliferation and fate. The activation of the canonical WNT pathway causes inhibition of the glycogen synthase kinase-3β (GSK-3β) via dishevelled (DVL) protein family members, thus leading the down-stream effector β-catenin to translocate into the nucleus and up-regulate specific target genes, including *CCND1* (cyclin D1) and *MYC* [2].

The non-canonical WNT pathways include the WNT/PCP (planar cell polarity) pathway and the WNT/Ca^{2+} pathway. The major function of the WNT/PCP pathway is the regulation of the cytoskeletal organization by activating Rac and Rho small GTPases and JNK (Jun N-terminal kinase) via DVL. The WNT/Ca^{2+} pathway is a heterotrimeric G-protein-dependent signaling pathway, which leads to the increase of diacylglycerol (DAG) and intracytosolic calcium, via the activation of phospholipase C (PLC). Calcium-initiated effects include the activation of calmodulin-dependent protein kinase II (CAMKII) and other calcium-related molecules (e.g., calpain and calcineurin), while calcium and DAG together work to activate protein kinase C (PKC). The specificity of function of the different WNT proteins, including the ability to activate canonical or non-canonical WNT pathways, likely depends on differences in WNT ligands and FZD receptors, as well as on the type of the target cell, its differentiation stage, and the cellular context [2].

The inappropriate activation of the canonical WNT signaling pathway has been implicated in the formation of several cancers [3]. Among brain tumors, the aberrant activation of canonical WNT pathway characterizes a subset of human medulloblastomas (MBs), the most common malignant brain tumors of childhood [4, 5]. This activation is roughly identified by the presence of nuclear CTNNB1 immunoreactivity in clinical samples, and it seems to be due to mutations in the *CTNNB1* gene in 60–70% of cases and to mutations in other WNT/CTNNB1 pathway components (*APC*, *AXIN1* and *AXIN2*) in part of the remaining cases. Aberrant activation of non-canonical WNT pathways might also play a role in cancer, but it is unknown whether it contributes to the formation of human MBs.

FZD receptors are upstream effectors of the WNT signaling pathways, as previously stated. Of note, their over-expression has been considered as an additional molecular abnormality leading to the activation of the canonical WNT signaling pathway [6], or it might activate the non-canonical WNT signaling pathways, which also seem to be tumorigenic. Alternatively, however, FZD over-expression might only be a consequence, rather than a cause, of the activation of the WNT signaling pathways, just as the over-expression of the *PTCH1* receptor may be a consequence of the activation of the SHH pathway. Indeed, *PTCH1* gene is a direct target of the transcription factor *GLI1*, which is a down-stream effector of the SHH pathway.

Only scant observations are available on the expression of FZD receptors in human MBs and in human cerebellum, and there is no evidence on the contribution of individual FZD proteins to human MB pathology (and, partly, to cerebellar development). A distinct but redundant expression of the Fzd receptor genes has recently been shown in the developing mouse brain [7]. To shed light on this topic, we have carried out real-time PCR experiments to examine the expression profile of the ten known members of the *FZD* gene family in human MBs. In particular, the aim of our work was to investigate the expression of the transcripts of the FZD receptors in a series of human MBs and in four human MB cell lines using real-time PCR; in addition, the level of expression of the FZD2 protein was investigated by western blot. Finally, we investigated the expression profile of the *ASPM* gene, a potential marker of proliferation expressed at a very low level in human adult cerebellum, with the additional aim of confirming the presence of aberrantly proliferating cells in each tumor sample [8].

Materials and methods

Tissue samples

Seventeen MB specimens surgically resected from children ($n = 8$) and adults ($n = 9$) were obtained from the Neurosurgery Department of the IRCCS Foundation, "Carlo Besta" Neurological Institute, Milan, and from the Division of Pediatric Neurosurgery, Policlinico "A. Gemelli", Rome, Italy. Specimens were snap-frozen in liquid nitrogen at the time of surgery and kept at −80°C until use; or they were immediately put into 15-ml tubes containing NaCl 0.9%, and sent at room temperature to the laboratory, where they were received within 24 h after surgery. In this latter case, each sample was then divided into two parts: one was cryopreserved and kept at −80°C until use, the other was processed by mechanical dissociation in the attempt to establish MB cell lines. Histologic diagnoses were confirmed by pathologic review according to the WHO criteria [9].

Cell lines

The established human MB cell line D283 Med (D283) and D341 Med (D341), both from the American Type Culture Collection, were maintained in Dulbecco's modified Eagle's medium (DMEM)/F12 with Glutamax™ (Invitrogen, #31331-028) with 20% (vol/vol) fetal bovine serum.

In contrast, the only two stably growing MB cell lines obtained from patients' tumors in our laboratory (MB4 and MB16) were maintained in DMEM/F12 with B27 supplement without vitamin A (Invitrogen, #12587-010), FGF2 (20 ng/ml, PeproTech EC, #100-18B), 5% (vol/vol) BSA Fraction V (Invitrogen, #15260-037). The latter cell lines express the MB markers *NEUROG1* and *OTX2* [10] and grow in suspension, forming spheroids (MB4) and aggregates (MB16) (Fig. 1S).

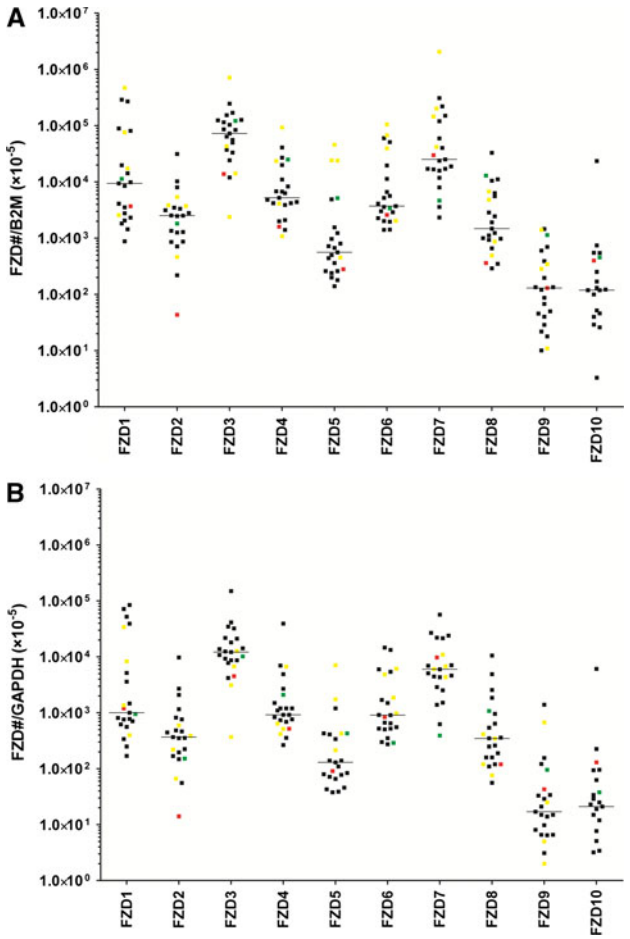

Fig. 1 Amount of FZD1-10 receptor transcripts in human cerebellum, and medulloblastoma tissues and cell lines. The levels of each *FZD* receptor mRNA are normalized by *B2M* (**a**) or *GAPDH* (**b**) in each tissue and cell line sample (log scale for the *y-axis*). The median is shown as a *line*. *Red dot* adult cerebellum; *green dot* fetal cerebellum; *yellow dots* cell lines. For instance, the amount of *FZD1* mRNA normalized by *B2M* (i.e. the ratio *FZD1*/*B2M*) in fetal cerebellum (*green dot*) is 11,300 × 10^{-5}, while it is 3,700 × 10^{-5} in adult cerebellum (*red dot*). The *dot plot* clearly shows that the median value for *FZD3* mRNA in medulloblastomas is the highest (72,700 × 10^{-5} and 12,200 × 10^{-5} after normalization with *B2M* and *GAPDH*, respectively), while the median value for *FZD10* mRNA is among the lowest ones (119 × 10^{-5} and 21 × 10^{-5} after normalization with *B2M* and *GAPDH*, respectively). Four data points corresponding to the amounts of FZD10 in the cell lines are outside the lower axis limit (that is 1.00), as they are equal to 0

RNA isolation and reverse transcription

RNA from human normal fetal cerebellum was purchased from BioChain Institute (http://www.biochain.com, #R1244040-50), while RNA from human normal adult cerebellum pooled from ten male/female Caucasians was purchased from Clontech (http://www.clontech.com, #636535). Total RNAs of the primary human MB tissues and cell lines were extracted using TRIzol® Reagent (Invitrogen, #15596-026), then treated with DNase I (Roche-Boehringer Mannheim) to remove any contaminating genomic DNA, and finally purified using RNeasy MinElute Cleanup columns (Qiagen, #74204). cDNA was synthesized from 1 μg of RNA in a volume of 20 μl, using the High-Capacity cDNA Reverse Transcription kit (Applied Biosystems, #4368814). For each sample, two independent reverse transcription reactions were carried out.

Quantitative real-time PCR analysis

Real-time PCR was performed on an ABI Prism 5700 Sequence detection system (Applied Biosystems) using the TaqMan technology (Applied Biosystems, #4304437). For each gene in a given sample, real-time PCR reactions were performed in duplicate from two independent cDNA preparations. Real-time PCR was also performed on non-retrotranscribed RNA samples, to exclude the possibility of positive results due to contaminating genomic DNA. This is of particular importance as seven FZD genes (i.e., FZD1, 2, 5, 7, 8, 9, and 10) are mono-exonic. The following human Assays-on-Demand (Applied Biosystems) were employed: Hs00259943_s1 (*FZD1*), Hs00361432_s1 (*FZD2*), Hs00184043_m1 (*FZD3*), Hs00201853_m1 (*FZD4*), Hs00258278_s1 (*FZD5*), Hs00171574_m1 (*FZD6*), Hs00275833_s1 (*FZD7*), Hs00259040_s1 (*FZD8*), Hs00268954_s1 (*FZD9*), Hs00273077_s1 (*FZD10*). Amplification was done essentially according to the manufacturer's instructions. Briefly, 20 μl of reaction mixture containing 45 ng of cDNA template were amplified as follows: incubation at 50°C for 2 min, denaturation at 95°C for 10 min, and 40 cycles at 95°C for 15 s and 60°C for 1 min. To obtain more robust data, we used two reference genes, *B2M* and *GAPDH*, to normalize the expression of each target gene (Assays-on-Demand Hs99999907_m1 for *B2M* and Hs99999905_m1 for *GAPDH*). Hence, for each sample, the level of expression of the target genes was normalized to both *B2M* and *GAPDH* mRNA contents, using the following formula: $2^{-\Delta C_t}$, where the ΔC_t = target gene C_t−reference gene C_t. Note that a difference of one in $\Delta C_{t\ values}$ corresponds to a twofold difference in the mRNA level. The relative expression of the target gene in each individual samples was then normalized

to its level of expression in the normal adult cerebellum. In addition, the *EEF1B2* gene (Assay-on-Demand Hs00253438_m1), which has been reported to be evenly expressed in MBs [11], was used as an endogenous control gene in four MB samples (#4, #7, #10, #17) and in the normal fetal and adult cerebella. A standard curve using serial dilutions of two different samples (#5 and #12) was carried out to assess the efficiency of the three reference genes (*B2M*, *EEF1B2*, and *GAPDH*). We used *ASPM* gene expression level as an index of cell proliferation to further confirm the presence of tumor cDNA in each sample (Assay-on-Demand Hs00411505_m1), and *MYC* gene expression level as a marker of WNT/CTNNB1 pathway activation (Assay-on-demand Hs00153408_m1). Results were expressed as the relative amount of the target gene to the reference gene (that is *B2M* or *GAPDH*) in each sample, and as the relative amount of the target gene in the sample compared with that of the calibrator sample, that is the adult human cerebellum.

Western blot analysis for FZD2

Protein extraction was performed with TRIzol® Reagent (Invitrogen, #15596-026), according to the manufacturer's protocol, but protein precipitation was carried out using acetone (and not isopropyl alcohol). Precipitated proteins were resuspended in a resuspension buffer containing Triton X-100, SDS, leupeptin, PMFS and pepstatin. Protein concentration was determined by the Micro BCA Protein Assay Kit (Thermo Scientific Pierce, #23235) using BSA as standard. Protein extracts were separated on NuPAGE Novex (4–12%) Bis–Tris Mini Gels (Invitrogen, #NP0336BOX) and blotted onto PVDF membranes by electroblotting. The membranes were blocked with 5% nonfat dry milk in Tris-buffered saline containing 0.1% Tween 20, and then probed with the primary rabbit anti-FZD2 antibody (1:125 in blocking solution; Zymed Laboratories; Invitrogen Immunodetection, #38-4700) for 2 h at room temperature. The secondary antibody (HRP-conjugated goat anti-rabbit IgG, #NA934VS; GE Healthcare/Amersham) was applied for 1 h at room temperature. Finally, the membrane was treated with SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific Pierce, #34080) and exposed to an Amersham Hyperfilm™ MP (GE Healthcare, #28906844). Specificity of the primary anti-FZD2 antibody was roughly tested using the KNRK nuclear extract (Santa Cruz, #sc-2141). Transfer blotting and protein amounts/loading were verified by Red Ponceau staining and α-tubulin detection.

Immunohistochemistry for CTNNB1

Immunohistochemistry for *CTNNB1* was carried out as previously described [10], using the purified mouse anti-β-catenin antibody (1:50, BD Transduction Laboratories™, #610153). Immunohistochemistry for *FZD2* (Sigma-Aldrich, rabbit anti-FZD2, #F3304) and *FZD3* (Sigma-Aldrich, rabbit anti-FZD3, #F3179) was performed, but did not yield satisfactory results, likely because the antibodies were not suitable for Carnoy-fixed, paraffin-embedded tissues.

MYC amplification analysis

The analysis of *MYC* amplification was assessed by Multiplex Ligation-dependent Probe Amplification (MLPA) in all MBs and MB cell lines overexpressing *MYC* and *FZD1* using SALSA MLPA kit P171 Gain-1 (MRC-Holland).

Statistical analyses

Statistical analyses were performed using GraphPad Prism 5.00 for Windows (GraphPad Software, San Diego, CA, USA, http://www.graphpad.com). Unsupervised hierarchical clustering analysis was done using the fold change in the expression levels of the ten *FZD* genes indicated in Table 1 and using the Euclidean distance metric as a measure of distance between genes/samples. The analysis was performed using R and Bioconductor (http://www.bioconductor.org).

Methodological remarks

The difference in threshold cycles (ΔC_t) between two ideal reference genes should be similar in all samples and the relative expression level of an ideal reference gene, normalized by another ideal reference gene, in two samples should be equal to or in the vicinity of one. Here, we have found that, although the results are generally comparable, the ΔC_t between these two common reference genes used for normalization in real-time PCR experiments, i.e. *B2M* and *GAPDH*, varies from sample to sample; indeed, the average ΔC_t was 2.6 with a standard deviation (SD) of 0.9. This variability cannot be due to different amplification efficiencies of the Assays-on-Demand, as found by the comparison of the standard curves. Of note, we observed even more variability using the *EEF1B2* gene [11], which was not considered for further analyses [i.e., the average differences in threshold cycles (ΔC_t) between *EEF1B2* and *B2M*, and between *EEF1B2* and *GAPDH* were 3.1 ± 1.91 SD, and 5.8 ± 2.06 SD]. As a consequence, we decided to consider each FZD receptor gene to be over- or under-expressed if its expression level was >3-fold higher or lower than that in human adult cerebellum, normalizing both by *B2M* and by *GAPDH* [12].

Table 1 *FZD* mRNA expression in human MBs

#[b]	Type[c]	Age	*N*-fold expression variation[a]									
			FZD1		*FZD2*		*FZD3*		*FZD4*		*FZD5*	
			B2M	*GAPDH*	*B2M*	*GAPDH*	*B2M*	*GAPDH*	*B2M*	*GAPDH*	*B2M*	*GAPDH*
FCrb	N/A	33 weeks	3.05	−1.28	*41.93*	*10.78*	8.82	2.27	*15.56*	*4*	*18.38*	*4.72*
1[d]	D	A	*23.92*	*70.03*	*235.57*	*689.78*	*11.16*	*32.67*	*25.81*	*75.58*	1.27	3.73
2[d,e]	LC	P	3.84	2.99	*5.06*	*3.94*	*4.06*	*3.16*	2.75	2.14	1.79	1.39
3[e]	D	P	−2.56	−4.76	*19.7*	*10.56*	1.77	−1.05	1.27	−1.47	−1.05	−1.96
4	D	A	−1.82	−1.59	*72.5*	*82.14*	2.71	3.07	1.29	1.46	−1.56	−1.39
5	C	A	1.1	−1.69	*29.65*	*15.89*	3.63	1.95	2.99	1.6	−1.15	−2.13
6	C	A	*−4.16*	*−7.14*	*19.97*	*11.96*	4.66	2.79	−1.17	−1.96	−1.43	−2.39
7[e]	C	P	*5.31*	*4.26*	*184.82*	*148.06*	*9*	*7.21*	*16.8*	*13.45*	*5.58*	*4.47*
8	C	A	−2.04	−2.17	*58.08*	*54.19*	*8.34*	*7.78*	2.43	2.27	−1.06	−1.13
9[d,e]	C	P	*72.5*	*32.45*	*31.12*	*13.93*	6.06	2.71	3.94	1.77	3.46	1.55
10[e]	An	P	*78.79*	*59.3*	*77.17*	*58.08*	*12.3*	*9.25*	*12.47*	*9.38*	*17.51*	*13.18*
11	C	A	−1.05	−1.96	*58.49*	*31.56*	*7.52*	*4.06*	2.62	1.41	1.58	−1.17
12	D	A	2.66	−1.22	*80.45*	*24.93*	6.23	1.93	4.26	1.32	2.57	−1.25
13	C	P	−1.33	−1.54	*29.04*	*25.11*	2.41	2.08	3.25	2.81	−2	−2.32
14	D	P	−1.61	−3.45	*56.89*	*25.99*	5.28	2.41	5.17	2.36	2.01	−1.08
15[e]	C	A	2.53	−1.5	*724.08*	*191.34*	*17.88*	*4.72*	6.68	1.77	4.41	1.16
16[d,e]	D	P	*21.71*	*43.41*	*16.45*	*32.9*	−1.15	1.74	2.6	5.21	2.38	4.76
17	D	A	2.28	1.21	*64.45*	*34.3*	*9.19*	*4.89*	4.17	2.22	2.85	1.52
D283	C	P	−1.45	−3.03	*88.65*	*42.22*	*−5.88*	*−12.5*	2.57	1.22	*163.14*	*77.71*
D341	C	P	20.53	1.13	*86.22*	*4.72*	51.98	2.85	14.62	−1.25	*85.63*	*4.69*
MB4[e]	D	P	*4.69*	*6.87*	*10.63*	*15.56*	1.03	1.51	−1.5	−1.01	1.61	2.36
MB16[e]	An	P	*127.12*	*28.25*	*123.64*	*27.47*	3.14	−1.43	*57.68*	*12.82*	*86.22*	*19.16*
rs			*0.94 ($p < 0.0001$)*		*0.66 ($p = 0.0008$)*		0.78 ($p < 0.0001$)		*0.66 ($p = 0.0008$)*		*0.84 ($p < 0.0001$)*	

#[b]	Type[c]	Age	*N*-fold expression variation[a]									
			FZD6		*FZD7*		*FZD8*		*FZD9*		*FZD10*	
			B2M	*GAPDH*	*B2M*	*GAPDH*	*B2M*	*GAPDH*	*B2M*	*GAPDH*	*B2M*	*GAPDH*
FCrb	N/A	33 weeks	1.33	−2.94	*−6.25*	*−25*	*35.51*	*9.13*	8.63	2.22	1.14	−3.4
1[d]	D	A	2.17	6.36	−1.19	2.46	*30.48*	*89.26*	*11*	*32.22*	−120.3	−41.1
2[d,e]	LC	P	1.39	1.09	−1.75	−2.27	2.73	2.13	−1.96	−2.5	*−8.8*	*−11.2*
3[e]	D	P	1.12	−1.66	*−8.33*	*−16.66*	2.55	1.37	5.28	2.83	*−3.3*	*−6.1*
4	D	A	−1.81	−1.61	−1.58	−1.40	2.69	3.05	−3.33	−2.94	−2.3	−2.1
5	C	A	1.43	−1.30	*−3.7*	*−7.14*	1.82	−1.03	*−7.69*	*−14.28*	1.4	−1.4
6	C	A	−1.85	−3.03	−1.25	−2.08	−1.25	−2.08	−2.63	−4.34	*−15.3*	*−25.6*
7[e]	C	P	*19.7*	*15.78*	*7.31*	*5.86*	*6.73*	*5.39*	*4.53*	*3.63*	*58.9*	*47.2*
8	C	A	*7.57*	*7.06*	−1.78	−1.92	−1.05	−1.12	*−6.25*	*−6.66*	*1.9*	*1.7*
9[d,e]	C	P	4.38	1.96	5.06	2.27	3.07	1.38	−2.94	−6.66	*−4*	*−8.9*
10[e]	An	P	*23.1*	*17.39*	−2.5	−3.33	*28.84*	*21.71*	1.02	−1.29	*−3.3*	*−4.4*
11	C	A	2	1.08	1.35	−1.36	4.06	2.19	−1.51	−2.77	*−3.1*	*−5.7*
12	D	A	2.55	−1.26	1.99	−1.61	5.24	1.62	1.02	−3.12	*−1.6*	*−5.1*
13	C	P	−1.31	−1.51	−1.88	−2.17	3.41	2.95	*−4.54*	*−5.26*	*−3.3*	*−3.9*
14	D	P	−1.28	−2.77	−1.75	−3.84	*90.51*	*41.36*	1.48	−1.47	*−7.7*	*−16.9*
15[e]	C	A	1.58	−2.38	10.48	2.77	*17.27*	*4.56*	2.95	−1.28	*−10*	*−37.8*
16[d,e]	D	P	−1.13	1.75	*−12.5*	*−6.25*	*7.84*	*15.67*	*−12.5*	*−6.66*	*−13.7*	*−6.9*
17	D	A	1.16	−1.61	4.17	2.22	*15.24*	*8.11*	−1.09	−2.04	1.4	−1.4

Table 1 continued

#[b]	Type[c]	Age	*N*-fold expression variation[a]									
			FZD6		*FZD7*		*FZD8*		*FZD9*		*FZD10*	
			B2M	*GAPDH*	*B2M*	*GAPDH*	*B2M*	*GAPDH*	*B2M*	*GAPDH*	*B2M*	*GAPDH*
D283	C	P	*15.24*	*7.26*	1.45	−1.45	1.35	−1.56	*−11.11*	*−25*	*ND*	*ND*
D341	C	P	40.5	2.22	20.53	1.13	18.51	1.01	2.14	−8.33	*ND*	*ND*
MB4[e]	D	P	−1.26	1.16	−2.38	−1.61	2.38	3.4	*10.63*	*15.56*	*ND*	*ND*
MB16[e]	An	P	*25.99*	*5.78*	2.01	−2.22	13.18	2.93	2.6	−1.72	*ND*	*ND*
rs			*0.77 ($p < 0.0001$)*		*0.83 ($p < 0.0001$)*		*0.78 ($p < 0.0001$)*		*0.87 ($p < 0.0001$)*		*0.91 ($p < 0.0001$)*	

A adult (≥18 years), *An* anaplastic, *C* classic, *D* desmoplastic, *FCrb* fetal cerebellum, *LC* large cell, *N/A* not applicable, *ND* not detected, *P* pediatric (<18 years); *rS* Spearman r

[a] Results are expressed as *N*-fold variation of the *FZD* gene in the tumor tissue or cell line sample compared to the normal adult cerebellum sample after normalizing both samples on the basis of their *B2M* and *GAPDH* mRNA content. A cutoff of threefold after normalizing by the two housekeeping genes was established to define differential expression (up or down). Significant increases (>3-fold) or decreases (<3-fold) in the expression level of the FZD receptor are shown in italics.

[b] MB4 is from sample #3; MB16 is from sample #10

[c] Pathological evaluation was performed in two distinct services

[d] This sample was investigated for β-catenin staining

[e] This sample was investigated for *MYC* mRNA expression

Results

Expression of FZD1-10 mRNAs in human cerebellum, medulloblastomas and MB cell lines

We quantified the transcripts of all *FZD* receptors (*FZD1-10*) in human adult and fetal cerebellum, in 17 cases of human MBs and in four MB cell lines. The amount of each *FZD* mRNA in each sample normalized with the amount of *B2M* or *GAPDH* in the same sample is summarized in Fig. 1; the amount of each FZD mRNA in fetal cerebellum, MBs, and MB cell lines relative to the normal adult cerebellum is indicated in Table 1, and represented by a heat map in Fig. 2.

Overall, the results obtained using both *B2M* or *GAPDH* as reference genes are comparable. In detail, real-time PCR showed that the amount of *FZD3* normalized by *B2M* and *GAPDH* in adult cerebellum was $13{,}800 \times 10^{-5}$ and $4{,}500 \times 10^{-5}$, respectively, while the amount of *FZD7* was $29{,}937 \times 10^{-5}$ and $9{,}740 \times 10^{-5}$. These results showed that *FZD7* mRNA in human adult cerebellum was the highest, followed by *FZD3*. On the other hand, the amount of *FZD2* normalized by *B2M* and *GAPDH* was 43×10^{-5} and 14×10^{-5}, respectively, and that of *FZD10* 399×10^{-5} and 130×10^{-5}, respectively. These results showed that *FZD2* mRNA in human adult cerebellum was the lowest, and *FZD10* mRNA was among the lowest expressed FZDs, which also include *FZD5*, *FZD8* and *FZD9* (Fig. 1, red dots). In human MB tissues and cell lines, the median values of *FZD3* and *FZD7* transcripts were the highest ones, while the median value of *FZD10* transcript was among the lowest ones, including *FZD2*, *FZD5*, *FZD8* and *FZD9*, as in normal adult cerebellum.

In contrast, when analyzing the expression of each *FZD* receptor (normalized by *B2M* or *GAPDH*) relative to the normal adult cerebellum, we found that *FZD2* transcript was constantly over-expressed in all MB tissues and cell lines, as well as in human fetal cerebellum. In fact, the ratios of *FZD2*/*B2M* and *FZD2*/*GAPDH* were between 220×10^{-5} and $31{,}425 \times 10^{-5}$, and between 56×10^{-5} and $9{,}740 \times 10^{-5}$. Therefore, *FZD2* mRNA normalized by *B2M* and *GAPDH* was ~5–724 and 4–690 times higher than that in normal adult cerebellum, respectively. In contrast, *FZD10* was absent in all MB cell lines and underexpressed in 11 out of 17 cases (~65%) in which the ratios of *FZD10*/*B2M* and *FZD10*/*GAPDH* were between 3.3×10^{-5} and 130.7×10^{-5}, respectively, and between 3.2×10^{-5} and 33.6×10^{-5}, respectively. Therefore, *FZD10* mRNA normalized by *B2M* and *GAPDH* in 11 MBs was ~3–131 and 3–34 times lower than in normal adult cerebellum, respectively. *FZD1*, *FZD3* and *FZD8* were over-expressed in 5, 8, and 7 out of 17 MBs, respectively, with FZD8 mRNA over-expressed also in foetal cerebellum. Finally, *FZD4*, *FZD5* and *FZD6* were over-expressed in 3, 2 and 3 out of 17 cases, while *FZD7* and *FZD9* were under-expressed in 3 and 4 cases, respectively.

FZD2 mRNA over-expression correlates with *ASPM* mRNA over-expression

ASPM (abnormal spindle-like microcephaly associated) mRNA was evaluated by qRT-PCR in all MB tissue

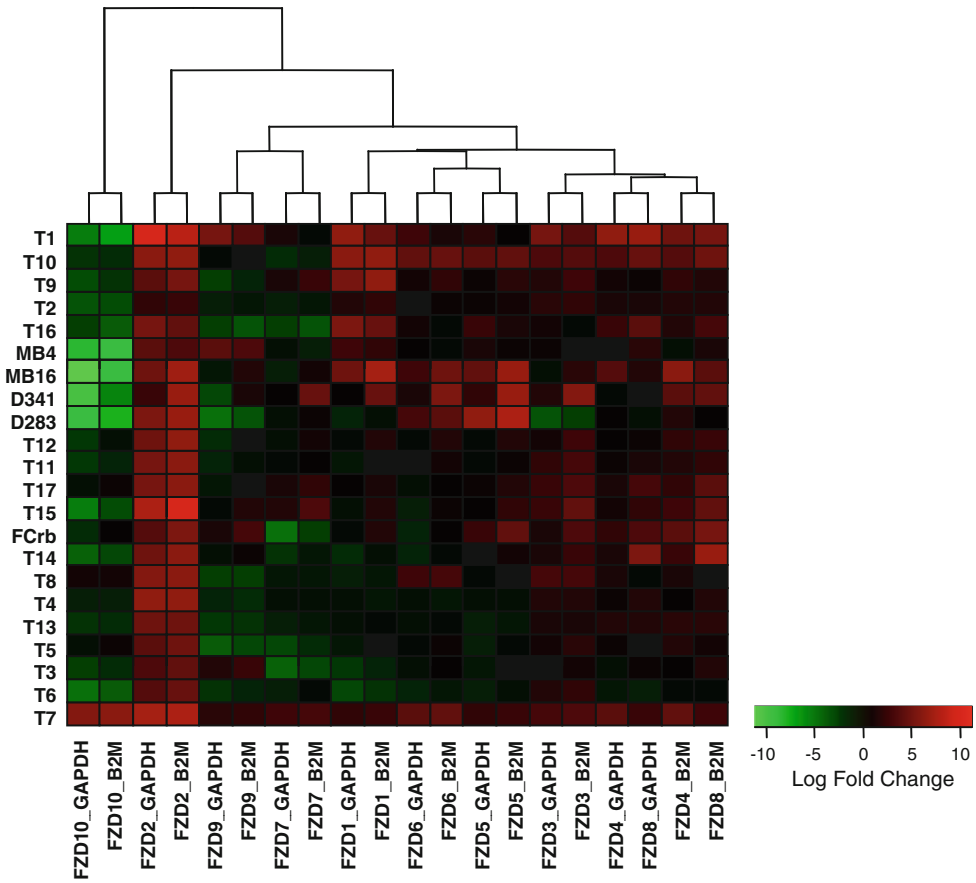

Fig. 2 Heat map representation of the 10 *FZD* transcript expression in a series of MBs and MB cell lines. Normalized expression values for each *FZD* gene relative to normal adult cerebellum are represented by a heat map: *red* higher values; *green* lower values. As expected and shown, the clustering of the *FZD* genes normalized by *B2M* or *GAPDH* reveals ten "first-level" clusters, each composed of the same *FZD* normalized by *B2M* or *GAPDH*, except for *FZD4* and *FZD8*; in addition, five "second-level" clusters were found (i.e., *FZD10*; *FZD2*; *FZD7* and *FZD9*; *FZD1*, *FZD5* and *FZD6*; *FZD3*, *FZD4* and *FZD8*) (see also supplementary Fig. 2S). No clustering of the samples has been presented, as the number of both samples and investigated genes is too small, and the power of the analysis is limited. *FCrb* fetal cerebellum, *MB* medulloblastoma cell line, *T* tumor sample

($n = 17$) and cell lines ($n = 4$), as well as in human cerebellum. The ratios of *ASPM/B2M* and *ASPM/GAPDH* in adult cerebellum were 14.8×10^{-5} and 4.8×10^{-5}, respectively, while the ratios of *ASPM/B2M* and *ASPM/GAPDH* in the fetal cerebellum were 1820×10^{-5} and 152×10^{-5}, respectively. The ratios of *ASPM/B2M* and *ASPM/GAPDH* in MBs ranged between $5{,}112 \times 10^{-5}$ and $89{,}503 \times 10^{-5}$, and between $1{,}152 \times 10^{-5}$ and $39{,}777 \times 10^{-5}$, respectively. Finally, the ratios of *ASPM/B2M* and *ASPM/GAPDH* in MB cell lines were between $23{,}982 \times 10^{-5}$ and $258{,}471 \times 10^{-5}$, and between $1{,}734 \times 10^{-5}$ and $88{,}884 \times 10^{-5}$, respectively. Therefore, *ASPM* mRNA is expressed at very low levels in human adult cerebellum, and is robustly over-expressed in human fetal cerebellum, MB tissues and cell lines. In particular, the amounts of *ASPM* mRNA normalized by *B2M* and *GAPDH* in human MBs were between 346 and 6,060 times and between 240 and 8,278 times higher than those in adult cerebellum, respectively.

We analyzed the correlation between *FZD2* and *ASPM* mRNA expression with the Spearman rank-correlation coefficient in human fetal cerebellum and MB tissues. *FZD2* mRNA levels were significantly correlated with *ASPM* mRNA levels using both *B2M* and *GAPDH* as reference genes ($r_s = 0.6371$, $P = 0.0045$, and $r_s = 0.4801$, $P = 0.0437$, respectively).

Expression of FZD2 protein in medulloblastomas and medulloblastoma cell lines

As expected, western blotting revealed the presence of a band of ~85 kDa, corresponding to the FZD2 receptor, in all MB cell lines and in the subgroup ($n = 4$) of MBs examined (#3, 4, 10, and 15; Fig. 3). No band was detected in the KNRK nuclear extract, as FZD2 is a cell membrane protein.

Overexpression of FZD receptors is not clearly associated with WNT/CTNNB1 pathway activation

WNT/CTNNB1 pathway activation was investigated looking at: (1) the nuclear β-catenin staining in 4 out of 17 MBs (samples 1, 2, 9, and 16 as indicated in Table 1 by an asterisk); (2) the mRNA expression of *MYC* gene, a β-catenin target, in 7 out of 17 MBs (samples 2, 3, 7, 9, 10, 15, and 16) and in the four MB cell lines. No nuclear β-catenin staining was observed in any of the four investigated MBs, but in two of them (samples 1 and 2), we

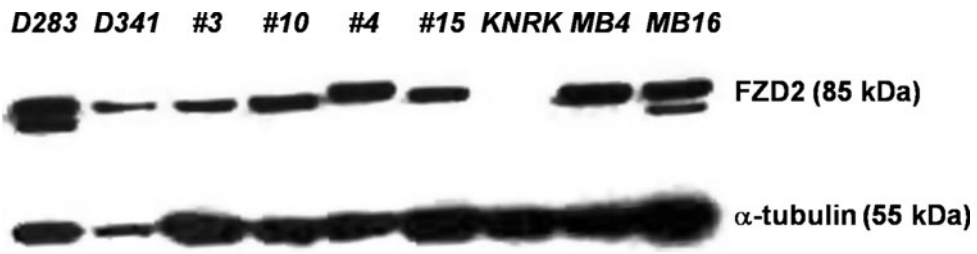

Fig. 3 Western blot showing the expression of FZD2 in human medulloblastoma tissues and cell lines. *FZD2* is expressed at protein level in all the investigated MB tissues (samples *3*, *4*, *10*, and *15*) and cell lines (*D283*, *D341*, *MB4*, and *MB16*). *KNRK* is a rat nuclear extract from K-Ras-transformed kidney cells, and was used as negative control

found cytoplasmic immunopositivity; *MYC* transcript was found to be overexpressed in four MBs (samples 7, 9, 10, and 16) and in all the MB cell lines; *MYC* gene amplification was found exclusively in D341 and, to a lesser extent, in D283. The activation of non canonical WNT pathways was not investigated.

Discussion

Altered expression of *FZD* genes may provide an alternative mechanism leading to the activation of oncogenic WNT signaling in human MBs. Thus, we have investigated the expression of all FZD receptors by real-time RT-PCR in normal human cerebellum (adult and fetal), in a series of human MBs and in four MB cell lines, two commercially available and two derived from primary human MBs surgically removed at our Institute. Some observations may be highlighted which suggest further investigations.

We found that *FZD2* mRNA is constantly and robustly over-expressed in human MBs and MB cell lines, as well as in human fetal cerebellum, in comparison with the expression level of normal adult cerebellum; the expression of FZD2 protein was confirmed by western blot. This means that the FZD2 receptor might electively play a role in promoting proliferation of both human MB cells and cerebellar progenitors from which these malignancies arise. Accordingly, *Fzd2* mRNA is over-expressed in the proliferating granule cell progenitors of mouse cerebellum at post-natal day 7 [P7; see Brain Gene Expression Map (BGEM) at http://www.stjudebgem.org], and we found a significant correlation between the mRNA levels of *FZD2* and *ASPM*, which may be considered as a marker of mitosis. Indeed, we found that *ASPM* is expressed at high levels in all MB tissues and cell lines, and in normal fetal cerebellum, but at very low levels in normal adult cerebellum. This substantial differential expression between proliferating tissues (malignant and normal) and normal adult parenchyma fits well with the potential mitotic role of ASPM, and suggests that ASPM may be an attractive therapeutic target in MBs, just as recently indicated for glioblastomas [13].

It should be emphasized that distinct FZD receptors were found to be specifically over-expressed in distinct tumors. Indeed, FZD2, FZD8 and FZD9 are over-expressed in gastric cancer [14], FZD5 and FZD8 in renal cell carcinoma [12], and FZD7 in hepatocellular carcinoma [6]. This means that over-expression of different FZDs—with the potential to activate WNT signaling pathways—might be tissue-specific for different malignancies.

It is unclear whether or not FZD2 over-expression causes the deregulation of non-canonical or canonical WNT signaling pathways. Fzd2 receptor seems to signal through the non-canonical WNT/cGMP/Ca^{2+} pathway, suppressing a cGMP-dependent protein kinase (PKG) and activating calcium release from the intracellular stores [15, 16]. Moreover, Fzd2 might lead to the strong activation of the mitogen-activated protein kinase p38 and, then, to proliferation [17]. It may also lead to contact-independent cell growth [18], a feature frequently seen in MB cell lines even in the presence of fetal bovine serum [19]. On the other hand, Verkaar et al. have recently demonstrated that human FZD2 does not couple to a G-protein-dependent signaling pathway, but activates the canonical Wnt signaling pathway, involving the transcriptional modulator β-catenin [20]. According to our data, the hypothesis that FZD2 over-expression in human MBs leads to the strong activation of the canonical WNT/CTNNB1 pathway is implausible for the following reasons: (1) we observed FZD2 over-expression in all cases, but nuclear localization of the CTNNB1 protein, an immunohistochemical marker of the canonical WNT pathway activation, has only been reported in 18–30% of human MBs [21]; (2) despite FZD2 over-expression being observed in all cases, we detected no nuclear CTNNB1 immunoreactivity in four investigated MBs, and found over-expression of *MYC*—a target of the canonical WNT pathway—in four of seven investigated MBs.

Unlike FZD2, the other FZD receptors showed a case-restricted over-expression or under-expression, and their

role in promoting deregulation of WNT signaling in MB tissue and cell lines is difficult to infer.

Intriguingly, it should be noted that: (1) only the four MBs (all pediatric) over-expressing *MYC*, also over-express *FZD1*, which is a FZD receptor that signals by the canonical WNT pathway; (2) *MYC* is also over-expressed in the two MB cell lines (MB4 and 16) over-expressing *FZD1*; (3) *MYC* over-expression in the four MBs and in the MB4 and MB16 cell lines is not due to *MYC* amplification, while *MYC* over-expression in the D283 and D341 cell lines, which do not over-express *FZD1*, is associated with *MYC* amplification [22]. The meaning of this finding remains uncertain for the following reasons: (1) the sample size is very small; (2) *MYC* over-expression may be due to canonical WNT pathway gene mutations (e.g., *CTNNB1*); (3) *MYC* over-expression may be independent from the canonical WNT pathway activation. However, this observation may also suggest that FZD1 over-expression leads to canonical WNT pathway activation. This might be true even if no nuclear β-catenin immunoreactivity was found in the two MBs that over-expressed both *FZD1* and *MYC* and were investigated for β-catenin positivity (samples 9 and 16 in Table 1). Indeed, it has been previously proved that negative immunohistochemical results do not eliminate the possibility of increased nuclear localization of CTNNB1—with consequent WNT/CTNNB1 pathway activation—because weak nuclear staining may be difficult to evaluate [23]. Moreover, the lack of nuclear β-catenin staining has been reported in tumors arising from WNT/CTNNB1 pathway deregulation, and a variety of WNT/CTNNB1 pathway target genes, such as *MYC*, may be up-regulated even in the absence of nuclear β-catenin immunoreactivity [12, 23]. Finally, as observed by Merle et al. in human hepatocellular carcinomas, over-expression of FZD receptors might lead to levels of nuclear β-catenin sufficient to induce target genes, but not strong enough to be detected by immunohistochemistry [6].

FZD10 transcript is under-expressed in a variety of human MBs and in all MB cell lines examined. FZD10 appears to interact with Wnt1 (but not Wnt3a) as a ligand:receptor pair, and promote neuronal differentiation [24]. Hence, the under-expression of *FZD10* in primary tumors and cell lines of human MBs might reflect the less differentiated state of tumor cells. However, it remains to be investigated whether this observation has some biological relevance, considering the low levels of *FZD10* also present in normal adult cerebellum, and whether FZD10 plays some role in the neuronal differentiation of cerebellar progenitors, from which MBs arise. Moreover, *FZD10* was found to be 'enriched' in the WNT subgroup of MBs (see table 1 in Northcott et al. [5]).

FZD3 expression levels were the highest in both normal cerebellum and human MBs compared to the other *FZDs*, but *FZD3* was found to be over-expressed only in a subset of human MBs, and, most importantly, in none of the MB cell lines. Thus, the meaning of *FZD3* over-expression in MB tumorigenesis is unclear. *Fzd3*, *Fzd4* and *Fzd7* transcripts were all found up-regulated together in MBs from Ptch1$^{+/-}$ mice, suggesting that the expression of these receptors might be associated with deregulation of the Sonic Hedgehog pathway [25]. However, in nine cases of MB over-expressing *PTCH1* and *GLI1* mRNA—PTCH1 and GLI1 are downstream effectors of the SHH pathway—we found *FZD3* over-expression only in two (samples 8 and 15, both of the adult age and with no *FZD4* nor *FZD7* over-expression associated) (unpublished data). Hence, our data do not convincingly support any association between SHH pathway activation and *FZD3*, *FZD4*, and/or *FZD7* over-expression in human MBs.

FZD8, which may activate the oncogenic WNT/CTNNB1 signalling pathway [14], was over-expressed in fetal cerebellum and in some cases of MBs, but not in MB cell lines, as for FZD3.

None of the other FZDs (FZD4, FDZ5, FZD6, FZD7 and FZD9) showed consistent over- or under-expression in human MBs and MB cell lines. However, we highlight that *FZD6*, which is another gene 'enriched' in the WNT subgroup of MBs [5], was found to be over-expressed in three MBs and in the MB cell line (MB16) derived from one of them (sample 10); in contrast, *FZD7*, which is a WNT pathway gene that was found to be 'enriched' in the SHH subgroup of MBs [5], was found to be under-expressed in three MBs, and over-expressed only in one.

Overall, our results indicate that FZD2—which may be associated to non-canonical WNT pathways—may play a more important role than the other FZD receptors in human MBs, and in the cerebellar progenitors from which these malignancies derive. In contrast, the role of the other FZD receptors in the formation and maintenance of these malignancies, if any, remains uncertain, and probably redundant.

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Conflicts of interest None.

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