Integrated Systems and Technologies

Genome-Wide Analysis of Alternative Splicing in Medulloblastoma Identifies Splicing Patterns Characteristic of Normal Cerebellar Development

Francesca Menghi1, Thomas S. Jacques2,3, Martino Barenco1, Ed C. Schwalbe4, Steven C. Clifford4, Mike Hubank1, and Jonathan Ham1

Abstract

Alternative splicing is an important mechanism for the generation of protein diversity at a post-transcriptional level. Modifications in the splicing patterns of several genes have been shown to contribute to the malignant transformation of different tissue types. In this study, we used the Affymetrix Exon arrays to investigate patterns of differential splicing between pediatric medulloblastomas and normal cerebellum on a genome-wide scale. Of the 1,262 genes identified as potentially generating tumor-associated splice forms, we selected 14 examples of differential splicing of known cassette exons and successfully validated 11 of them by reverse transcriptase PCR. The pattern of differential splicing of three validated events was characteristic for the molecular subset of sonic hedgehog (Shh)-driven medulloblastomas, suggesting that their unique gene signature includes the expression of distinctive transcript variants. Generally, we observed that tumor and normal fetal cerebellar samples shared significantly lower exon inclusion rates than normal adult cerebellum. We investigated whether tumor-associated splice forms were expressed in primary cultures of Shh-dependent mouse cerebellar granule cell precursors (GCP) and found that Shh caused a decrease in the cassette exon inclusion rate of five of the seven tested genes. Furthermore, we observed a significant increase in exon inclusion between postnatal days 7 and 14 of mouse cerebellar development, at the time when GCPs mature into postmitotic neurons. We conclude that inappropriate splicing frequently occurs in human medulloblastomas and may be linked to the activation of developmental signaling pathways and a failure of cerebellar precursor cells to differentiate. Cancer Res; 71(6); 2045–55. ©2011 AACR.

Introduction

Medulloblastoma is a malignant embryonal tumor of the cerebellum which most commonly affects children (1). Despite recent advances in the clinical management of this disease, approximately one third of the cases remain incurable and the majority of survivors suffer from long-term side effects caused by the therapeutic treatment (2). During the last few years, whole-genome gene expression studies have provided strong molecular evidence supporting the notion that medulloblastomas represent a heterogeneous disease and discrete medulloblastoma molecular subgroups have been identified on the basis of the expression profile of specific sets of genes (3, 4).

Alternative splicing (AS) is key post-transcriptional mechanism for the generation of protein diversity. It has been estimated that more than 50% of human protein coding genes undergo AS (5) and that splicing events greatly contribute to tissue- and development-specific protein expression (6). Inappropriate mRNA splicing has been described in many types of human cancer (7–10) and has been shown to affect the global pattern of protein expression within tumor tissues through the generation of novel protein variants or the unbalanced expression of normal protein isoforms (11). In both cases, AS has the potential to initiate and/or sustain tumor growth. A few studies of human medulloblastomas have described alternative splice forms of individual genes which are associated with specific molecular subsets of medulloblastoma (12), normal cerebellar development (13), or tumor recurrence (14). To comprehensively study the potential contribution of alternative transcript forms to medulloblastoma pathogenesis, we used the Affymetrix Human Exon array and measured gene expression at the level of individual exons on a genome-wide scale for 14 medulloblastoma and 5 normal cerebellar samples. We identified and validated differential splicing events occurring between the normal cerebellum and medulloblastoma and between different medulloblastoma molecular subgroups. We also showed that medulloblastomas share splicing patterns with mouse cerebellar granule cell
precursors (GCP), the proposed cells of origin for a molecular subset of medulloblastomas (15). This suggests that higher levels of specific transcript variants are maintained from the early stages of cerebellar development through the malignant transformation of precursor cells and could represent important pathways for medulloblastoma tumorigenesis.

Materials and Methods

Clinical samples
The exon array study included 14 snap-frozen pediatric medulloblastoma samples obtained from the Histopathology Department of Great Ormond Street Hospital (GOSH), with the approval of the local Research Ethics Committee (ref. no. 06/Q0508/57). A histopathologic review of the medulloblastoma cases was undertaken by a clinical pediatric neuropathologist (T.S.J.). Tumor features of anaplasia, nodularity, and desmoplasia were graded for each tumor sample, and histologic classification was carried out according to published criteria (16, 17). A sample of normal cerebellar tissue was obtained from a patient who had undergone brain surgery at GOSH for the removal of a pilocytic astrocytoma. Additional normal cerebellar total RNA samples were purchased from commercial sources.

A further set of 20 primary medulloblastoma and 10 normal cerebellar samples was used to independently validate tumor-associated splicing events by reverse transcriptase PCR (RT-PCR). All tumor samples had been previously profiled using Affymetrix 3’ arrays and classified into medulloblastoma molecular subgroups as reported in Kool and colleagues (3). Control samples included 6 normal adult cerebellar samples obtained from the UCL Institute of Neurology Queen Square Brain Bank, London, UK; and 4 additional RNA samples purchased from commercial sources. Details about sample classification and source can be found in Supplementary Table S1.

RNA extraction and array hybridization
Total RNA was isolated from frozen tissues by using an RNeasy mini kit (Qiagen) following the manufacturer’s instructions. RNA quality was assessed on an Agilent Bioanalyzer. One microgram of total RNA was processed and labeled using the Affymetrix GeneChip Whole Transcript Sense Target Labeling Assay as outlined in the manufacturer’s instructions. Hybridization to Affymetrix Human Exon 1.0 ST arrays was carried out for 16 hours at 45°C.

Analysis of array data
Analysis of AS was done as described by Shah and Pallas (18). Briefly, exon- and gene-level signal estimates were generated using the Robust Multichip Average algorithm (19) implemented in the Expression Console software (Affymetrix) and including only core, non–cross-hybridizing probe sets. Signal values were then analyzed using R and Bioconductor. To reduce the generation of false-positive results which can be caused by uniformly nonexpressed genes/exons, probe sets and transcript clusters falling into the lowest quartile of the expression signal distribution across all samples were excluded from the data set. The Splice Index algorithm was applied to identify exons with significantly different inclusion rates between groups of samples. Exon/gene signal ratios were compared using the moderated t statistic of the LIMMA package (20) and observations were selected for false discovery rate (FDR) adjusted values of $P < 0.01$.

The necessity to filter Splice Index results to remove those probe sets with the highest likelihood of introducing false-positive results has been reported in several publications (6, 18, 21, 22). We therefore applied a series of postanalysis filters: (i) we discarded hits corresponding to the last probe set or the final 2 probe sets at each end of a transcript cluster. Probe sets mapping to the 5’ and 3’ regions of a gene often fail to respond to expression changes as efficiently as those that map to the remainder of the gene and are therefore more likely to generate false-positive results (21). (ii) We removed all genes with a greater than 4-fold change in their level of expression between sample subgroups, as these have a tendency to produce false-positive results (6, 18, 23). (iii) We removed genes represented by fewer than 5 probe sets because their exon expression patterns are often more difficult to interpret (22). (iv) We focused on known genes by filtering out transcript clusters with no HUGO gene symbol. (v) We selected only probe set hits with a fold change in gene-normalized expression values between sample subgroups that was higher than 1.5. All filtered results were then subjected to a detailed visual inspection to assess the likelihood that individual events represented true positive results. This was achieved by examining probe set expression plots and analyzing candidate exons within their genomic context by using the X:Map Genome Browser (24). As previously described by Whistler and colleagues (22), each selected probe set was classified as yes (Y)—strong evidence of differential splicing; the candidate probe set maps to a known alternatively spliced exon or more than one adjacent probe sets behave similarly; probable (P)—single probe set mapping to an unknown alternatively spliced exon; unlikely (U)—unclear evidence of differential splicing; minor changes in probe set signal intensity or high-signal variance, probe set with ambiguous transcript cluster assignment, probe set mapping to regions of overlapping transcript clusters; no (N)—expression pattern indicative of nonrespon- sive, uniformly nonexpressed, or saturated probe set and no other indications of differential splicing. In some cases, inspection of candidate gene expression plots allowed the identification of probe sets whose profile was strongly indicative of differential splicing, even though they did not meet the requirement for statistical significance. These probe sets were considered false-negative results and were considered for further analysis. Candidate splicing events which were selected for further validation by RT-PCR all belonged to the list of probe sets designated as Y.

Exon array data are available from the ArrayExpress database (www.ebi.ac.uk/arrayexpress) under accession number E-MTAB-292.

Reverse transcriptase PCR
Total RNA (0.5 µg) was reverse transcribed into first-strand cDNA using SuperScript II reverse transcriptase (Invitrogen)
and oligo(dT). Approximately, 5 ng of cDNA template was amplified using Taq DNA polymerase (Qiagen). Primer sequences are provided in Supplementary Table S2. PCR reactions were conducted for 28 to 34 cycles, and PCR products were resolved on 2% agarose gels. PCR-amplified DNA bands were quantified using the ImageQuant TL v2005 software (GE Healthcare). Student’s t test was used to compare log2 ratios between splice variants across sample groups. To verify the sequence of the different PCR products, individual PCR bands were purified from agarose gels using a QiAquick Gel Extraction kit (Qiagen) and sequenced using an Applied Biosystems 3730x Genetic analyzer. PCR products that gave ambiguous sequencing results were cloned into the pGEM-T Easy vector (Promega), according to the manufacturer’s instructions. Following bacterial transformation, several clones were sequenced as described earlier.

Calculation of the Splicing Indicator value
To better visualize alternative transcript expression, we calculated the ratio between the difference and the average of the signal intensities corresponding to the 2 alternative splice forms. This formula generates a value between −2 and 2, which we called the Splicing Indicator. Values of −2 and 2 correspond to exclusive amplification of the skipped exon isoform and the retained exon splice form, respectively.

Mouse GCP culture and mouse total cerebellum RNA samples
GCPs were isolated from 7-day-old C57BL6/J mice as described by Wechsler-Reya and colleagues (25) and as outlined in the Supplementary Materials and Methods.

Medulloblastoma cell lines
The DAOY cell line was purchased from the American Type Culture Collection (ATCC). The UW228-2 and D425 Med cell lines were the kind gift of Prof. Silvia Marino (Barts and The London School of Medicine and Dentistry, London, UK). Cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% FBS, 2 mmol/LL-glutamine, and 1% penicillin–streptomycin (all from Invitrogen). Total RNA was isolated using TRIzol. The medulloblastoma cell lines were regularly checked for Mycoplasma contamination with a MycoProbe Mycoplasma Detection kit (R&D Systems), according to the manufacturer’s instructions. The MycoProbe assay detects Mycoplasma contaminants with a colorimetric signal amplification system which targets Mycoplasma 16S ribosomal RNA. The cells were last tested in May 2010, 1 month before submission of the manuscript. The cell lines were authenticated as follows: The DAOY cell line was authenticated by the ATCC according to its standard protocols, and the cells were used within 6 months of receipt for the experiments in this article. The DW228-2 and D425 cell lines are not stocked in cell banks and therefore could not be compared with a reference cell line. Both cell lines are human cells, and we carried out RT-PCR experiments with RNA extracted from the 2 cell lines and PCR primer pairs specific for 20 different human genes. In all cases, the PCR products were sequenced and all corresponded to known human DNA sequences. No PCR products corresponding to other mammalian species were detected.

Results

Molecular classification of tumor samples
We used Affymetrix Human Exon arrays to analyze gene expression and splicing in 14 pediatric medulloblastomas and 5 samples of normal cerebellum. Patients’ clinical features are presented in Supplementary Table S1 and summarized in Figure 1. We initially investigated gene-level expression profiles to identify samples expressing gene signatures characteristic of previously described medulloblastoma molecular subgroups. Unsupervised hierarchical cluster analysis of sonic hedgehog (Shh) signature genes (ref. 4 and Supplementary Table S3) identified a well-defined group of 3 medulloblastomas which significantly overexpressed genes typically belonging to the Shh signaling pathway or associated with a GCP status (Fig. 1). These 3 samples were therefore classified as Shh-driven medulloblastomas (i.e., SHH subgroup). The remaining tumor samples did not show a similarly distinct gene signature and were grouped together as medulloblastoma subgroup 2 (MB2).

Identification of tumor-associated splicing events
To identify splicing patterns associated with medulloblastomas, we compared exon/gene expression ratios between different sample subgroups. Several different comparisons were carried out to identify splicing events associated either with medulloblastoma in general or with SHH tumors specifically (Fig. 2A). A total of 1,262 genes with potential alternatively spliced exons were identified, of which 750 passed the post-analysis filters. A detailed breakdown of the post-analysis filtering steps is reported in Supplementary Table S4. On visual inspection, 174 candidate events were classified as Y: yes (23.2%); 285 as P: probable (38%); 242 as U: unlikely (32.3%); and 49 as N: no (6.5%). Examples for each individual category are shown in Supplementary Figure S1. On the basis of the characterization of transcript structures by using the X:Map database, 14 splicing events corresponding to known alternatively spliced cassette exons and belonging to the Y designation were selected for validation by RT-PCR on a subset of the profiled samples. At this initial point, the selected candidate events were not evaluated for their biological relevance or potential implication in cancer pathogenesis and included 2 events classified as false-negative results (Supplementary Fig. S2). Differential splicing occurring between different sample subgroups was confirmed for 11 of the 14 examples studied (Fig. 2B and Table 1). In the 3 nonconfirmed cases, only one PCR product was detected, suggesting that only one transcript form was expressed (data not shown). These events were not analyzed further.

For 3 of the 11 experimentally validated genes (DAAM1, EHBP1, and TRRAP), the splicing pattern distinguished between SHH tumors and all of the other samples. In the remaining cases, all of the medulloblastomas shared a similar exon pattern which differed from that of normal adult cerebellar samples. Interestingly, normal fetal cerebellar...
samples typically produced splicing patterns which were more similar to the tumors than to the normal adult samples (Fig. 2B).

Identification of novel splice forms

For some genes, we observed additional PCR products to those predicted by the exon array analysis. In 2 cases (ATP2B1 and R3HDM1), we found that an extra transcript variant was generated via an alternative 5' splice site located within the candidate cassette exon (Fig. 2B). Both novel transcript forms have been eventually annotated in EnsEMBL release 56. In the case of the TRRAP gene, the unexpected PCR product corresponded to a previously unreported splice form in which exon 22 is excluded and exon 23 is retained (Fig. 2B). We also identified a novel 9-nucleotide long exon inserted upstream of cassette exon 34 in the MADD gene (Fig. 2C). Direct sequencing showed that this novel miniexon was heterogeneously expressed across the sample set (data not shown). All of the other extra bands detected corresponded to heteroduplexes forming between alternative PCR products (26, 27), as confirmed by PCR subcloning and sequencing.

Validation of differential splicing events in an independent set of primary medulloblastomas and in medulloblastoma cell lines

To confirm that the splicing patterns of the selected genes were characteristic of medulloblastoma in general, we analyzed an independent set of 10 normal cerebellum and 20 medulloblastoma samples by RT-PCR (Fig. 3). The tumors were classified into either the SHH subgroup or the MB2 subgroup according to whether or not they showed a gene expression signature indicative of Shh signaling pathway activation (data not shown). Typically, the novel independent set of samples (test set) replicated the same patterns of splicing we identified in the initial set of samples (training set). We found statistically significant differences in log2 ratios of transcript forms between sample subgroups (Table 1). We also analyzed splicing patterns in 3 human medulloblastoma cell lines (DAOY, D425 Med, and UW228-2) and found that they were similar to those of primary medulloblastomas, with the cell lines preferentially expressing tumor-associated transcript forms (Fig. 3).

To effectively visualize alternative transcript expression, we calculated Splicing Indicator values on the basis of quantified signal intensities of the alternative PCR products resolved on agarose gels (as described in Materials and Methods). In cases where more than 2 transcript forms were amplified, the 2 most prominent were taken into account. Unsupervised cluster analysis was carried out using the Splicing Indicator values generated for each individual sample analyzed by RT-PCR (Fig. 4). Most of the adult normal cerebellar samples formed a homogeneous cluster and predominantly expressed transcript forms in which the candidate exons were retained. An exception to this being the MADD gene, whose exon 34 seemed to be mostly excluded. Interestingly, the majority of SHH tumors shared similar splicing patterns, including a unique profile for those genes which were initially identified as undergoing SHH-associated splicing (DAAM1, EHRP1, and TRRAP). We also confirmed that normal fetal cerebellar samples generally expressed patterns of splicing more similar to those of tumor samples than those found in normal adult cerebella.
Tumor-associated splicing patterns are observed during the normal development of mouse cerebellum

Cerebellar GCPs are the proposed cells of origin of Shh-driven medulloblastomas (15). We therefore investigated whether transcripts which showed characteristic splicing patterns in medulloblastomas were also differentially spliced in cultured GCPs. GCP primary cultures were incubated either in the presence or absence of Shh for 48 hours. In the absence of Shh, GCPs differentiate into postmitotic cerebellar granule neurons. In the presence of Shh, GCPs continue to proliferate and maintain a neuronal precursor state (25). We analyzed splicing variations in GCP cultures for 7 of the candidate genes whose genomic organization was conserved between human and mouse [according to the XMap database (version 2.4)]. In the presence of Shh, the splicing patterns for 5 of the 7 genes analyzed by RT-PCR (Damm1, Ehbp1, Magi1 exon 24, Trrap, and Wdr7) recapitulated the patterns observed in the human tumor samples (Fig. 5A and B); that is, Shh increased the amount of cassette exon skipping that occurred. In the 2 remaining cases (MADD and Nav2), both transcript forms were expressed independently of Shh treatment (Supplementary Fig. S3).

The analysis of different stages of early postnatal mouse cerebellar development showed that the switch between the 2
Table 1. Genes alternatively spliced in medulloblastoma identified by exon array analysis and validated by RT-PCR

<table>
<thead>
<tr>
<th>Probe set ID</th>
<th>Gene symbol</th>
<th>Exon set location*</th>
<th>Transcript ID</th>
<th>Comparisonb</th>
<th>Exon array</th>
<th>RT-PCR validationc</th>
<th>Alternative exon</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>3464995</td>
<td>ATP2B1</td>
<td>Exon 20</td>
<td>ENST00000359142</td>
<td>MB vs. CB</td>
<td>0.003</td>
<td>&lt;0.001</td>
<td>Frameshift (154 aa), calmodulin-binding domain, early stop codon, CNS-specific</td>
<td>40</td>
</tr>
<tr>
<td>3329783</td>
<td>MADD</td>
<td>Exon 34</td>
<td>ENST00000311027</td>
<td>MB vs. CB</td>
<td>&gt;0.01†</td>
<td>0.01</td>
<td>Frameshift (23 aa), late stop codon, CNS-enriched</td>
<td>49</td>
</tr>
<tr>
<td>2680306</td>
<td>MAGI1</td>
<td>Exon 24</td>
<td>ENST00000330909</td>
<td>MB vs. CB</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>Frameshift (29 aa), early stop codon, cytoplasmic localization, CNS-enriched</td>
<td>36-38</td>
</tr>
<tr>
<td>3323129</td>
<td>NAV2</td>
<td>Exon 6</td>
<td>ENST00000360655</td>
<td>MB vs. CB</td>
<td>0.009</td>
<td>0.02</td>
<td>In frame (23 aa)</td>
<td>32, 34, 36</td>
</tr>
<tr>
<td>2507435</td>
<td>R3HDM1</td>
<td>Exon 15</td>
<td>ENST00000364160</td>
<td>MB vs. CB</td>
<td>0.004</td>
<td>0.04</td>
<td>In frame (85 aa), CNS-enriched</td>
<td>Fast DB (50)</td>
</tr>
<tr>
<td>3615612</td>
<td>TJP1</td>
<td>Exon 20</td>
<td>ENST00000346128</td>
<td>MB vs. CB</td>
<td>0.002</td>
<td>0.007</td>
<td>In frame (80 aa)</td>
<td>36</td>
</tr>
<tr>
<td>3789479</td>
<td>HDR7</td>
<td>Exon 17</td>
<td>ENST00000254442</td>
<td>MB vs. CB</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>In frame (33 aa), CNS-enriched</td>
<td>Fast DB (50)</td>
</tr>
<tr>
<td>2680331</td>
<td>MAGI1</td>
<td>Exon 15</td>
<td>ENST00000330909</td>
<td>SHH vs. CB</td>
<td>&gt;0.01†</td>
<td>0.03</td>
<td>In frame (27 aa), CNS-specific</td>
<td>32</td>
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<td>3538263</td>
<td>DAAM1</td>
<td>Exon 16</td>
<td>ENST00000351081</td>
<td>SHH vs. CB</td>
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<td>0.01</td>
<td>In frame (10 aa), formin-homology domain, CNS-enriched</td>
<td>32, 34, 35</td>
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<td>Exon 8</td>
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<td>SHH vs. MB2</td>
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<td>3014461</td>
<td>TRRAP</td>
<td>Exons 22/23</td>
<td>ENST00000456197</td>
<td>SHH vs. MB2</td>
<td>0.003</td>
<td>0.004</td>
<td>In frame (24 aa), CNS-enriched</td>
<td>Fast DB (50)</td>
</tr>
</tbody>
</table>

Note: FAST DB is an AS database (50).
Abbreviations: MB, medulloblastoma (including both SHH and MB2); SHH, Shh-driven medulloblastoma; CB, normal cerebellum; CNS, central nervous system; aa, amino acids.
*Exons are numbered according to the Ensembl transcripts listed to the right (EnsEMBL release 56).
†In several cases, probe sets were identified as differentially spliced for more than one comparison. Here, we report the comparison that generated the most significant difference.
‡Fold change in gene-normalized probe set signal values.
§Fold change in gene signal values.
P values were calculated on the basis of the log2 ratio between skipped and retained transcript forms as quantified from the agarose gels.
 Events with an adjusted P > 0.01 but a profile suggestive of AS (Supplementary Fig. S2).
alternative transcript forms of the 5 validated mouse genes occurred between postnatal days 7 and 14, at the time when GCPs mature into postmitotic neurons (Fig. 5A and B). Thus, during the postnatal development of the mouse cerebellum, as the number of proliferating GCPs decreases, the amount of cassette exon retention that occurs increases.

Discussion

Alternative pre-mRNA splicing makes a major contribution to the generation of protein variety in a tissue- and development-specific manner. Alterations in the normal pathways of AS have been associated with the growth and maintenance of...
several tumor types and have been indicated as candidate biomarkers of tumor progression, metastasis, and patient survival (7, 28, 29). In this study, we applied genome-wide exon array technology to identify splicing variations that distinguish between medulloblastoma and normal cerebellum. We identified 1,262 unique genes containing at least 1 candidate exon whose inclusion rate differed between sample subgroups. The use of postanalysis filters and visual inspection of candidate event expression plots and exon structures provided us with a list of 174 events with the highest likelihood of representing real positive hits (Supplementary Table S4). This number is comparable with the number of real positive hits described in other reports in which tumor-enriched splice variants have been investigated using Affymetrix Exon arrays (8, 9). Eleven of a total of 14 examined cassette exons belonging to this list were confirmed to undergo differential splicing by RT-PCR. All of the validated cassette exons encoded a polypeptide which would be included in the final protein product and did not contain nonsense codons (Table 1).

Although in most cases the functional significance of the AS has not yet been clarified, many of the validated genes have been previously implicated in key biological processes such as neuronal differentiation and cancer progression. Several of the splicing events that we experimentally validated occurred in genes involved in cytoskeleton remodeling, cell morphology regulation, and cell–cell interaction. DAAM1, EHBP1, and NAV2 all contain actin-binding domains and have been suggested to mediate extensive reorganization of actin structures likely affecting basic cellular functions such as neurite growth and endocytic trafficking (30–32). DAAM1 plays an important role in the regulation of actin assembly during axon formation and outgrowth (33). Skipping of exon 16, which we confirmed to frequently occur in Shh-driven medulloblastomas, would remove 10 amino acids (DFFVNSNSKQ) from a functionally important linker sequence located between the lasso and knob structures in a formin-homology-2 domain which is required for the actin assembly activity of the DAAM1 protein (32, 34). Interestingly, the AS of DAAM1 exon 16 has been shown to be regulated during neuronal development as part of a general network of brain-enriched alternatively spliced exons whose expression contributes to promoting cell differentiation (35).

MAGI1 and TJP1 (also known as ZO-1) have been described as tight junction proteins colocalizing in the apical region of several tumor types and have been indicated as candidate biomarkers of tumor progression, metastasis, and patient survival (7, 28, 29). In this study, we applied genome-wide exon array technology to identify splicing variations that distinguish between medulloblastoma and normal cerebellum. We identified 1,262 unique genes containing at least 1 candidate exon whose inclusion rate differed between sample subgroups. The use of postanalysis filters and visual inspection of candidate event expression plots and exon structures provided us with a list of 174 events with the highest likelihood of representing real positive hits (Supplementary Table S4). This number is comparable with the number of real positive hits described in other reports in which tumor-enriched splice variants have been investigated using Affymetrix Exon arrays (8, 9). Eleven of a total of 14 examined cassette exons belonging to this list were confirmed to undergo differential splicing by RT-PCR. All of the validated cassette exons encoded a polypeptide which would be included in the final protein product and did not contain nonsense codons (Table 1).

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polarized epithelial cells (36). However, they are expressed in nonepithelial cells, particularly in neuronal cell types, where they may be involved in different cell processes such as transduction of signals from the cell surface to the nucleus (36, 37). Indeed, both MAGI1 and TJPL1 can localize to the nucleus of cells and are predicted to interact with a variety of protein partners (38, 39). Specifically, the exclusion of exon 24 from the mature MAGI1 mRNA results in a frameshift which generates a unique COOH-terminal sequence containing 2 bipartite nuclear localization signals. This form is predominantly found in the nucleus of MDCK (Madin–Darby canine kidney) cells, as opposed to the 2 alternative transcript forms which retain exon 24 and encode proteins that mostly localize to the cytoplasm (38). Both cassette exons 15 and 24 of the MAGI1 gene are almost exclusively expressed in the brain, and it has been suggested that they might be important for neuronal specification (36).

The ATP2B1 (or PMCA1) gene encodes a plasma membrane calcium pump which is responsible for the expulsion of Ca^{2+} from the cytosol of eukaryotic cells and is implicated in the modulation of neurotransmitter release (40). AS of ATP2B1 exon 20 affects the COOH-terminal tail of the protein, whereby inclusion of the cassette exon results in a frameshift which introduces an earlier translational stop codon and affects the establishment of a variety of protein–protein interactions (40). Of note, ATP2B1 exon 20 is a known target of the neuronal splicing factor Nova, which is implicated in the regulation of splicing events associated with several neuronal activities (41).

TRRAP is a highly conserved, large nuclear adaptor protein containing HEAT repeats and the fifth LXXLL motif in the TRRAP protein. The sequence of unknown function which is inserted between the equivalent mouse exons was seen in Shh-treated GCPs than in controls (Fig. 5). Also, more skipping of the equivalent mouse exons was seen in Shh-treated GCPs than in normal cerebellum development (Fig. 5). Several studies have reported the role of AS in inducing and regulating neuronal differentiation (30, 35, 49). This occurs through the transcriptional regulation of a variety of neuronal splicing factors and results in a switch in the splice forms that are expressed in the adult brain as compared with the developing brain, with many neuron-specific exons being incorporated into the mature mRNAs, and eventually leading to the neuronal differentiation of progenitor cells. It is plausible that the splicing pattern of at least some of the candidate genes identified through the exon array analysis results from the failure of cerebellar precursor cells to undergo neuronal differentiation, caused by the aberrant activation of oncogenic pathways such as the Shh signaling pathway. It will therefore be interesting to investigate whether the characteristic patterns of cassette exon exclusion that we identified in human medulloblastomas may be related to a possible role of Shh in controlling the overall activity of the splicing machinery in the developing cerebellum. It will also be of interest to investigate whether subgroup-specific patterns of differential splicing also characterize the medulloblastoma subgroup with hyperactivation of the WNT signaling pathway and the other discrete medulloblastoma molecular subgroups.

In summary, by profiling exon expression in normal and malignant cerebellum, we identified splice variants that are generally enriched in medulloblastomas or specifically associated with the Shh-driven medulloblastoma molecular subgroup. We showed that in some cases, medulloblastoma-associated splicing patterns were Shh dependent and indicative of a normal cerebellar undifferentiated phenotype. This suggests that activation of oncogenic pathways during the development of the cerebellum may lead to a failure of neuronal differentiation in part through the disruption of AS programs.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.
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References


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Correction: Genome-Wide Analysis of Alternative Splicing in Medulloblastoma Identifies Splicing Patterns Characteristic of Normal Cerebellar Development

In this article (Cancer Res 2011;71:2045–55), which was published in the March 15, 2011 issue of Cancer Research (1), the last sentence of the grant support is incomplete and should read as follows: J. Ham was supported by a Wellcome Trust Senior Research fellowship (grant number 057700) and the work was also funded by a grant from Research in Childhood Cancer.

Reference


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