Upregulation of SOX2, NOTCH1, and ID1 in supratentorial primitive neuroectodermal tumors: a distinct differentiation pattern from that of medulloblastomas

Clinical article

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Object. Supratentorial primitive neuroectodermal tumor (PNET) and medulloblastoma are highly malignant embryonal brain tumors. They share morphological similarities, but differ in their differentiation patterns and global gene expression. The authors compared the expression of specific genes involved in neuroglial differentiation in supratentorial PNETs and medulloblastomas to define the distinct characters of these tumors.

Methods. The mRNA expression of 8 genes (SOX2, NOTCH1, ID1, ASCL-1, NEUROD1, NEUROG1, NEUROG2, and NRG1) was evaluated in 25 embryonal tumors (12 supratentorial PNETs and 13 medulloblastomas) by quantitative real-time polymerase chain reaction. The expression levels of the transcripts of these genes were compared between the tumor groups. Activation of the JAK/STAT3 pathway was assessed by immunoblotting. Relative expression levels of STAT3 and phosphorylated STAT3 proteins were compared.

Results. Supratentorial PNETs expressed significantly higher levels of SOX2, NOTCH1, ID1, and ASCL-1 transcripts, whereas the transcription of proneural basic helix-loop-helix factors, NEUROD1, NEUROG1 (significantly), and NEUROG2 (not significantly) was upregulated in medulloblastomas. The proportion of phosphorylated STAT3α relative to STAT3α was significantly greater in supratentorial PNETs than in medulloblastomas, indicating activation of the JAK/STAT3 pathway in supratentorial PNETs.

Conclusions. These results indicate that supratentorial PNET predominantly has glial features and medulloblastoma largely follows a neuronal differentiation pattern. These divergent differentiation patterns may be related to the location and origin of each tumor. (DOI: 10.3171/2010.2.PEDS1065)

Key Words • supratentorial primitive neuroectodermal tumor • medulloblastoma • SOX2 • NOTCH1 • ID1 • basic helix-loop-helix protein

Since the inception of brain tumor classification, the diversity of brain tumors has been considered to stem from their different cellular origins.21 However, with the discovery of neural stem cells, the common precursors of both neurons and macroglia throughout the CNS, maldevelopment or deranged differentiation has become the focus of research on the development and heterogeneity of brain tumors.23

Supratentorial PNET and medulloblastoma are highly malignant embryonal brain tumors that arise predominantly in children. These tumors have long been thought to represent a common spectrum of PNETs because they share morphological features of the primitive neuroepithelium, and are essentially indistinguishable by light-microscopic analysis. However, besides their location propensities, these tumors have quite different responses to adjuvant therapies and prognoses.10,24 Recent

Abbreviations used in this paper: bHLH = basic helix-loop-helix; GAPDH = glyceraldehyde-3-phosphate-dehydrogenase; GFAP = glial fibrillary acidic protein; phospho-STAT3 = phosphorylated STAT3; PNET = primitive neuroectodermal tumor; PCR = polymerase chain reaction; RT-PCR = reverse transcription PCR; TBS = Tris-buffered saline.

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studies have also demonstrated substantial differences in the chromosomal aberrations and global gene expression in embryonal tumors.\textsuperscript{13,19,28,31,33}

One of the key histopathological features of medulloblastoma is its predominantly neuronal differentiation. Many neuronal markers, such as synaptophysin and class 3 \(\beta\)-tubulin, are highly expressed in the majority of medulloblastoma. These tumors sometimes exhibit extensive neuronal differentiation.\textsuperscript{15} Conversely, supratentorial PNETs show more diverse differentiation patterns, such as neuronal, glial, ependymal, and so on. The expression of cell lineage markers and transcription factors is highly variable in supratentorial PNETs.\textsuperscript{35}

Neurogenesis and gliogenesis are complex processes directed by both intrinsic and extrinsic factors.\textsuperscript{29} In the mammalian CNS, neurogenesis precedes gliogenesis. To begin the neurogenesis, neural stem cells have to commit themselves to a neuronal fate, exiting from the cycle of self-renewal; SOX2 is a key transcription factor that maintains the self-renewal of neural stem cells, and downregulation of SOX2 is a prerequisite of neurogenesis.\textsuperscript{8} Neurogenesis is controlled by the proneural proteins, many of which have a basic helix-loop-helix (bHLH) motif. NeuroD, Neurogenin, and achaete-scute families are typical of the proneural bHLH transcription factors.\textsuperscript{5} At the end of neurogenesis, gliogenesis begins. Many different molecular signals contribute to this gliogenic switch.\textsuperscript{29}

Activation of Notch signaling and JAK/STAT pathway plays a crucial role in gliogenesis. ID1 is an inhibitor of bHLH transcription factor and behaves as a suppressor of the proneural transcription factors.\textsuperscript{29}

In our previous study, we found a strikingly different SOX2 expression in immunohistochemistry between supratentorial PNET and medulloblastoma.\textsuperscript{32} We postulated that the difference came from the divergent differentiation patterns of the tumors. In this study, we extend the interest to other genes deeply involved in neurogenesis and gliogenesis, to assess the hypothesis that the divergent differentiation pattern is an important molecular signature of the embryonal brain tumors.

\section*{Methods}

\subsection*{Tissue Samples}

Freshly frozen embryonal tumor tissues from 25 patients were obtained from the Brain Bank at Seoul National University Hospital, Seoul. Supratentorial PNET was diagnosed in 12 of the patients and medulloblastoma arising from the cerebellar vermis in 13. The pathological slides were reviewed by a neuropathologist (S.H.P.). Brain tissues with focal cortical dysplasia, obtained from a patient treated with epilepsy surgery, were used as controls (Table 1). This study protocol was approved by the Institutional Review Board of Seoul National University Hospital.

\subsection*{Extraction of RNA and use of RT-PCR}

Total RNA was extracted from 50–100 mg of tumor and brain tissues, using TRIzol reagent (Invitrogen), according to the manufacturer’s instructions. First-strand complementary DNA was synthesized from 1 \(\mu\)g of RNA using a SuperScript III First-Strand Synthesis SuperMix kit (Invitrogen), according to the manufacturer’s instructions. The complementary DNA (1 \(\mu\)g) was amplified for 30 cycles with primers for SOX2, NOTCH1, NEURODI, NEUROGI, and GAPDH with an AccuPower PreMix kit (Bioneer, Daejeon). The primer sequences, annealing temperatures, and the size of expected PCR products are summarized in Table 2. The PCR products were separated on 1.2% agarose gel. After being stained with ethidium bromide, they were visualized under ultraviolet illumination.

\subsection*{Quantitative Real-Time PCR}

For the real-time PCR reactions, a master mix of the following components was prepared: 6.25 \(\mu\)l water, 2.5 \(\mu\l of the forward and reverse primer (9 \(\mu\M) set, 12.5 \(\mu\l of TaqMan PCR 2X master mix. The complementary DNA solution (5 \(\mu\l) was added as the PCR template. Real-time PCR amplification was performed with the ABI PRISM 7000 Sequence Detection System (Applied Biosystems). Assays-on-Demand TaqMan probes (Applied
were used to measure SOX2, NOTCH1, ID1, ASCL1, NEUROD1, NEUROG1, NEUROG2, and NRG1 expression (Table 3). All PCR reactions were performed in triplicate. The expression levels of the target genes were normalized to the values of brain tissue (obtained in Patient 26) and levels of internal GAPDH expression, and are represented as the relative expression.23

Protein Extraction, Immunoblotting, and Quantitation

Total protein was extracted from 10–50 mg of tumor and brain tissues, using PRO-PREP solution (Intron Biotech), according to the manufacturer’s protocol. Protein concentrations of the soluble lysates were determined using a BCA protein assay kit (Thermo Fisher Scientific Inc.). For immunoblotting, 10 μg of protein was mixed with sodium dodecyl sulfate (SDS) sample buffer (Invitrogen), boiled for 5 minutes at 95°C and then separated by 10% SDS polyacrylamide denaturing gel and transferred to a polyvinylidene fluoride membrane (Amersham Pharmacia Biotech). After the membrane had been blocked with TBS containing 5% nonfat dry milk, it was incubated overnight at 4°C with the primary antibody in TBS containing 0.1% Tween 20. After the blot was washed, it was incubated with horseradish-peroxidase–conjugated species-specific secondary antibody (1:5000) for 1 hour at room temperature. After the blots had been washed several times in TBS with 0.1% Tween 20, they were developed with enhanced chemiluminescence reagent (Amersham Pharmacia Biotech) and exposed to film.

Statistical Analysis

Statistical analyses were performed with SPSS 17.0 software (SPSS, Inc.). The expression levels of mRNAs and proteins of each tumor group were compared using a nonparametric Mann-Whitney U-test because of the small sample size of each group. All tests were 2-sided and the significance level was 5%.

Results

Analysis of mRNA Expression by RT-PCR

In a pilot study, we compared the mRNA expression of SOX2, NOTCH1, NEUROD1, and NEUROG1 in a small number of supratentorial PNETs and medulloblastomas developed with enhanced chemiluminescence reagent (Amersham Pharmacia Biotech) and exposed to film. The primary antibodies and their dilution factors were as follows: anti–phospho-STAT3 (Tyr705, 1:2000, Cell Signaling Technology), anti-STAT3 (1:2000, Cell Signaling Technology), and anti–β-actin (1:10000, Sigma-Aldrich). Quantification of the blot density was performed using TINA 2.0 software (Raytest GmbH). The blot densities of the target proteins were normalized to the values of brain tissue (Patient 26) and levels of internal β-actin expression and were represented as the relative intensity values.

TABLE 2: Primer sequences, annealing temperatures, and the size of the expected PCR products in RT-PCR experiments*

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer</th>
<th>Sequence (5′→3′)</th>
<th>AT (°C)</th>
<th>Size (base pairs)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SOX2</td>
<td>forward</td>
<td>CCCCCGGCGGCAATAGCA</td>
<td>65</td>
<td>448</td>
</tr>
<tr>
<td></td>
<td>reverse</td>
<td>TCGGCGCCGGGAGATACAT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NOTCH1</td>
<td>forward</td>
<td>GCCGCCTTTGCTCCTTCCT</td>
<td>61</td>
<td>300</td>
</tr>
<tr>
<td></td>
<td>reverse</td>
<td>CCGTGTTGCTGGCTGGCTC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NEUROD1</td>
<td>forward</td>
<td>GCCCCAGGGATTAGACATACACT</td>
<td>60</td>
<td>523</td>
</tr>
<tr>
<td></td>
<td>reverse</td>
<td>CCGCAAGCCAGCGATGTTTCTT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NEUROG1</td>
<td>forward</td>
<td>CCGACGACCAAAGCTCCTCA</td>
<td>54</td>
<td>337</td>
</tr>
<tr>
<td></td>
<td>reverse</td>
<td>GGAATGAAACAGCGGT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GAPDH</td>
<td>forward</td>
<td>CGTGGAAAGCTCATGAC</td>
<td>48</td>
<td>447</td>
</tr>
<tr>
<td></td>
<td>reverse</td>
<td>CAAATTGTTGCTACACCAG</td>
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</tr>
</tbody>
</table>

* AT = annealing temperature.
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(5 tumors of each type) by RT-PCR (Fig. 1). Higher \textit{SOX2} mRNA expression was observed in the supratentorial PNETs, as reported in a previous immunohistochemical study.\textsuperscript{32} Transcripts of \textit{NOTCH1}, which codes for a receptor of the Notch signaling pathway, were also expressed more highly in supratentorial PNETs. Both \textit{NEUROD1} and \textit{NEUROG1} are proneural bHLH transcription factors of the \textit{NEUROD} family. Higher \textit{NEUROD1} and \textit{NEUROG1} mRNA expression was observed in the medulloblastomas.

\textbf{Expression Analysis by Quantitative Real-Time PCR}

The expression of \textit{SOX2} mRNA and that of other factors involved in neuroglial differentiation was measured by quantitative real-time PCR in 25 embryonal tumor samples (Fig. 2). There was significantly greater expression of \textit{SOX2} and \textit{NOTCH1} mRNAs in supratentorial PNETs than in medulloblastomas (\textit{p} < 0.0001 for both). Transcripts of \textit{ID1}, which codes for a suppressor of bHLH transcription factors, and transcripts of \textit{ASCL1} were also expressed more highly in the supratentorial PNETs (\textit{p} = 0.0478 and \textit{p} = 0.0032, respectively). The medulloblastomas expressed more \textit{NEUROD1} and \textit{NEUROG1} mRNAs than did the supratentorial PNETs (\textit{p} = 0.0004 and \textit{p} = 0.0015, respectively). The expression of \textit{NEUROG2} mRNA was also stronger in medulloblastomas, but not to a statistically significant extent (\textit{p} = 0.0729). Expression of \textit{NRG1} mRNA did not differ between the 2 types of tumor (\textit{p} = 0.6742). The fold differences in transcript expression were in the order of thousands for \textit{NEUROG1}, of hundreds for \textit{NEUROD1}, and of scores for \textit{SOX2} and \textit{NOTCH1}.

\textbf{Immunoblotting for STAT3 and Phospho-STAT3}

\textit{STAT3} is an important transcription factor involved in diverse cellular processes, such as cell differentiation and immune modulation. \textit{STAT3} is activated by phosphorylation at Tyr 705 residue. We compared \textit{STAT3} and phospho-\textit{STAT3} expression in embryonal tumors by immunoblotting. \textit{STAT3} has 2 alternatively spliced isoforms,
STAT3α and STAT3β. STAT3α is a full-length isoform and STAT3β is a truncated variant.27 STAT3α is the major isoform in most cell types, but expression of the 2 isoforms is known to depend on specific cellular context.7 In our study, STAT3α was the major isoform in both supratentorial PNET and medulloblastoma (Fig. 3A). Especially, phosphorylation of STAT3 at the Tyr 705 residue occurred predominantly in STAT3α. Therefore, we quantitated the band intensities of STAT3α and phospho-STAT3α and compared them between the tumor groups.

Supratentorial PNETs showed a significantly higher level of STAT3α expression than medulloblastomas (Fig. 3B, p = 0.010). The difference in the expression levels of phospho-STAT3α was more striking (Fig. 3C, p < 0.001). The proportion of phospho-STAT3α relative to STAT3α (phospho-STAT3α/STAT3α) can reflect the true levels of STAT3 activation in each tumor. The proportion of phospho-STAT3α relative to STAT3α was significantly greater in supratentorial PNETs than in medulloblastomas, indicating activation of the JAK/STAT3 pathway in supratentorial PNETs (Fig. 3D, p < 0.001).

**Discussion**

The histogenesis of embryonal brain tumors has been controversial since the time of Bailey and Cushing.3,21 Young age of onset, primitive histological features, highly malignant behavior, and multilineage differentiation patterns suggest that these tumors originate from stem cells or early progenitor cells. The discovery of tumor-initiating cells in specimens from many brain tumors, including medulloblastomas, also supports this suggestion.38 At the heart of this hypothesis of a stem cell–cancer connection, are the concepts that these malignant tumors develop from the aberrant differentiation of stem/progenitor cells and that their specific differentiation patterns determine the specific nature of the tumors. Medulloblastomas show predominantly neuronal differentiation. Genetically engineered mouse models with defects in the sonic hedgehog and/or p53–Rb pathways develop medulloblastomas from the cerebellar granule cell layer.26,41 Medulloblastomas also express transcription factors specific for cerebellar granule neurons, such as ZIC and NSCL1.33 Glial differentiation is observed in many medulloblastomas, but only in a small region of the tumors. Intermittent GFAP-expressing cells are sometimes regarded as entrapped astrocytes, rather than true glially differentiated tumor cells.14

The most salient evidence of neural differentiation is that many neural transcription factors are highly expressed in medulloblastomas. A pioneering study showed with northern blotting that transcripts of the bHLH transcription factors, NEUROD1, NEUROD2, and NEUROD3 (NEUROG1) are expressed in medulloblastomas, whereas supratentorial PNETs expressed the ASCL1 transcripts.36

The bHLH transcription factors are important regulators of cell-fate determination in the development of the nervous system and neuroendocrine system.18,30 Many bHLH proteins act within a transcriptional network, and this machinery often functions under cancerous conditions.12,17 In the present study, the transcripts of proneural bHLH factors NEUROD1 and NEUROG1 were highly expressed in supratentorial PNETs. The expression of these factors in supratentorial PNETs suggests that these tumors originate from neural progenitor cells.
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expressed in medulloblastomas, whereas supratentorial PNETs robustly expressed ASCL1 and ID1 mRNAs. The higher expression of ASCL1, a proneural bHLH, appears to betray the scheme of divergent differentiation in embryonal brain tumors. ASCL1, also known as HASH1, is the best-characterized proneural bHLH factor, and specifies neuronal identity in the telencephalic ventricular zone and other regions of the CNS. Intriguingly, NEUROG1/NEUROG2 and MASH1, a mouse homolog of ASCL1, are expressed in distinct, nonoverlapping regions in the rodent cerebellar ventricular zones.\(^{40}\) Furthermore, the rhombic lip, which is the second germinal matrix of the developing cerebellum and where the classic type of medulloblastoma is known to arise, depends on MATH1 (ATOH1) rather than on MASH1 for neural fate commitment.\(^{11,40}\) In fact, ATOH1 is a known marker of a subset of human medulloblastoma.\(^{37}\) Therefore, the higher expression of ASCL1 in supratentorial PNETs can be regarded as a result of region-specific gene expression: supratentorial PNETs originate from telencephalic ventricular zone stem cells, whereas medulloblastomas arise from rhombic lip progenitors in the cerebellum.

Inhibitor of differentiation 1 (ID1) is a suppressor of bHLH proteins. It is expressed during astrocyte differentiation and it has been postulated to have an oncogenic or cancer-promoting role.\(^{1,20,29}\) The higher expression of ID1, together with the lower expression of NEUROD1 and neurogenins, observed in supratentorial PNETs indicates that a proneural bHLH gene network is suppressed in these tumors. SOX2, a strong inhibitor of neuronal differentiation, is also highly expressed in supratentorial PNETs. SOX2 is reportedly expressed in mature astrocytes and glial lineage tumors.\(^{4,32}\) Therefore, the expression of SOX2 and ID1 may prevent supratentorial PNETs from following a neuronal differentiation pathway and therefore confer a glial character on the tumors. The activation of the JAK/STAT3 pathway and the elevation of NOTCH1 transcripts in supratentorial PNETs support this assumption, because the activation of these pathways is critical in gliogenesis.\(^{29}\)

Neural stem cells are considered to have a glial nature.\(^{5,9}\) Radial glial cells expressing glial markers are legitimate stem cells in the developing CNS.\(^{5}\) Furthermore, mitotically active GFAP- and nestin-positive subventricular-zone astrocytes are thought to represent stem cells in the adult mammalian brain. SOX2 is also a well-established neural stem cell marker, despite its expression in mature astrocytes.\(^{4}\) If the granular-neuron–specific markers and neuronal differentiation patterns of medulloblastomas imply that the origins of these tumors are granular neuron precursors, as has been proposed by many authors, the glial signature of supratentorial PNETs, which includes SOX2, NOTCH1, and ID1, may reflect their origin from the stem cells in the telencephalic ventricular zone or subventricular zone. It is also noteworthy that persistent JAK/STAT3 signaling and ID1 upregulation are related to cancer progression and drug resistance in various human cancers.\(^{10,22,24}\) Patients with a supratentorial PNET show far less response to chemotherapy and have shorter survival than patients with a medulloblastoma. Further research is required on this intriguing phenomenon in embryonal tumors.

Conclusions

The expression of SOX2, NOTCH1, and ID1—genes involved in gliogenesis—is upregulated and JAK/STAT3 pathway is activated in supratentorial PNET. By contrast, the expression of proneural bHLH transcription factors is upregulated in medulloblastoma. These results indicate that supratentorial PNET predominantly has glial features and medulloblastoma largely follows a neuronal differentiation pattern. These divergent differentiation patterns may be related to the location and origin of each tumor.

Disclosure

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The authors report no conflict of interest concerning the materials or methods used in this study or the findings specified in this paper.

Author contributions to the study and manuscript preparation include the following. Conception and design: JH Phi, JH Kim. Acquisition of data: JH Phi, KM Eun, KH Park, SA Choi, YY Kim. Analysis and interpretation of data: JH Phi, JH Kim, SA Choi, SH Park. Drafting the article: JH Phi, KH Park. Critically revising the article: SK Kim, KC Wang, BK Cho. Reviewed final version of the manuscript and approved it for submission: all authors.

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References