A patient with medulloblastoma in its early developmental stage

Case report

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Medulloblastoma is the most frequent malignant brain tumor of the posterior fossa in children and is considered an embryonal tumor. It has been suggested that medulloblastomas be categorized into 4 distinct molecular subgroups—WNT (DKK1), SHH (SFRP1), Group 3 (NPR3), or Group 4 (KCNA1)—since each subgroup is distinct and there is no overlap. The authors report on a 13-year-old boy with medulloblastoma. He presented with sudden-onset nausea and vomiting due to intratumoral hemorrhage. The medulloblastoma was thought to be in an early developmental stage because the tumor volume was extremely small. Immunohistochemical analysis showed that the tumor was mainly composed of DKK1- and NPR3-positive areas. The individual areas of the tumor stained only for DKK1 or NPR3, with no overlap—that is, DKK1 and NPR3 expression were mutually exclusive. Samples obtained by laser microdissection of individual areas and subjected to mass spectrometry confirmed that the expression patterns of proteins were different. Fluorescence in situ hybridization for chromosome 6 showed there were 2 distinct types of cells that exhibited monosomy or disomy of chromosome 6. These results demonstrated that distinct subtypes of medulloblastoma may be present within a single tumor, an observation that has not been previously reported. Our findings in this case indicate that early-stage medulloblastoma may include more than 1 distinct subtype and hint at factors involved in the origin and development of medulloblastomas.

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Key Words • medulloblastoma • intratumoral hemorrhage • tumor development • genetic subgroup • heterogeneity • oncology

Medulloblastoma is the most frequent malignant brain tumor of the posterior fossa in children and is classified as an embryonal tumor.⁷ Adjuvant therapies, including radio- and chemotherapy, have improved the outcome for patients with these tumors, and the 5-year survival rate now exceeds 80%.¹¹,¹² The age at onset, the extent of resection, and the presence of dissemination are established prognostic factors. There is also evidence that medulloblastomas should be classified into 4 distinct molecular subgroups—WNT (DKK1), SHH (SFRP1), Group 3 (NPR3), and Group 4 (KCNA1)—and that the subgroup is a significant prognostic factor.⁸,⁹

Abbreviations used in this paper: FFPE = formalin-fixed, paraffin-embedded; FISH = fluorescence in situ hybridization; LC = liquid chromatography; MS = mass spectrography; Rsc = ratio from spectral counting.

We report on a 13-year-old boy whose medulloblastoma was detected at an early developmental stage; the tumor was mainly composed of hematomas, and its volume was extremely small. Immunohistochemical analysis showed that it consisted primarily of DKK1- and NPR3-positive areas, and DKK1 and NPR3 expression within the tumor were mutually exclusive. The samples obtained by laser microdissection of individual areas and subjected to mass spectrometry confirmed that the expression patterns of the proteins detected were different. Moreover, fluorescence in situ hybridization (FISH) for chromosome 6 convinced us that the medulloblastoma was composed of 2 distinct types of cells, one type of cells exhibiting monosomy 6 was thought to be the WNT subtype while the other type of cells with disomy was considered to be the Group 3 subtype. Our results in this single case indicate that in the early stage, a single medulloblastoma...
can include 2 distinct molecular subtypes. Yokota et al. demonstrated that lung cancers involving Myc family oncogenes have shown molecular heterogeneity and suggested that activation of c-Myc in the tumor cells occurs during tumor progression and the cells with c-Myc activation have outgrown the other cells without it. Recently, Clavería et al. demonstrated the selection of an epiblast cell population by Myc-driven endogenous cell competition in the early mammalian embryo. In this context, our medulloblastoma may reflect the stage that precedes the evolution into a monoclonal entity selected by cell competition, because it was composed of 2 distinct entities, i.e., WNT and Group 3, with different levels of Myc expression.

**Methods**

Written prior informed consent was obtained from the patient’s family members. Our protocol for gene and molecular analysis and for the establishment of cancer cells from specimens of patients with brain tumors was approved by the Research Ethics Committee of the Institutional Review Board of Kumamoto University Hospital, where the genetic and molecular analyses were conducted.

**Immunohistochemistry**

Immunohistochemistry was performed with validation of positive and negative controls as previously described. Formalin-fixed, paraffin-embedded (FFPE) tissue samples were used, and 4-μm-thick serial sections were stained. According to Northcott et al., the same primary antibodies were used for detecting subgroups of medulloblastoma: anti-DKK1 (WNT) (H00022943-M11, mouse, clone 2A5, 1:250, Abnova), anti–natriuretic peptide receptor C (NPR3) (ab32433, rabbit, 1:200, Abcam), anti-SFRP1 (SHH) (ab4193, rabbit, 1:100, Abcam), anti-Kv1.1 (KCNA1) (ab32433, rabbit, 1:200, Abcam), and antic-Myc (ab32072, rabbit, clone Y69, 1:200, Abcam). The other primary antibodies used in this study were anti–human synaptophysin (ab32433, rabbit, clone Y69, 1:200, Abcam), anti-Kv1.1 (KCNA1) (ab32433, rabbit, 1:200, Abcam), and anti–Myc (ab32072, rabbit, clone Y69, 1:200, Abcam). The other primary antibodies used in this study were anti–human synaptophysin (A0010, rabbit, 1:50, Dako), anti–human GFAP (Z0334, rabbit, 1:4000, Dako), and anti–human Ki 67 (M7240, mouse, clone MIB-1, 1:100, Dako) antibodies.

**Protein Extraction**

Proteins were extracted from FFPE samples as previously described. Briefly, 4-μm-thick tissue samples were placed on membrane slides (Leica Microsystems), air dried, deparaffinized, and stained with hematoxylin and eosin. Tumor areas were microdissected using a laser microdissection system (LM7D000, Leica Microsystems) and placed into tube caps containing 10 mM Tris, 1 mM EDTA, and 0.002% Zwittergent 3–16 (Calbiochem). Collected tissues were heated at 98°C for 90 minutes. After 60-minute sonication in a water bath, samples were digested overnight at 37°C with 1.5 μl of 1 mg/ml trypsin (Promega).

**Proteome Analysis**

Proteome analysis was performed as previously described. Briefly, the digests were reduced with dithiothreitol, dried, and redissolved with 40 μl of mass spectrometry (MS)–grade water containing 0.1% trifluoroacetic acid and 2% acetonitrile. Peptide mixture samples were analyzed with a liquid chromatography (LC)–MS/MS system (LTQ Velos Pro, Thermo Fisher Scientific; Advance Splitless Nano-Capillary LC dual solvent delivery system, Bruker-Michrom; HTS-xt PAL autosampler, CTC Analytics; XYZ nanoelectrospray ionization source, AMR). The samples were injected into a peptide L-trap column (Chemical Evaluation Research Institute). The peptides were separated on capillary reversed-phase C18 columns (Chemical Evaluation Research Institute), gradient eluted, and injected into a mass spectrometer using an ion spray. The spray voltage was 2.3 kV. The peptide and fragment mass tolerances were 2.0 D and 0.8 D, respectively. Trypsin specificity was applied with a maximum of 2 missed cleavages. All MS/MS spectral data were searched against Homo sapiens entries in the Swiss-Prot database using the SEQUEST database search program. For variable peptide modifications, methionine oxidation and N-formylation including formyl (K), formyl (R), and formyl (N-terminus) were taken into account. The search results were filtered with a moderate peptide confidence value.

**Semiquantitative Comparisons Using Spectral Counting**

To compare protein expression levels from the results of shotgun analysis we used the label-free spectral counting method. The number of peptide spectra with moderate confidence was used as the spectral count value. Fold changes in expressed proteins at each area (WNT/Group 3 area) on a base-2 logarithmic scale were calculated using the protein ratio from spectral counting (Rsc). Rsc > 1 or < –1 corresponded to fold changes > 2 or < 0.5.

**Fluorescence in Situ Hybridization**

To evaluate the status of chromosome 6 in the tumor cells, fluorescence in situ hybridization (FISH) was performed using FFPE samples and centromeric probes (CEP 6 [D6Z1] SpectrumGreen probes for the locus 6p11.1-q11 [Abbott Laboratories]) in the Cytogenic Testing Group, Molecular Genetic Testing Department, Clinical Laboratory Center of Mitsubishi Chemical Medience Corporation, Tokyo, Japan.

**Case Report**

**History and Presentation.** This 13-year-old boy experienced transient vomiting at gymnastics practice and was brought to a local hospital. A mass lesion in the fourth ventricle was identified and he was referred to our hospital in November 2012.

**Examination.** The results of neurological examination at the time of admission were normal, although the patient was suffering from nausea. CT scans and MR images showed a mass lesion measuring 1.0 × 1.5 × 1.0 cm, with intratumoral hematomas occupying the fourth ventricle (Fig. 1). No obstructive hydrocephalus was observed. A presumptive diagnosis of cavernous angioma was made.
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Operation. The patient underwent a suboccipital craniectomy with total resection of the intraventricular tumor via the subvermis midline approach. The mass was reddish and contained numerous hematomas. There was intraoperative bleeding from microvessels feeding the well-circumscribed tumor, which was easily and safely dissected from the floor of the fourth ventricle. The tentative intraoperative diagnosis was medulloblastoma, and the tumor was removed completely.

Postoperative Course. The patient’s postoperative clinical course was uneventful and he underwent radiochemotherapy according to our Packer-based protocol.11 His Karnofsky Performance Status remained 100%. Follow-up MR images obtained at the end of February 2014 revealed no regrowth and no dissemination into the spinal region.

Light Microscopy. Microscopic examination of the tumor showed that it consisted of round cells characterized by round nuclei with scant cytoplasm, corresponding to classic medulloblastoma. An area of high cellularity and a large hemorrhagic area were observed (Fig. 2A). There was clear evidence of mitoses, indicating that the neoplasm was of a malignant nature. Calcification was also noted.

Immunohistochemistry. Immunohistochemically most of the tumor cells were strongly positive for synaptophysin (Fig. 2B) and negative for GFAP (data not shown). The MIB-1 labeling index, representing the percentage of positively stained tumor nuclei, was more than 20.0%. Interestingly, the tumor mainly stained for DKK1 (Fig. 2C and D) and NPR3 (Fig. 2E and F), which are representative of the WNT subtype and Group 3 subtype, respectively. Even more interestingly, individual regions of the tumor stained only for DKK1 or NPR3, but not both—that is, DKK1 and NPR3 expression were mutually exclusive (Fig. 2C–F). Few tumor cells were immunoreactive for SFRP1; none were immunostained for Kv1.1 (data not shown). These results indicated that the tumor was composed of 2 distinct subtypes. Since c-Myc overexpression is frequently observed in the Group 3 subtype,8 we examined the expression of c-Myc, and confirmed the cells stained for c-Myc localized only in the region in which NPR3 expression was high (Fig. 2G–J).

Laser Microdissection and Mass Spectrometry. To confirm that the tumor cells consisted of 2 different subtypes we performed laser microdissection in regions positive for DKK1 (WNT subtype) or NPR3 (Group 3 subtype). Mass spectrometry, performed according to our standard protocol,15 showed the expression patterns of proteins were different at individual regions positive for DKK1 or NPR3 in the tumor (Fig. 3). The mass spectrometry...
Fluorescence in Situ Hybridization for Chromosome 6. Almost all cases of the WNT subtype exhibit monosomy 6, which is very rarely found in the other subgroups. We performed FISH for chromosome 6 and found 2 types of cells. One type of cell showed 1 green signal in the nucleus, which indicated monosomy 6, consistent with the WNT subtype, whereas the other type of cell showed 2 signals in the nucleus, consistent with the Group 3 subtype (Fig. 4). This finding also confirmed that the tumor comprised 2 different subtypes.

Discussion

It is of note that the initial manifestation of medulloblastoma in our patient was symptomatic intratumoral hemorrhage. While approximately 5% of the patients in previously reported cases presented with histological or radiological evidence of intratumoral hemorrhage, medulloblastoma with symptomatic hemorrhage is extremely rare. In their review of the literature, Fukai et al. found few patients with symptomatic hemorrhage thought to be attributable to mechanisms such as vascular invasion by tumor cells or venous congestion. Our case demonstrates that intratumoral hemorrhage can make it possible to detect even very small medulloblastomas and that the tumors may be resected easily and safely. The tumor in our patient was found at a very early stage of development, as evidenced by its being composed mainly of hematomas and its extremely small size.

According to the molecular classification system, medulloblastomas can be categorized as distinct subtypes—WNT, SHH, Group 3, or Group 4—since each subtype is independent and there is no overlap among subtypes based on genetic analysis. Immunohistochemical analysis showed that our patient’s tumor mainly consisted of areas positive for DKK1 and NPR3, representative markers for the WNT and Group 3 subtypes, respectively. Moreover, the areas of expression of DKK1 and NPR3 were mutually exclusive, not overlapping. Mass spectrometry after laser microdissection of individual areas confirmed that the expression patterns of proteins in each area were different. Furthermore, each area also had some proteins that were representative for each subtype, as Northcott et al. reported. Indeed, the number of representative proteins was smaller than that of genes reported by use of assays based on polymerase chain reaction (PCR). The reason is that PCR is able to identify a large number of different genes by amplification even if the expression levels are quite low, while mass spectrometry has limitations in identifying small quantities of proteins since there is no methodology to amplify proteins. We additionally performed FISH for chromosome 6 because most cases of the WNT subtype exhibit monosomy 6, which is nearly absent in the other subgroups, and we found that the tumor consisted of 2 types of cells, with monosomy or disomy of chromosome 6. These results strongly indicate that this single tumor comprised distinct subtypes of medulloblastoma. This observation has not been previously reported. The most likely explanation for this finding is simultaneous growth of each subgroup from 2 different cells of origin. However, we have to take into ac-

Fig. 2. Photomicrographs of serial sections of the tumor. A: H & E, original magnification ×100. B: Synaptophysin, original magnification ×100. C and D: DKK1, original magnifications ×100 and ×200, respectively. The rectangle in C indicates the area shown in D. E and F: NPR3, original magnifications ×100 and ×200, respectively. The rectangle in E indicates the area shown in F. G: NPR3, original magnification ×50. H: c-Myc, original magnification ×50. I and J: c-Myc, in the area strongly positive for NPR3 (I), and weakly stained for NPR3 (J), original magnification ×100 (both). The arrows indicate corresponding areas of H that are shown at higher magnification in I and J.
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count that the tumor in our case was an extremely small medulloblastoma, which is rare and indicates that the tumor was at the extremely early stage of development. We speculate that medulloblastomas may be composed of different cells of origin at very early stages of development. It is well known that a high expression level of Myc is observed in Group 3 tumors. Interestingly, the tumor in the present case displayed heterogeneity in the expression of c-Myc, and the cells stained for c-Myc were observed only in the region strongly stained for NPR3. Yokota et al. demonstrated that lung cancers involving Myc showed molecular heterogeneity. Interestingly, in 1 of 12 squamous cell carcinomas, c-Myc was amplified in metastases but not in the primary tumor. In 2 cases of 17 small cell carcinomas, amplification or rearrangement of c-Myc was found only in the cell lines, but not in the original tumors obtained from the same individuals. Their results suggest that activation of these oncogenes in small cell carcinomas and squamous cell carcinomas occurs during tumor progression and the cells with c-Myc activation have outgrown the other cells without it. Recently Claveria et al. demonstrated the selection of an epiblast cell population by endogenous cell competition in the early mammalian embryo under different Myc expression levels. Group 3 tumors express a higher level of Myc than do other subgroups. Thus, we can speculate that our medulloblastoma may reflect a stage that precedes the evolution into a monoclonal entity selected by cell competition.

In conclusion, our findings in one case suggest that in the early stages of development, medulloblastoma may include cells of more than one medulloblastoma subtype and suggest factors involved in the origin and development of these tumors.
Disclosure

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