Implications of DNA methylation classification in diagnosing ependymoma

Implications of DNA Methylation Classification in Diagnosing Ependymoma

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BACKGROUND: **Ependymoma is a central nervous system (CNS) tumor that arises from the ependymal cells of the brain's ventricles and spinal cord. The histopathology of ependymomas is indistinguishable regardless of the site of origin, and the prognosis varies. Recent studies have revealed that the development site and prognosis reflect the genetic background. In this study, we used genome-wide DNA methylation array analysis to investigate the epigenetic background of ependymomas from different locations treated at our hospital.**

METHODS: **Four cases of posterior fossa ependymomas and 11 cases of spinal ependymomas were analyzed.**

RESULTS: **DNA methylation profiling using the DKFZ methylation classifier showed that the methylation diagnoses of the 2 cases differed from the histopathological diagnoses, and 2 cases could not be classified. Tumor that spread from the brain to the spinal cord was molecularly distinguishable from other primary spinal tumors.**

CONCLUSIONS: **Although adding DNA methylation classification to conventional diagnostic methods may be helpful, the diagnosis in some cases remains undetermined. This may affect decision-making regarding treatment strategies and follow-up. Further investigations are required to improve the diagnostic accuracy of these tumors.**

INTRODUCTION

Ependymoma is a central nervous system (CNS) tumor that arises from the ependymal cells of the brain's ventricles and spinal cord.[1] This tumor occurs at any age and has an age-adjusted incidence rate of 0.42 (0.41–0.43), accounting for 1.6% of all CNS tumors.[2] Its prognosis is variable, and although attempts have been made to separate malignancy grades using WHO grades, histopathological findings and prognosis are not always concordant.[3,4] Ependymomas do not differ in histopathological findings according to the site of origin, but their prognoses are different. Recent studies have revealed that the development site and prognosis reflect genetic and epigenetic backgrounds.[5-13]

The 2016 World Health Organization (WHO) classification relies primarily on histological findings. Although there have been several reports on the genetic background of ependymal tumors,[11] only RELA-fused ependymomas have been included in the WHO classification, considering the availability of testing methods.[14] The situation changed dramatically when the 2016 WHO classification was revised, and the 2021 WHO classification was launched. Tumors were classified according to their location and genetic background. Supratentorial ZFTA and YAP1 fusions were established. Posterior fossa (PF) ependymomas are classified into groups A or B depending on the methylation pattern. MYCN amplification has recently been identified in spinal ependymomas.[15,16] Although the ependymomas of each site have similar histopathology, their molecular biology is heterogeneous, and their behavior is remarkably different from that of the same classification. Ependymomas, as a

Key words
- DNA methylation
- Ependymoma
- Epigenomics
- Myxopapillary ependymoma
- Spinal cord

Abbreviations and Acronyms
CNS: Central nervous system
CNV: Copy number variation
FFPE: Formalin-fixed paraffin-embedded
MC: Methylation classifications
NEC: Not elsewhere classified
PF: Posterior fossa
t-SNE: t-distributed stochastic neighbor embedding

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histopathological entity, maybe a phenotype of a group of related diseases.[7] Thus, information on genetic abnormalities is very important for diagnosing new-generation ependymomas. Furthermore, the 2021 WHO classification recommends that diagnosis be made by integrating histopathological findings and molecular diagnosis. However, it is challenging to clearly classify in some cases.

Capper et al. performed a comprehensive methylation analysis of 2801 CNS tumors and used these reference data to establish a random forest classifier using machine learning. This platform can be freely accessed by anyone on the website, and data are still being accumulated using this platform.[17]

This study applied DNA methylation array analysis to verify the consistency between methylation profiling and the clinical diagnosis of ependymomas treated at our hospital and to inspect the accuracy by referring to previous reports and investigating some irregularities.

MATERIALS AND METHODS

Patient Cohort

We collected cases of spinal tumors resected and diagnosed as ependymoma at the Spine and Spinal Center of Juntendo University Hospital from 2010 to 2020 and cases of PF tumors resected and diagnosed as PF ependymoma at the Neurosurgery Center of the hospital from 2013 to 2020. Formalin-fixed paraffin-embedded (FFPE) specimens were available, and at least 250 ng of DNA was extracted.

Processing of DNA and Analysis of DNA Methylation Microarray

DNA was extracted from FFPE tumor tissues using the GeneReadTM DNA FFPE Kit (Cat. No.180134 Qiagen, Manchester, UK) and quantified using a Quantus FLUOROMETER (Promega Corp., Madison, WI, USA). At least 250 ng of DNA was extracted from the FFPE samples. The samples were treated with bisulfite to identify the methylation sites where unmethylated cytosine was converted to thymine. DNA methylation microarray analysis was performed using the Infinium Methylation EPIC BeadChip Kit (8xBead Chip Cat; WG-317−1001) (Illumina, San Diego, CA, USA), following the manufacturer's instructions. No DNA repair kits were used during this process.

DNA Methylation Profiling and Analysis

Raw methylation array data (IDAT files) generated from Capper's 2801 reference data were uploaded to a methylation profiling platform (www.molecularneuropathology.org) to obtain methylation

Table 1. Details of the Patient Cohort

Case	Age	Sex	Location	Pathology	Resection	Follow-up (Months)	Recurrence	Remarks
Spinal								
1	63	M	Cervical	Ependymoma	GTR	114	(−)	
2	39	M	Cervical	Ependymoma	GTR	95	(−)	
3	53	F	Cervical	Ependymoma	GTR	64	(−)	
4	31	M	Cervical	Ependymoma	GTR	27	(−)	
5	70	M	Cervical	Ependymoma	GTR	26	(−)	
6	27	M	Cervical	Ependymoma	GTR	N/A	(−)	History of surgery for PF ependymoma
7	69	F	Conus	Ependymoma	GTR	40	(−)	
8	33	M	Cauda equina	Myxopapillary ependymoma	GTR	69	(−)	
9	40	M	Cauda equina	Myxopapillary ependymoma	GTR	28	(−)	
10	28	M	Cauda equina	Myxopapillary ependymoma	GTR	24	(−)	
11	21	F	Cauda equina	Myxopapillary ependymoma	GTR	32	(−)	
Intracranial								
12	3	F	PF	Ependymoma PFB	STR	30	(+)	PFS : 13 months OS : 30 months
13	11	M	PF	Ependymoma PFB	GTR	106	(−)	
14	30	M	PF	Ependymoma PFB	GTR	48	(−)	
15	36	F	PF	Ependymoma PFB	STR	52	(+)	PFS : 22 months

M, Male, F, Female, GTR, Gross total resection, OS, Overall survival, PF, Posterior fossa, PFS, Progression-free survival, STR, Subtotal resection.

classifications (MC) using publicly available v11b4 and v12.5 versions of brain classifiers.

Ependymoma samples were compared with reference data, and each sample classification was calculated using a calibrated score that provided a confidence index for MC agreement. As recommended in previous reports, results can be considered consistent with the reference MC if the calibration score is 0.9 or higher.[17]

An unsupervised clustering t-distributed stochastic neighbor embedding (t-SNE) analysis was performed using the R package "Rtsne" using 939 references of ependymal and glial tumors and normal tissue control selected from 2,801 cases of Capper.

RESULTS

Details of the Patient Cohort

Table 1 presents a list of the patient cohort. This study included 15 patients. There were 7 spinal ependymomas, 4 arising from the cauda equina, and 4 PF ependymomas. All lesions occurred for the first time at each site.

The age range was 3–70 years old (median, 33 years old), the male-female ratio was 2.0 (M/F ratio: 2.0), and the follow-up period was 24–114 months (median, 44 months). Only 2 cases of PF ependymoma were partially resected, and recurrence was confirmed during the follow-up period. The times to recurrence were 13 and 22 months, respectively—none of the patients who underwent gross total resection experienced recurrence during the follow-up period.

Methylation Profiling

The methylation profiles of the 15 patients were investigated (Table 2). Eleven cases (11/15, 73.3%) had a calibrated score of 0.9 or higher, consistent with the histopathological diagnosis. Two spinal ependymomas differed from the clinical diagnosis. Case 6 was diagnosed with PFB ependymoma caused by MC instead of spinal ependymoma, and the patient had a history of surgical removal of the PF ependymoma. Case 7 was an ependymoma that occurred in the conus medullaris of the spinal cord. The histopathological diagnosis was spinal ependymoma, but methylation profiling diagnosed it as myxopapillary ependymoma.

Two of the 4 cases of PF ependymoma were unclassifiable. The methylation superfamily of case 12 was identified as ependymal tumors, but the calibrated score was 0.38. The methylation subclass suggested PFA; however, the calibrated score was less than 0.05. The methylation superfamily in case 13 was a low-grade glial/

Table 2. Results of Methylation Classifier

Case	Pathology	Location	v12.5 Super Family	Calibrated Score	v12.5 Subclass	Calibrated Score	Remarks
Spinal							
1	EPN	Cervical	Ependymal tumours	0.99	MC Spinal ependymoma	0.99	
2	EPN	Cervical	Ependymal tumours	0.99	MC Spinal ependymoma	0.99	
3	EPN	Cervical	Ependymal tumours	0.99	MC Spinal ependymoma	0.99	
4	EPN	Cervical	Ependymal tumours	0.99	MC Spinal ependymoma	0.99	
5	EPN	Cervical	Ependymal tumours	0.99	MC Spinal ependymoma	0.99	
6	EPN	Cervical	Ependymal tumours	0.99	MC Posterior fossa group B (PFB) ependymoma, subclass 1(novel)	0.99	History of surgery for PF EPN
7	EPN	Conus	Ependymal tumours	0.99	MC Myxopapillary ependymoma	0.99	
8	MPE	CE	Ependymal tumours	0.99	MC Myxopapillary ependymoma	0.99	
9	MPE	CE	Ependymal tumours	0.99	MC Myxopapillary ependymoma	0.99	
10	MPE	CE	Ependymal tumours	0.99	MC Myxopapillary ependymoma	0.99	
11	MPE	CE	Ependymal tumours	0.99	MC Myxopapillary ependymoma	0.99	
Intracranial							
12	EPN PFB	PF	Ependymal tumours	0.38	MC Posterior fossa group A (PFA) ependymoma, subclass 1 b(novel)	0.05	PFS : 13 months OS : 30 months
13	EPN PFB	PF	Low-grade glial/glioneuronal/ neuroepithelial tumours	0.87	MC Pilocytic astrocytoma, infratentorial	0.73	
14	EPN PFB	PF	Ependymal tumours	0.99	MC Posterior fossa group B (PFB) ependymoma, subclass 5(novel)	0.99	
15	EPN PFB	PF	Ependymal tumours	0.99	MC Posterior fossa group B (PFB) ependymoma, subclass 1(novel)	0.99	PFS : 22 months

CE, Cauda equina, EPN, Ependymoma, MC, Methylation class, MPE, Myxopapillary ependymoma.

glioneuronal/neuroepithelial tumor with a calibrated score of 0.87. The methylation subclass suggested pilocytic astrocytoma; however, the calibrated score was 0.73, which was lower than 0.9.

Copy Number Variation (CNV)

Fifteen cases of CNV have been reported previously. In spinal ependymomas, the loss of chromosome 22 was observed in all cases, and the loss of chromosomes 3, 13, 14, and 21 were observed (Figure 1). Gains were confirmed on chromosomes 4, 5, 7, 9, 16, 18, and 20 in myxopapillary ependymomas. No loss was observed (Figure 2). The CNVs in PF-B ependymoma cases were diverse and did not show a consistent pattern (Figure 3).

Case 12 had an unclassifiable tumor, and CNV detected the loss of only chromosome 14 (Figure 4A). Case 13 was also an unclassifiable tumor, and the CNV amplified only part of the q arm of chromosome 7, similar to pilocytic astrocytoma (Figure 4B).

t-SNE

Cases 1 to 5 were plotted near "EPN, SPINE" and were consistent with the histopathological diagnosis and methylation profiling of spinal ependymoma. Case 6 was plotted near the "EPN, PF B" clusters in the t-SNE plot, which was thought to be a PFB ependymoma, as revealed by MC. Cases 7 to 11 were plotted near the "EPN, MPE" clusters diagnosed by MC as myxopapillary ependymoma. The closest match to the case 12 plot was a subependymoma of the PF (SUBEPN). However, it did not fall within any cluster and was unclassifiable at the MC. Case 13 was also an unclassifiable tumor in MC, and the plot in t-SNE was quite close to that of the low-grade glioma subclass midline pilocytic

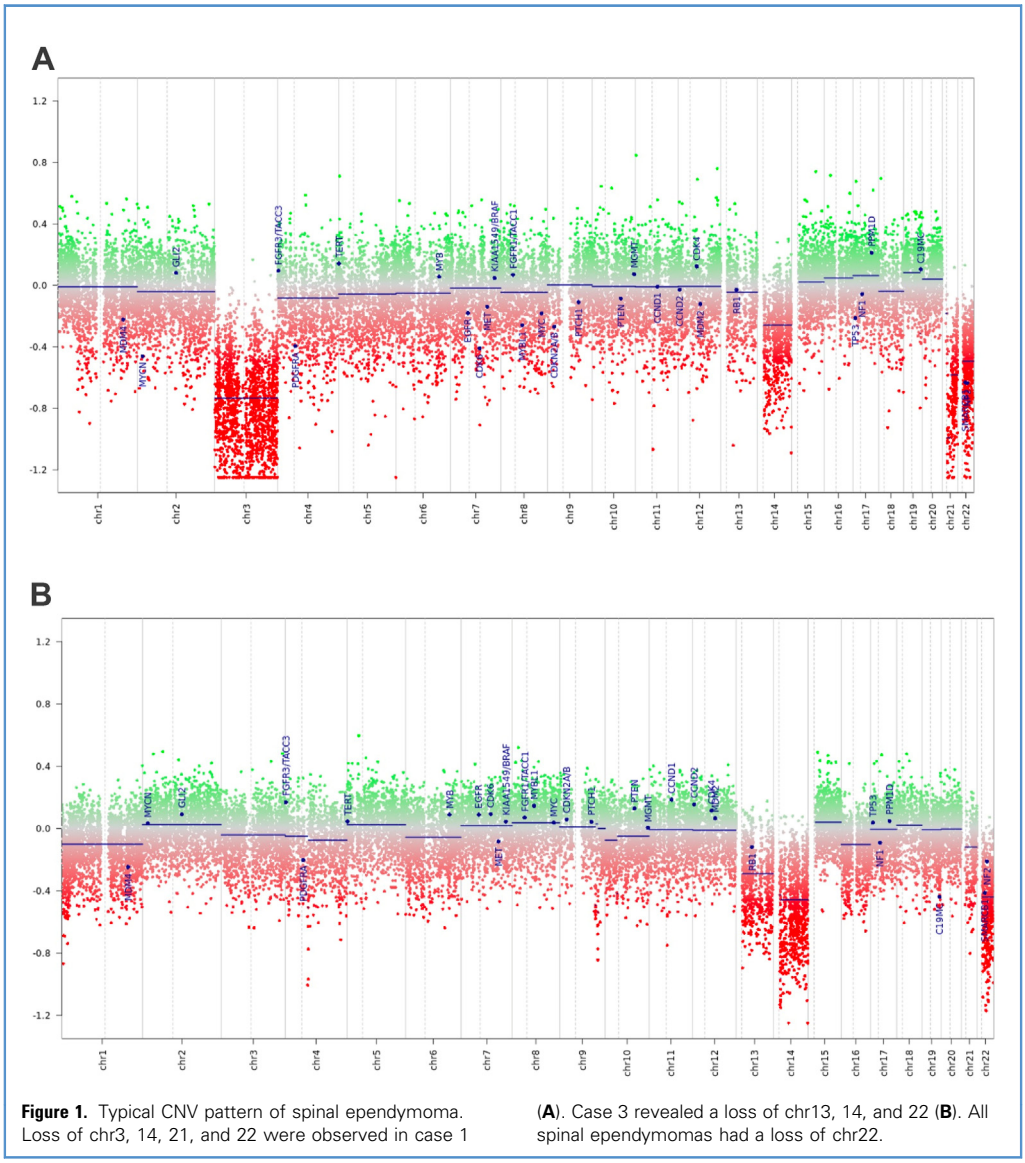

Figure 1. Typical CNV pattern of spinal ependymoma. Loss of chr3, 14, 21, and 22 were observed in case 1 (**A**). Case 3 revealed a loss of chr13, 14, and 22 (**B**). All spinal ependymomas had a loss of chr22.

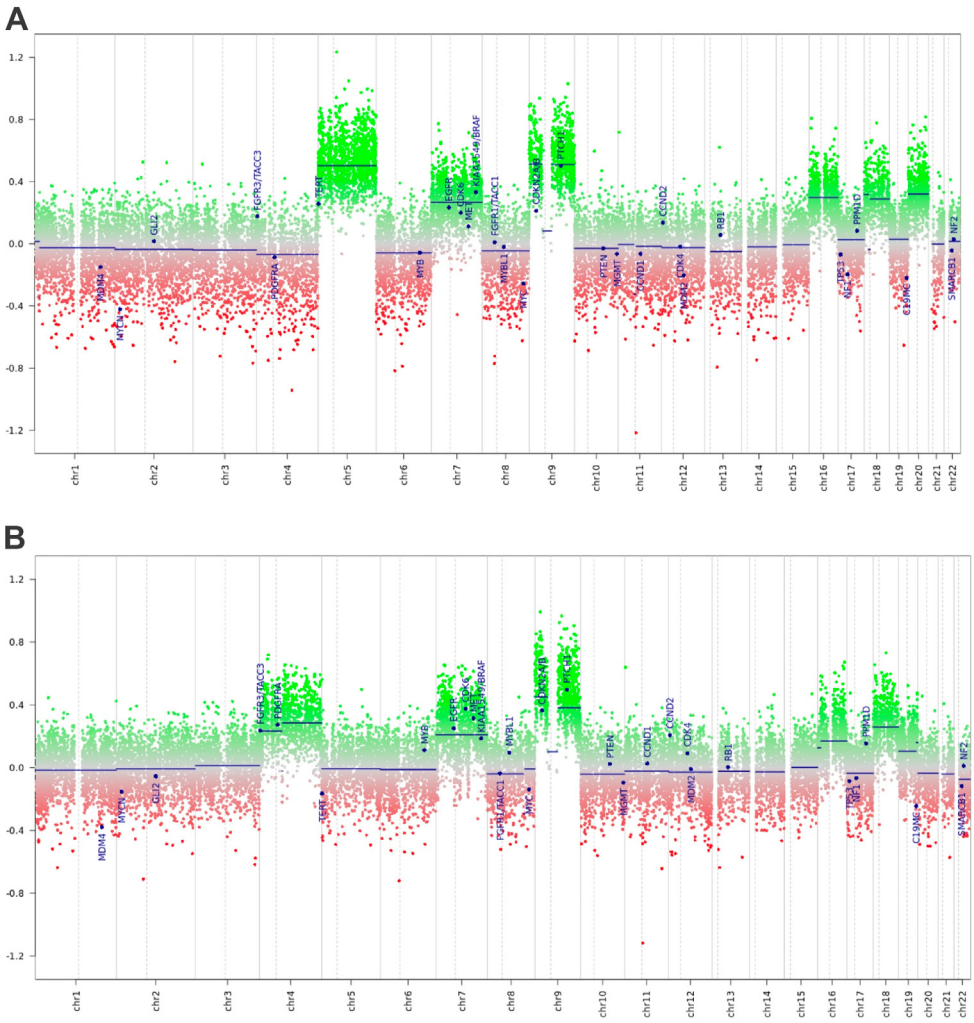

Figure 2. Typical CNV pattern of myxopapillary ependymoma. Gains were confirmed on chr4, 5, 7, 9, 16, 18, and 20 in myxopapillary ependymomas. No chromosomal loss was observed. [(**A**): Case 8; (**B**): Case 9].

astrocytoma (LGG, PA MID) but not well within the cluster. Cases 14 and 15 were plotted around the "EPN, PFB" and were consistent with histopathology and MC results (Figure 5).

Clinical, Radiologic, and Histopathologic Details of Specific Cases

Case 6 was a 27-year-old man with an intramedullary tumor, mainly located in the cervical spinal cord. He had a previous history of removal of a posterior fossa ependymoma (PFB). The histopathological findings showed a typical ependymoma pattern (Figure 6).

Case 7 was a 69-year-old woman with a tumor located in the conus of the spinal cord. Radiologic findings suggested that it was an extramedullary tumor. The differential diagnosis was myxopapillary ependymoma, but the pathologist's diagnosis at the time was ependymoma. Histopathological findings were characteristic of ependymoma, and immunohistochemical features were positive for GFAP and Vimentin, but negative for EMA, Olig-2, and AE1/AE3 (Figure 7).

Case 12 was a 3-year-old girl with a tumor in the fourth ventricle. The histopathologic diagnosis was WHO grade 2 ependymoma; histopathologically matched ependymoma, with typical findings including perivascular pseudorosette. No atypical histology was confirmed (Figure 8).

Case 13 was an 11-year-old boy with a tumor in the fourth ventricle who underwent total resection without recurrence. The histopathological diagnosis was WHO grade 2 ependymoma, but this was not a typical pathological finding. Immunohistochemical features showed that Synaptophysin and GFAP were focally positive, and negative for EMA and Vimentin (Figure 8).

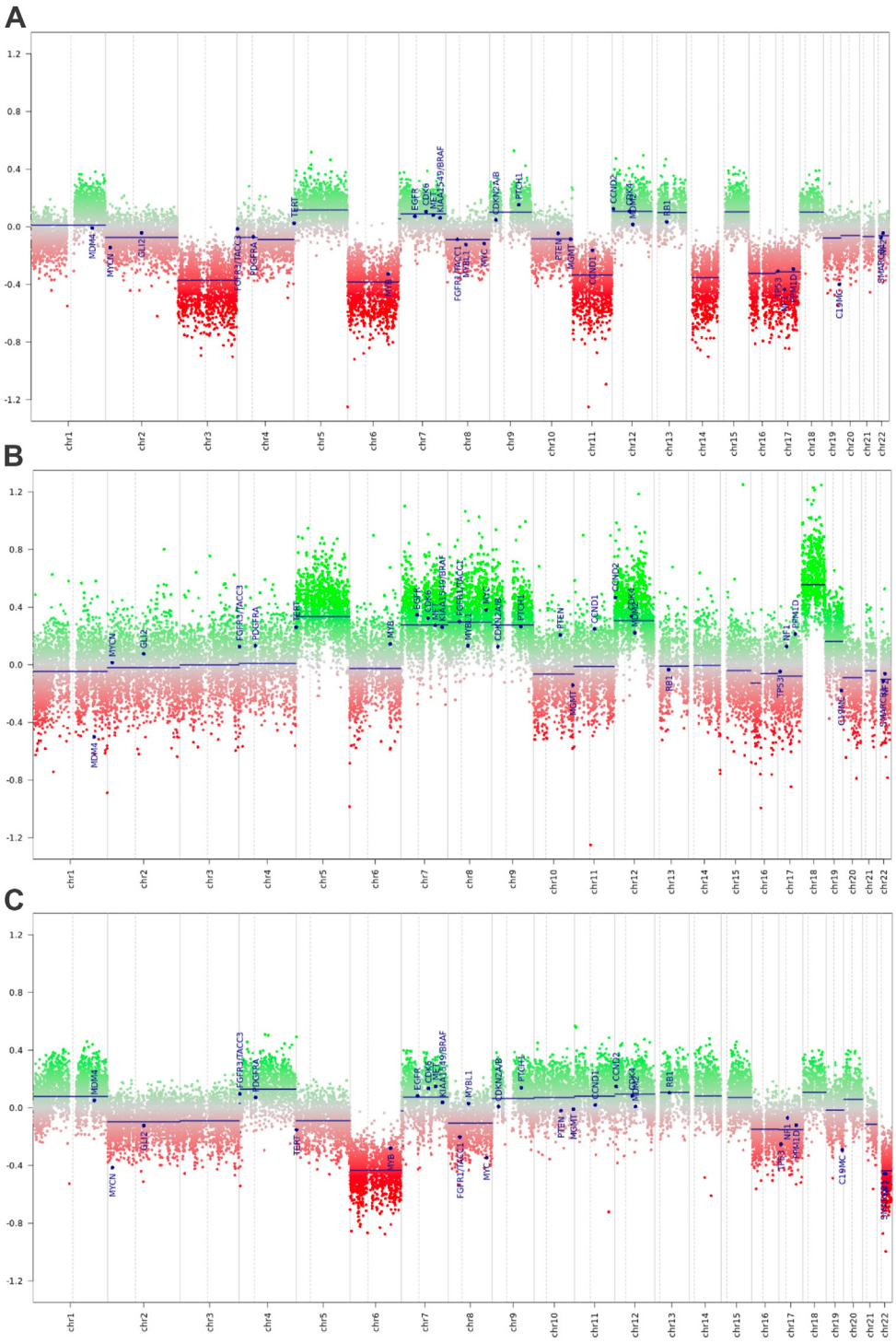

Figure 3. CNV of PF-B ependymoma. No fixed patterns were identified in these 3 cases [(**A**): Case 6; (**B**): Case 14; (**C**): Case 15].

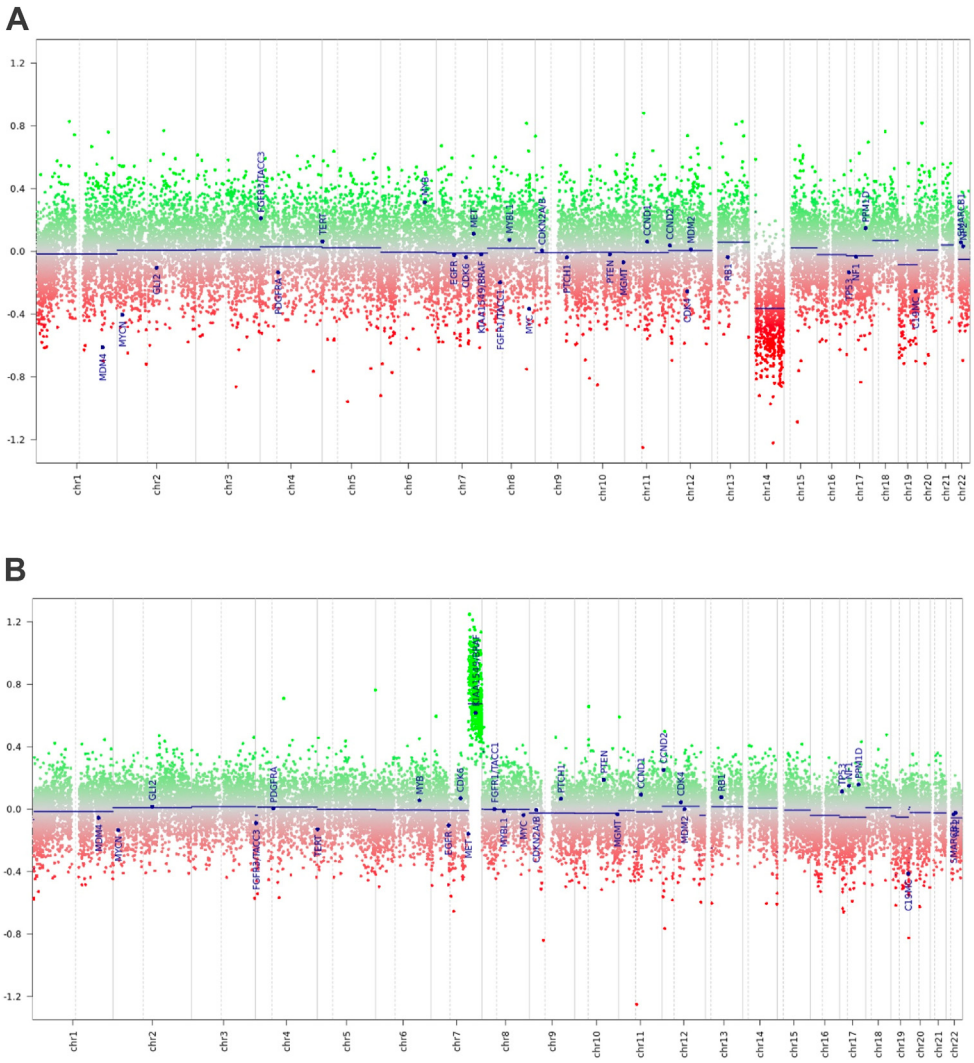

Figure 4. CNV of unclassifiable cases. Case 12 was an unclassifiable tumor, and the CNV showed a loss of only chromosome 14 (**A**). Case 13 was also an unclassifiable tumor, and the CNV amplified only in a local region on chromosome 7q, similar to pilocytic astrocytoma (**B**).

DISCUSSION

As a result of an epigenetic investigation using DNA methylation array analysis of resected ependymomas at our institution, we obtained some suggestions and found a rare case.

Although the number of cases was limited to 15, clinical diagnosis based on histopathologic findings and diagnosis based on methylation profiling agreed in 11 out of 15 cases (11/15, 73.3%) with a calibrated score of 0.9 or higher. Two cases in which the histopathology and MC were inconsistent were observed: Cases 6 and 7.

Methylation profiling clearly showed that case 6 was a disseminated spinal cord lesion of the ependymoma PFB, and the methylation status of the tumor was considered to reflect the cell of origin.[17-19] Thus, methylation profiling may help determine whether the tumor has metastasized from other sites. In addition, clarifying whether the tumor is a simultaneous multiple ependymoma or a disseminated tumor may help predict prognosis and aid in clinical judgment.[20-25] Case 7 was a tumor that developed in the conus medullaris of the spinal cord, and the histopathological diagnosis was ependymoma. However, methylation profiling revealed that this tumor was definitely a myxopapillary ependymoma. It has also been reported that myxopapillary ependymomas can be classified into further subtypes based on their methylation status.[26] Myxopapillary ependymomas tend to metastasize to the cerebrospinal fluid more frequently than other spinal ependymomas, recur repeatedly, and sometimes show poor

Figure 5. Result of t-SNE. Unsupervised clustering, t-SNE analysis was performed by R package "Rtsne," using 939 references of ependymal and glial tumors and normal tissue control selected from 2801 cases of Capper. Each reference cluster is color-coded, and our case is displayed as a black point with a CASE number.

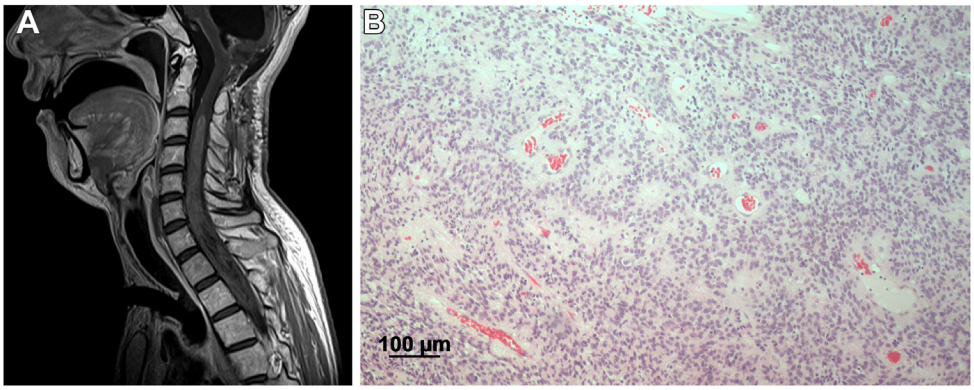

Figure 6. Contrast-enhanced MRI and hematoxylin-eosin stained (HE) specimen of Case 6 are shown. Case 6 was a 27-year-old man with a history of surgery for posterior fossa ependymoma who developed an intramedullary tumor in the cervical spinal cord (**A**). This case had perivascular pseudorosettes and was diagnosed with ependymoma WHO grade 2 according to the diagnostic criteria of the time (**B**).

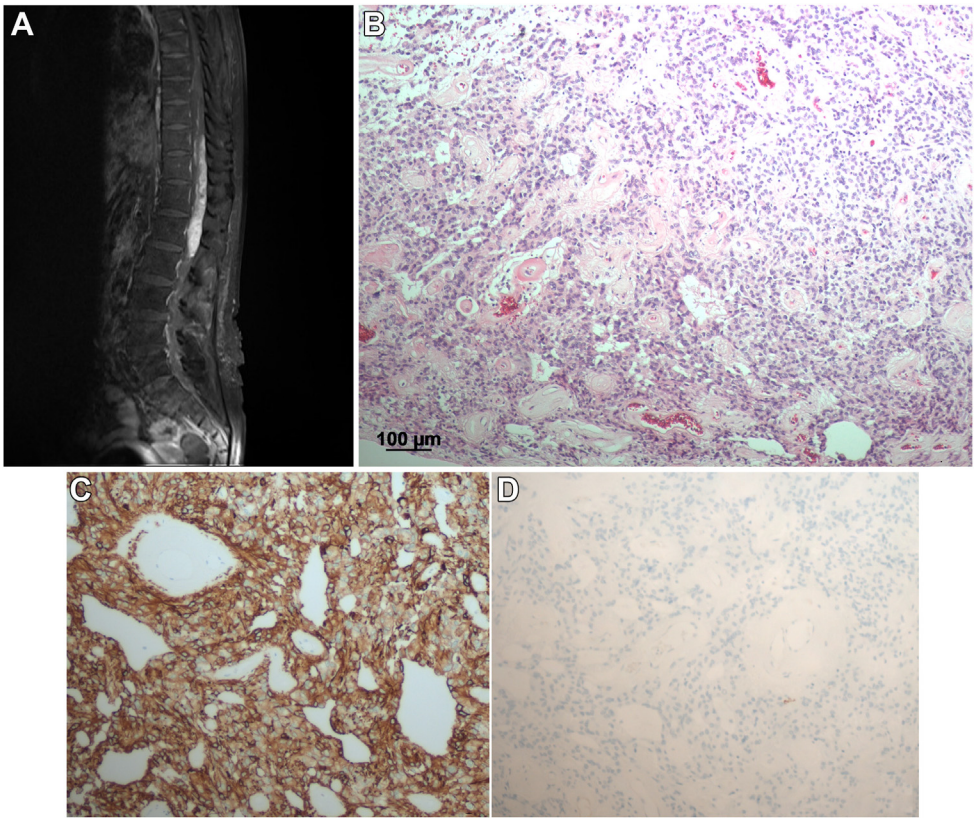

Figure 7. Radiologic and histopathologic features of Case 7 are shown. Case 7 was a tumor that occurred in the conus medullaris, as shown by contrast-enhanced MRI (**A**). The HE specimen showed findings that appeared to be ependymoma with perivascular pseudorosette. Papillary architecture, perivascular myxoid change, and focal myxoid cysts characteristic of myxopapillary ependymoma were not observed (**B**). GFAP was positive (**C**) and EMA was negative (**D**).

prognosis. The ability to distinguish myxopapillary ependymomas by methylation profiling may help in making decisions, such as patient follow-up.[27]

Interestingly, the other 2 cases were unclassifiable. Case 12 involved a 3-year-old girl with a tumor in the fourth ventricle. It was difficult to remove the part attached to the floor of the fourth ventricle; therefore, subtotal resection was performed. PFS and OS were 13 and 30 months, respectively. The histopathologic diagnosis was WHO grade 2 ependymoma. Methylation profiling was completely unclassifiable, suggesting that it may be an ependymal tumor. However, the calibrated score was 0.38, which was undiagnosable. In CNV, only Chr.14 loss was observed. t-SNEs tended to be similar to subependymomas of the PF but did not completely match. They plot far from other ependymomas and high-grade gliomas with poor prognoses. Although this tumor had an ependymal tumor-like behavior, it may be a type of tumor that has not been reported to date. If an integrated diagnosis is given for this case, it should be "tumor with ependymoma features, NEC." Further investigation is required to accumulate data in such cases. Case 13 involved an 11-year-old boy with a tumor in the fourth ventricle who underwent total resection without recurrence. The diagnosis was WHO grade 2 ependymoma; however, this was not a typical histopathological finding. The pathologist considered the possibility of a clear-cell ependymoma. However, it was difficult to conclude and comprehensively determine whether the diagnosis was ependymoma compatible, considering the patient's age, tumor location, and clinical information. When re-evaluated using methylation profiling, the superfamily was low-grade glial/glioneuronal/neuroepithelial tumors (calibrated score, 0.87), and the subclass suggested the possibility of infratentorial pilocytic astrocytoma (calibrated score, 0.73). The CNV was characteristic, amplifying only a particular part of Chr7q and showing a pilocytic astrocytoma-like pattern; however, the area of amplification was much higher than that exhibited by the true pilocytic astrocytoma KIAA1549 BRAF fusion, as previously reported.[28,29] In t-SNE, this tumor plotted near the pilocytic astrocytoma but was not complete in the cluster. Although pilocyte-like cell parts were present in the tissue retrospectively, it was presumed that it was difficult to conclude based only on the morphological diagnosis at that time. This tumor was similar to pilocytic astrocytoma with gene amplification of a large region,

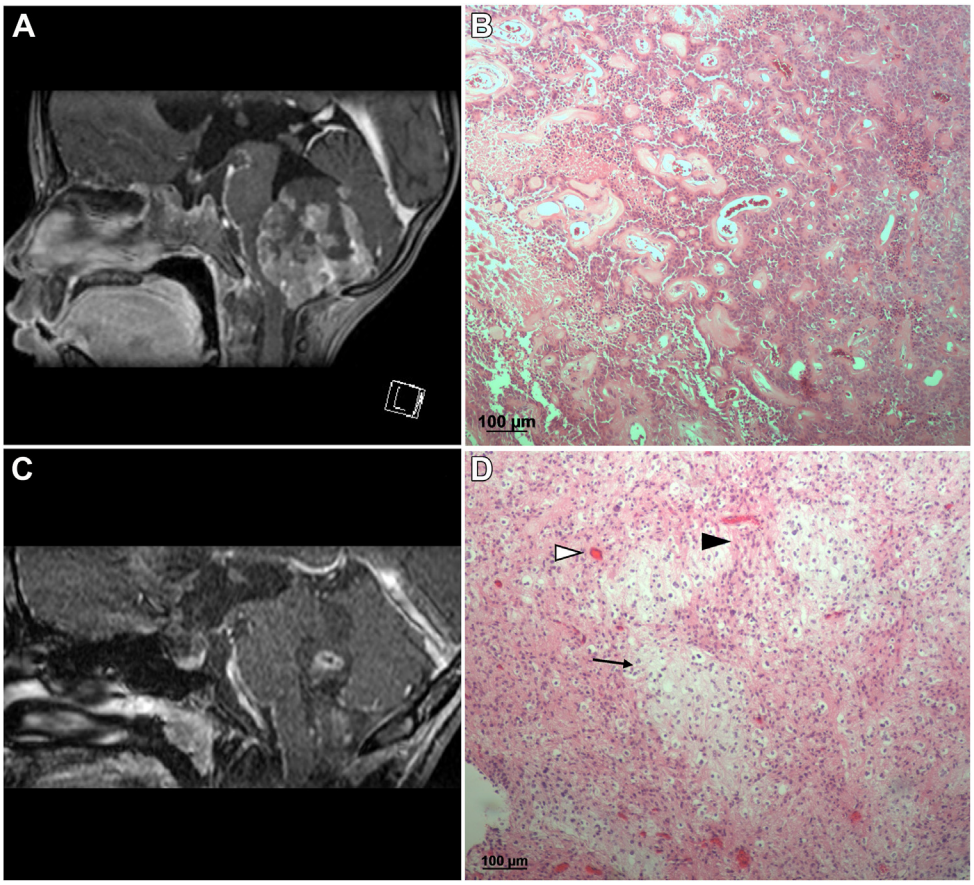

Figure 8. Radiologic and histopathologic features of Case 12 and Case 13 are shown. Case 12 was a patient with an intra-fourth-ventricular tumor [(**A**): contrast-enhanced MRI). Case 12's histopathology showed perivascular pseudorosettes and was diagnosed as ependymoma WHO grade 2 according to the diagnostic criteria of the time (**B**). Case 13 was also an intra-fourth-ventricular tumor [(**C**): contrast-enhanced MRI], and had an atypical histopathology for an ependymoma (**D**). Retrospectively, this case also included a pilocytic compartment [(**D**): arrowhead] and a clear cell-like part [(**D**): arrow). A part considered to be a perivascular pseudorosette-like pattern was also observed [(**C**): white arrowhead].

including the BRAF and KIAA1549 genes of Chr 7q; however, the possibility that it is a new category or tumor spectrum cannot be denied. Therefore, if an integrated diagnosis was to be made for this case, it might be "clear cell ependymoma with pilocytic astrocytoma features, NEC." In the previous version of the WHO classification, it was necessary to confirm the classification only by histopathological diagnosis. However, by conducting genetic investigations, including DNA methylation profiling, the borders of some tumor classes may become spectrum-like rather than separated. Of course, if the calibrated score is low, it is also necessary to consider the influence of sample quality. However, these 2 CNVs had no noise and passed the quality check.

An accurate diagnosis of ependymoma is difficult because reports from a single institution include 20% misdiagnoses. Difficulties in ependymoma diagnosis have been reported previously, leading to questions about the accuracy of studies based on large databases such as the Surveillance, Epidemiology, and End Results (SEER) database.[30] Therefore, methylation profiling may play a role in improving the diagnostic accuracy of research and statistics, although it is not yet approved for clinical diagnosis. The histopathologic diagnosis and MC were sufficiently consistent in 73.3% of the patients in our study.

CONCLUSION

DNA methylation profiling is a powerful tool for tumor diagnosis. Although there were only 15 cases in our study, DNA methylation array analysis for ependymoma suggested that it is possible to change the diagnosis from the previous diagnosis, which may help improve diagnostic accuracy and prevent misdiagnosis. However, unclassifiable cases were also discovered, indicating that this tool may be

useful for identifying new tumor types. At the same time, further investigation and accumulation of data are necessary in the future.

CRediT AUTHORSHIP CONTRIBUTION STATEMENT

Eiji Abe: Writing — original draft, Validation, Methodology, Investigation, Formal analysis, Conceptualization. **Mario Suzuki:** Investigation. **Koichi Ichimura:** Supervision, Methodology, Conceptualization. **Atsushi Arakawa:** Investigation. **Kaishi Satomi:** Investigation. **Ikuko Ogino:** Validation, Methodology. **Takeshi Hara:** Supervision. **Hirokazu Iwamuro:** Supervision. **Yukoh Ohara:** Supervision. **Akihide Kondo:** Writing — review & editing, Supervision, Conceptualization.

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