

# Methylation and molecular profiles of ependymoma: Influence of patient age and tumor anatomic location

HWA JIN CHO<sup>1\*</sup>, HA YOUNG PARK<sup>1\*</sup>, KWANGSOO KIM<sup>2</sup>, HEEJOON CHAE<sup>3</sup>, SUN HA PAEK<sup>4</sup>, SEUNG-KI KIM<sup>4</sup>, CHUL-KEE PARK<sup>4</sup>, SEUNG-HONG CHOI<sup>5</sup> and SUNG-HYE PARK<sup>6</sup>

<sup>1</sup>Department of Pathology, Inje University Busan Paik Hospital, Busan 47392; <sup>2</sup>Division of Clinical Bioinformatics, Biomedical Research Institute, Seoul National University Hospital, Seoul 03080; <sup>3</sup>Division of Computer Science, Sookmyung Women's University, Seoul 04310; Departments of <sup>4</sup>Neurosurgery, <sup>5</sup>Radiology and <sup>6</sup>Pathology, Seoul National University Hospital, Seoul National University College of Medicine, Seoul 03080, Republic of Korea

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**Abstract.** Ependymomas are tumors of the central nervous system that can occur in patients of all ages. Guidelines from the World Health Organization (WHO) for the grading of ependymomas consider patient age, tumor resection range, tumor location and histopathological grade. However, recent studies have suggested that a greater focus on both tumor location and patient age in terms of transcriptomic, genetic, and epigenetic analyses may provide a more accurate assessment of clinical prognosis than the grading system proposed by WHO guidelines. The current study identified the differences and similarities in ependymoma characteristics using three different molecular analyses and methylation arrays. Primary intracranial ependymoma tissues were obtained from 13 Korean patients (9 adults and 4 children), after which whole-exome sequencing (WES), ion-proton comprehensive cancer panel (CCP) analysis, RNA sequencing, and Infinium HumanMethylation450 BeadChip array analysis was performed. Somatic mutations, copy number variations, and fusion genes were identified. It was observed that the methylation status and differentially expressed genes were

significantly different according to tumor location and patient age. Several novel gene fusions and somatic mutations were identified, including a *yes-associated protein 1* fusion mutation in a child with a good prognosis. Moreover, the methylation microarray revealed that genes associated with neurogenesis and neuron differentiation were hypermethylated in the adult group, whereas genes in the homeobox gene family were hypermethylated in the supratentorial (ST) group. The results confirmed the existence of significantly differentially expressed tumor-specific genes based on tumor location and patient age. These results provided valuable insight into the epigenetic and genetic profiles of intracranial ependymomas and uncovered potential strategies for the identification of location- and age-based ependymoma-related prognostic factors.

## Introduction

Ependymomas can develop at all ages. However, their incidence is greatly affected by location (1), with 90% of tumors in pediatric cases occurring intracranially, and, particularly, 70% occurring in the posterior fossa (PF) (2). Supratentorial (ST) tumors affect both pediatric and adult patients (1); in adults, PF and spinal ependymomas develop with almost the same frequency (3).

The classification of tumors of the central nervous system (CNS), as per the World Health Organization (WHO) guidelines, delineates four important prognostic factors: Patient age, the extent of resection, location, and histopathological grade (1). Pediatric cases tend to be more severe because ependymomas commonly occur in the PF in children, whereas those in adults are more frequently found in the spinal cord (1). Of note, a multi-institutional retrospective analysis of patients with pediatric ependymomas revealed that anaplastic histopathological features and incomplete tumor resection led to a poor outcome in patients younger than 3 years of age (4).

Molecular genetic studies have also revealed distinct features associated with ependymoma subgroups. Intracranial and spinal ependymomas showed mutually exclusive gene mutations (5). Moreover, differential gene expression patterns among different CNS regions were recently identified (6). In addition, Taylor *et al* (6) found distinct patterns of gene

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*Correspondence to:* Professor Sung-Hye Park, Department of Pathology, Seoul National University Hospital, Seoul National University College of Medicine, 103 Daehak-ro, Jongno-gu, Seoul 03080, Republic of Korea  
E-mail: shparknp@snu.ac.kr

\*Contributed equally

**Abbreviations:** CCP, comprehensive cancer panel; CNS, central nervous system; GO, gene ontology; MAF, minor allele frequency; nsSNV, non-synonymous SNV; PF, posterior fossa; PFA, PF group A; PFB, PF group B; RELA, V-rel reticuloendotheliosis viral oncogene homolog A; SNV, single-nucleotide variant; ST, supratentorial; WES, whole-exome sequencing; WHO, World Health Organization

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expression and chromosome alterations among distinct ependymoma subsets that correlated well with the anatomic location of the tumor, but not with the clinical parameters or the tumor histological grade. Of note, ependymomas recapitulate the gene expression profiles of regionally specified radial glial cells, explaining the clinical heterogeneity among histologically similar tumors (6). This suggests that ependymoma treatments should target the appropriate cell-signaling pathways based on the tumor location.

Mack *et al* (7) studied epigenomic alterations in PF ependymomas, often associated with somatic single-nucleotide variants (SNVs); of note, these tumors are relatively rare in comparison with other organ malignancies. They divided PF ependymomas into two groups according to the patient age: PF group A (PFA) and PF group B (PFB) with PFA predominantly found in infants and associated with a poor prognosis even in the context of highly aggressive therapies, and PFB occurring in older children and adults, exhibiting a more favorable prognosis. PFA and PFB represent two very distinct molecular subgroups based on the unsupervised clustering of CpG-methylation sites, with certain genes, including *CRIP1*, *CYP26C1*, and *PKP1*, exhibiting increased CpG methylation in most PFA tumors.

Therefore, subgroups classified according to tumor location and patient age are associated with distinct transcriptomic, genetic, epigenetic, and clinical features; in fact, these classifications could be more informative for prognostic prediction than the WHO grading alone (1). Via the confirmation of the correct grouping parameters and the selection of the corresponding genes in each group, clinicians might be able to identify the major risk groups for recurrence and identify patients most suitable for adjuvant therapy. Indeed, Pajtler *et al* (8) assessed 500 cases of ependymal tumors, including cases of subependymoma and myxopapillary ependymoma, that were found across all CNS compartments, age groups, and histological grades. Through DNA-methylation profiling, they identified nine molecular subgroups, with the PFA and V-rel reticuloendotheliosis viral oncogene homolog A (RELA)-positive ependymoma groups exhibiting a very poor prognosis, indicating that risk stratification by molecular subgrouping is superior to histologic grading.

Here, we analyzed the genomic and epigenomic alterations in Korean patients with intracranial ependymomas using three different methods of molecular analysis and Illumina 450K methylation arrays. We attempted to identify differences and similarities in ependymoma characteristics according to each method. Our findings may support the development of an accurate classification system based on molecular subgroups to facilitate the prognosis and promote the use of personalized medicine approaches.

## Materials and methods

**Study subjects and experimental design.** Fresh-frozen samples were collected from 13 patients who underwent ependymoma resection surgery at the Seoul National University Hospital (Seoul, South Korea): Nine adults and four children younger than 7 years of age. The female-to-male ratio was 8:5. Tumors in nine patients were located in the PF, whereas the four other patients had ST ependymomas; no cases of spinal

ependymomas were identified in the cohort. Through a pathological review, six tumors were diagnosed as grade II and seven were diagnosed as grade III anaplastic ependymoma in accordance with the WHO guidelines for the classification of CNS tumors (1). The following identification criteria were further used to classify the tumors: Increased cellularity, frequent mitosis, abundant necrosis, and microvascular proliferation. High mitotic activity was defined when at least five mitotic figures were detected based on the results of a European clinical trial (9). Ki-67 immunohistochemical staining was also performed on all samples to assess the proliferation index of the tumor using an image analysis system (ScanScope XT; Aperio).

Ependymomas in two adults and three children were recurrent, with one resulting in death. The four surviving patients harbored grade-III tumors, all of which were located in the PF.

Of the 13 samples, some specimens did not pass DNA or RNA quality tests and showed insufficient DNA or RNA concentrations. Of note, experiments were only conducted with specimens that passed quality checks: 12 were used for methylation profiling, five were used for WES, seven were applied for targeted sequencing using an ion-proton CCP, and five were subjected to RNA sequencing.

### DNA methylation

**DNA extraction and preparation.** Genomic DNA ( $\geq 500$  ng) was isolated from all tissues. Bisulfite conversion of genomic DNA was achieved using the EZ DNA Methylation-Gold Kit (Zymo Research Corporation). Briefly, bisulfite treatment changes unmethylated cytosine nucleotides to thymidines, whereas methylated cytosines remain unchanged. This difference allows for the detection of C/T nucleotide polymorphisms at each CpG site. Genomic DNA was sent to Macrogen for hybridization on Illumina 450K methylation arrays (Illumina, Inc.). The Illumina Infinium human methylation 450 BeadChip kit (Illumina, Inc.) was used to evaluate the coverage of CpG sites throughout the genetic regions according to the manufacturer's instructions. BeadChips were scanned with an Illumina iScan apparatus (Illumina, Inc.).

**Data processing and analysis.** Following image analysis, data processing was performed using the Illumina GenomeStudio software version 2011.1 (Methylation Module v1.9.0; Illumina, Inc.) and the R package, version 3.0.2 (<http://www.r-project.org>). Data preprocessing and background correction were performed using the R library and dye-bias equalization. The detected CpGs were filtered (per sample) according to the P-value. On average, between 485,139.3 and 485,262.6 CpGs were detected with a cut-off value of  $P < 0.05$ . Next, the beta mixture quantile in the R package was used to correct any probe-design bias. To perform data transformation, the  $\beta$ -value, M-value, and  $\delta$ -mean were calculated.  $\beta$ -value represents the ratio of methylated probe intensity to the sum of methylated and unmethylated probe intensities. M-value is calculated as the  $\log_2$ -ratio of the intensities of the methylated probes vs. those of the unmethylated probes (10).  $\delta$ -mean represents the difference between the average  $\beta$ -value of the experimental results and that of the controls. The odds ratio was calculated via the transformation of the  $\delta$ -mean into the M-value and the

measurement of the ratio between the unmethylated intensity and methylated intensity for the test sample against that of a control. Fold expression changes were calculated as the ratio of methylation rates between test and control specimens.

*Hierarchical clustering analyses.* Using the M-values from significant data, 16,312 CpGs in different age groups and 5,034 CpGs in different grade groups were obtained with cut-off values of  $P < 0.05$  and a  $\delta$ -mean  $> 0.2$ . Among them, 500 CpGs were randomly selected. A heatmap of hierarchical clustering was plotted based on the distance similarity for samples and CpGs according to the Euclidean distance and complete linkage.

*Assessment of differential methylation patterns.* To determine the difference in DNA methylation profiles among samples, especially according to patient and clinical attributes such as age and tumor location, two basic approaches were adopted. First, the methylation status of CpG sites in the regulatory regions of target genes was classified: Hyper- or hypomethylated regions were defined depending on their methylation levels— $\beta$ -values of  $> 0.7$  and  $< 0.3$  were adopted as the cut-offs for hypermethylation and hypomethylation, respectively. For the second approach, we determined the differences in the absolute DNA methylation status between samples, regardless of their classification as hypermethylated or hypomethylated. For instance, if the  $\beta$ -value from a pediatric patient's sample was 0 and that of an adult patient's sample was 0.6, the first approach would not detect this difference as statistically significant, despite the difference of 0.6 in the  $\beta$ -value. In some cases, the differences obtained using the second method were greater than those using the first approach. Therefore, to capture the absolute differences in DNA methylation, the  $\beta$ -values between samples for the same CpG sites were subtracted from both the age and tumor location groups. In this approach, a  $\beta$ -value cut-off of 0.4 was set to identify significant differences between groups.

*Gene enrichment and functional annotation analyses.* Through these two analysis approaches, we successfully extracted target genes with a significant difference in DNA methylation in their regulatory regions (especially within the promoter and gene body regions). To examine gene ontology (GO), gene set analysis was performed using ToppGene (<https://toppgene.cchmc.org/>), a gene list enrichment analysis and candidate gene prioritization system based on functional annotations and protein interaction networks (11).

#### WES

*DNA sequencing.* DNA from five resected fresh-frozen ependymoma samples was extracted using the EZ DNA methylation gold kit (Zymo Research Corporation). DNA extracted from blood samples of the same patients was used as germline control. To enrich the coding regions, the SureSelect target-enrichment system capture process (Agilent Technologies, Inc.) was used. Sequence reads were generated using the Illumina HiSeq 2500 platform (12).

*Data analysis.* Raw sequence reads were mapped against the hg19 reference genome using a Burrows-Wheeler aligner (13).

After creating a BAM file, PCR duplicates were removed using Picard (<https://broadinstitute.github.io/picard/index.html>). Insertion and deletion (indel) realignment and base quality score recalibration were performed using the Genome Analysis Tool Kit (14,15). The VarScan2 software was used to detect somatic SNVs and small indels (16). ANNOVAR was used to annotate variants. To identify confident and rare somatic variants in the coding regions and splice sites, the following filtering criteria were applied: i) tumor total allele count  $\geq 10$ , tumor altered allele count  $\geq 3$ , and normal altered allele count = 0; ii) rare variants were based on a minor allele frequency (MAF)  $< 0.5\%$  in the Exome Aggregation Consortium Version 0.3 (ExAC03; <http://exac.broadinstitute.org>) (17).

*Comprehensive cancer panel.* To identify the mutation status in 409 cancer-related genes, targeted sequencing of seven tumor samples was performed using the Ion AmpliSeq Comprehensive Cancer Panel (CCP; Life Technologies; Thermo Fisher Scientific, Inc.). Mutations on the list showing non-synonymous SNVs and indels in coding regions with an altered allele frequency  $\geq 0.1$ , and rare variants based on a MAF  $< 0.5\%$  in the Exome Aggregation Consortium Version 0.3 were used for further analysis.

*RNA sequencing.* Using the Illumina TruSeq RNA sample preparation kit (Illumina, Inc.), mRNA was converted into a template library for subsequent cluster generation. Purified mRNA was fragmented, and reversely transcribed into cDNA. DNA polymerase I and RNase H were then used to construct second-strand cDNA. cDNA fragments had an additional adenines added to their 3'-end and the adapters were then ligated. Products were then enriched by PCR and HiSeq 2000 (Illumina, Inc.) was used for bridged-amplification reactions and imaging. Images of single-base extensions at a specific cluster were generated.

Sequence quality checks were performed using Fast (v0.10.0; [www.bioinformatics.babraham.ac.uk/projects/fastqc/](http://www.bioinformatics.babraham.ac.uk/projects/fastqc/)). The alignment was performed via TopHat (v1.3.3; <https://ccb.jhu.edu/software/tophat/index.shtml>), a fast splice-junction mapper for RNA-seq reads, with hg19 (18). Cufflinks (v2.0.2; <http://cole-trapnell-lab.github.io/cufflinks/>) was used for transcript assembly and to test for the differential expression and regulation of RNA-seq samples (19). Gene expression was measured in terms of fragments per kilobase of transcript per million mapped reads.

To detect SNVs and make annotations, SAMtools was used; alignments were manipulated in the SAM format (<http://samtools.sourceforge.net/cns0.shtml>). ANNOVAR (<http://www.openbioinformatics.org/annovar/>) and DeFuse (v0.4.3; <http://sourceforge.net>) were used to annotate functional genetic variants and to discover fusion genes, respectively.

Differentially expressed genes were detected via the comparison of the mean of the original value for each group against the mean of the Z-score for each group. Genes exhibiting the largest differences in expression were analyzed and validated via a literature search for their relevance to ependymoma, and included in the human disease database (MalaCards; <http://malacards.org/card/ependymoma?limit;>

Table I. Summary of the clinicopathologic and genomic characteristics of the patients included in the present study.

Variable	P1	P2	P3	P4	P5	P6	P7	P8	P9	P10	P11	P12	P13
Age (years)	32	48	53	48	25	41	29	48	6	2	3	3	38
Sex	F	M	F	F	F	M	F	M	F	M	M	F	F
Tumor location	ST	ST	PF	PF	PF	PF	PF	ST	ST	PF	PF	PF	PF
Recurrence	N	N	N	N	N	N	Y	Y	N	Y	Y	Y	N
Death	N	N	N	N	N	N	N	N	N	N	Y	N	N
Histologic grade	GII	GII	GII	GII	GIII	GIII	GIII	GIII	GII	GIII	GIII	GIII	GII
No. of SNVs by WES	NA	NA	9	NA	9	12	NA	NA	3	NA	NA	9	NA
No. of fusion genes by RNA sequencing	NA	NA	2	NA	3	0	3	NA	1	NA	NA	NA	NA
CCP	A	A	NA	A	NA	NA	NA	A	NA	A	A	NA	A
Methylation	A	A	A	A	A	A	A	A	A	A	A	A	NA

P, patient; F, female; M, male; ST, supratentorial; PF, posterior fossa; N, No; Y, Yes; G, grade; SNVs, single nucleotide variants; WES, whole exome sequencing; NA, not applicable; A, analyzed; CCP, comprehensive cancer panel.

[MaladiesGenes]=128#related\_genes). The Student's *t*-test was used to identify expression differences.

## Results

**Histopathological features.** Of the thirteen samples, six were classified as grade II ependymoma (low-grade; LG) and seven were classified as grade III anaplastic ependymoma (high-grade; HG). Increased cellularity was observed in five grade II ependymomas and in all grade III ependymomas. The mitotic count ranged from 0 to 4 (mean, 2.5) in grade II, and from 11 to 26 (mean, 19.0) in grade III ependymoma samples. Microvascular proliferation was observed in only one grade III ependymoma. Abundant necrosis was observed in four cases of grade III ependymoma and focal necrosis was observed in three cases of grade II ependymoma. The Ki-67 staining index ranged from <1 to 11.64% (mean, 5.0%) in grade II ependymomas, and from 4.48 to 32.97% (mean, 16.2%) in grade III ependymomas. The clinicopathological information of the patients, the experimental design and the summarized genomic characteristics are shown in Tables I and SI.

### DNA methylation

**Hierarchical clustering.** Significant differences in the methylation rates associated with patient age, tumor grade, and location were assessed via hierarchical clustering. Hierarchical clustering according to age respected the significance criterion ( $P < 0.05$ ) with an absolute  $\delta$ -mean value of  $>0.2$  using 500 randomly selected CpGs (Fig. 1A). In each clustered age-group, unsupervised clustering by anatomic location (ST and PF) was prominently observed. On the other hand, other factors, including grade and prognosis (recurrence), were not categorized in the same groups.

Hierarchical clustering by grade also showed clustering by location in each grade group (Fig. 1B). The HG group was separated into the ST group, including one patient, and the PF group, including six patients. Children and adults were clustered as separate groups for HG tumors in the PF. Overall, the methylation patterns showed meaningful

differences with respect to both tumor location and patient age.

**Methylation profile based on age and location.** We next counted the number of genes exhibiting significant differences in methylation based on patient age and tumor location. Many genes were identified to have a  $\beta$ -value difference of  $>0.4$  between adults and children (Table SII); of note, 66 genes were included in the adult-hypomethylated group and 425 genes were included in the adult-hypermethylated group. Three of these genes (*PDE4C*, *HOXA9*, and *TBX5*) in the adult-hypermethylated and child-hypomethylated groups showed the most substantial age-related differences, in line with data previously reported by Koch and Wagner (20) in an epigenetic study. These genes showed higher methylation in adults vs. children. Thus, using the absolute  $\beta$ -value is preferred over using the difference in the methylation rate to determine target genes in each age or location group due to the potential interference of age-related methylation. Of note, we used  $\beta$ -values of 0.7 and 0.3 as the hypermethylation and hypomethylation cut-off values, respectively, because we could not obtain significant data using the cutoff points of 0.8 or 0.2 proposed by Rogers *et al.* (2).

GO analysis revealed that a high proportion of genes displaying hypermethylation in adults was included in GO sets; 20 of the 1,638 genes associated with neurogenesis were identified in our samples, as well as 16 of the 1,405 genes associated with neuron differentiation (Fig. 2A). Many genes belonging to the HOX family were observed in several pathways. We also found many genes associated with the development of multicellular organisms (neurogenesis and embryo development) in the biological process category of GO enrichment analysis (Fig. 2B). Moreover, the analysis of Reactome pathways revealed that genes related to G protein-coupled receptors and amine compound solute carrier transporters were enriched (Fig. 2C). Importantly, these pathways are crucial targets for drug development.

Moreover, many genes showed  $\beta$ -values  $>0.4$  between the PF and ST groups (Table SIII). Sixty-one genes were included in the PF-hypomethylated group and fourteen genes

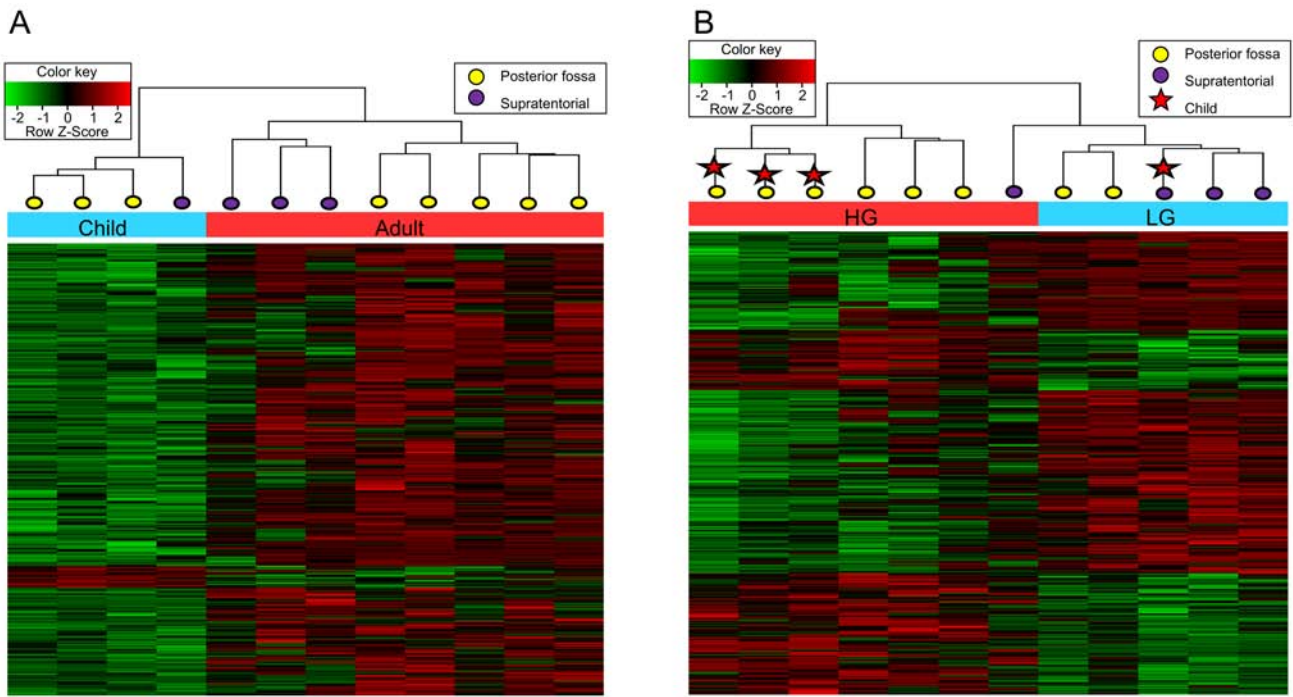


Figure 1. Two-way hierarchical clustering dendrogram of methylation patterns between adults and children. (A) and LG and (B) HG tumors. (A) unsupervised hierarchical clustering by location (supratentorial and PF) was prominently observed via supervised hierarchical clustering by age using 500 randomly selected CpGs. (B) Clustering by location in each grade group was observed via supervised hierarchical clustering by grade. In the HG (n=7) and PF (n=6) groups, clustering according to both age and location was observed. LG, low grade; HG, high grade; PF, posterior fossa.

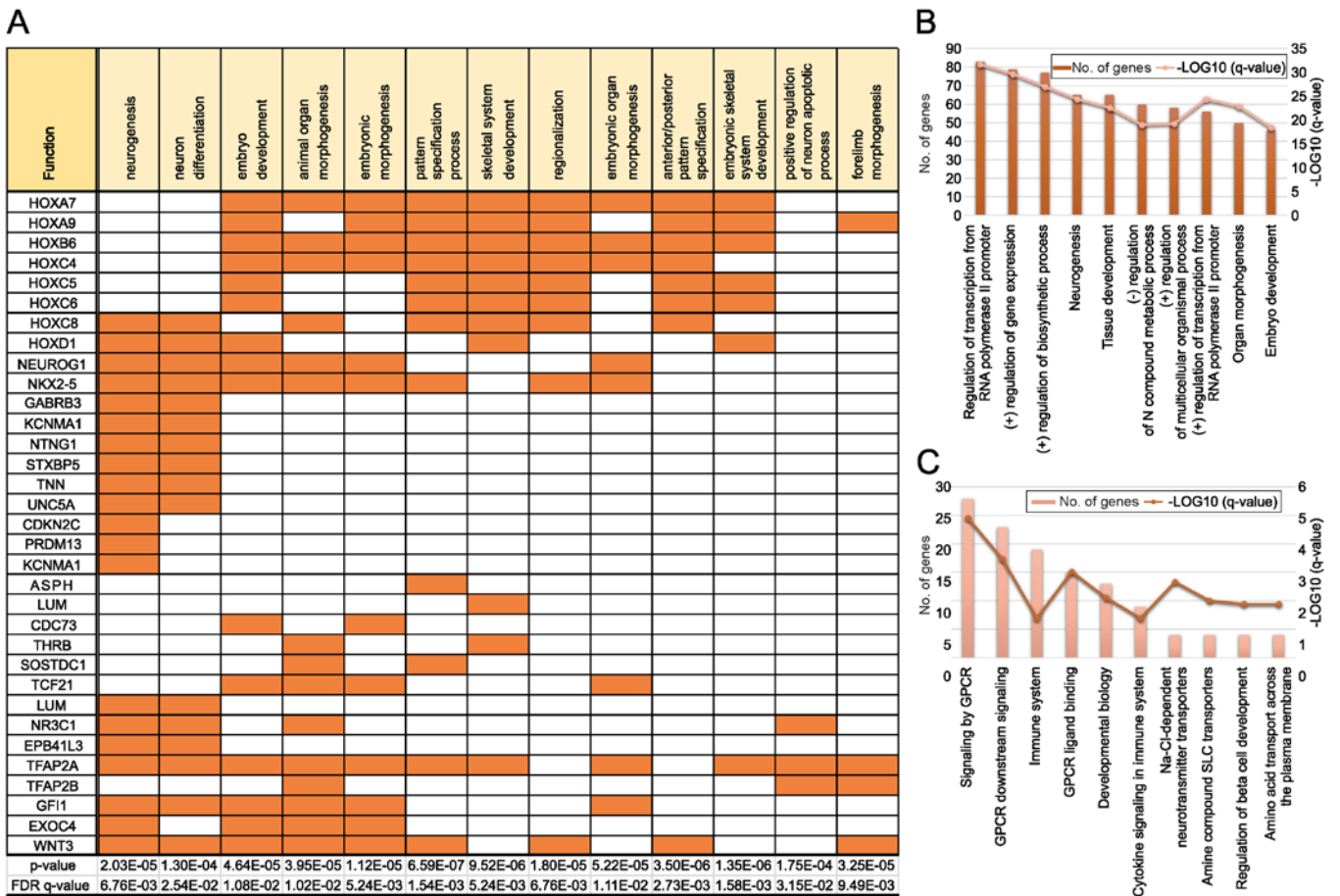


Figure 2. Functional enrichment of hypermethylated genes in the adult group. (A) Genes that were hypermethylated in the adult group were sorted per gene ontology functions. (B) The top 10 gene sets in the biological process category of gene ontology enrichment analysis. (C) The top 10 reactome pathways enriched in hypermethylated genes. GPCR, G-protein coupled receptor; SLC, solute carrier.

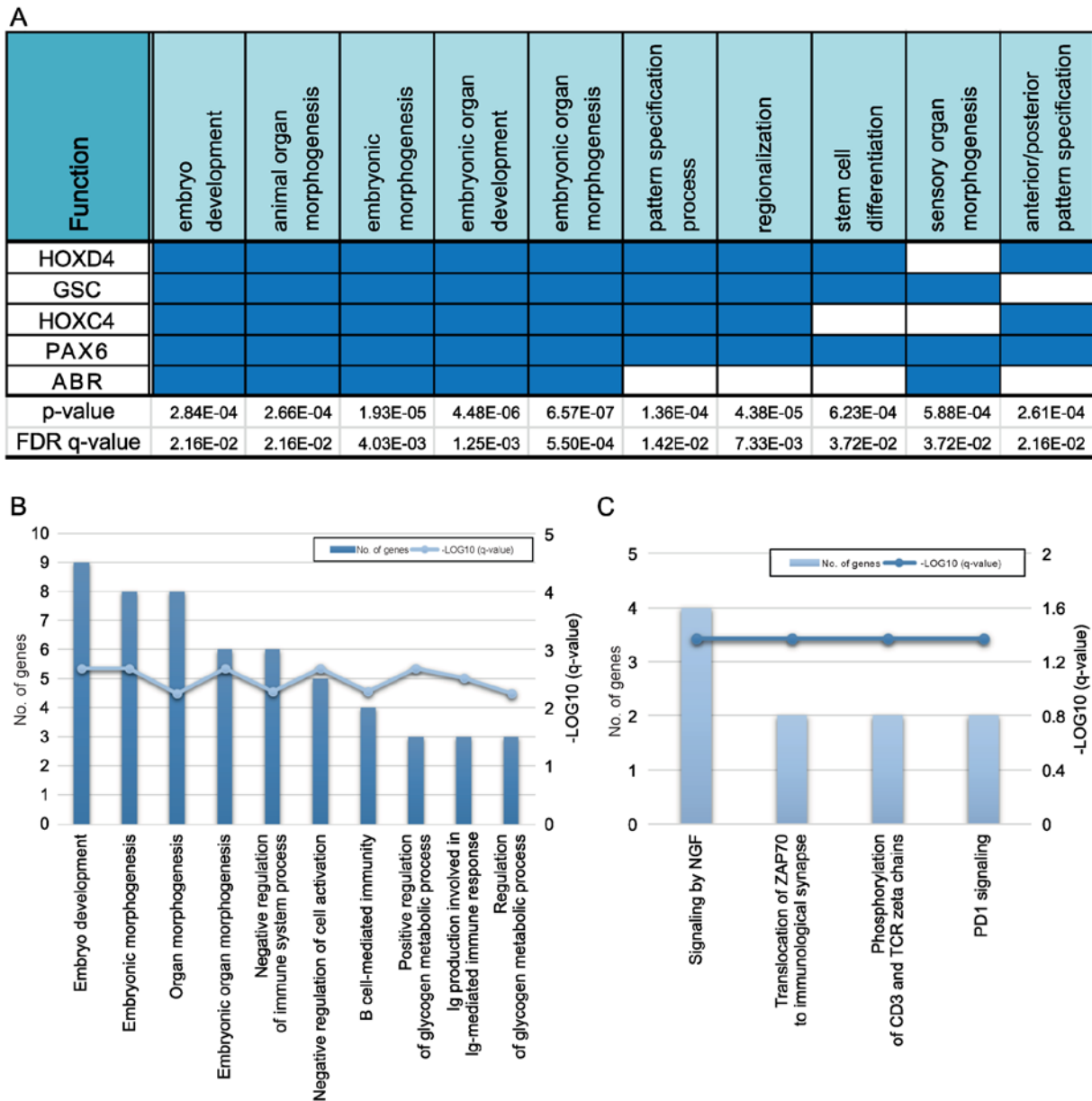


Figure 3. Functional enrichment of hypermethylated genes in the ST group. (A) Genes that were hypermethylated in the ST group sorted per gene ontology functions. (B) The top 10 gene sets in the biological process category of gene ontology enrichment analysis. (C) Reactome pathways enriched in hypermethylated genes. ST, supratentorial; NGF, nerve growth factor; ZAP70, zeta-chain-associated protein kinase 70; TCR, T-cell receptor; PD1, programmed death 1.

were included in the PF-hypermethylated group. However, no significant genes were detected in the PF-hypermethylated group, as per the GO analysis.

GO pathway analysis revealed that five genes (*HOXD4*, *GSC*, *HOXC4*, *PAX6*, and *ABR*) overlapped in a single biological process category (Fig. 3A). Among them, *HOXD4*, *HOXC4*, and *PAX6*, identified in the PF-hypomethylated/ST-hypermethylated group, were associated with hindbrain development during early embryogenesis. Moreover, many genes associated with the development of multicellular organisms (embryo development and embryonic morphogenesis) and the development of embryonic organs (organ morphogenesis and embryonic organ morphogenesis) were identified in the biological processes' categorization of the GO enrichment analysis (Fig. 3B). Finally, nerve growth factor signaling-associated genes were enriched, as per the Reactome pathway analysis (Fig. 3C).

*WES*. We performed *WES* for five paired tumor-normal tissue samples, including two LG tumors and three HG tumors. Three patients were adults and two were children. Tumors of four of these patients were located in the PF and the tumor of one patient was located in the ST.

Fifty-eight non-synonymous SNVs (nsSNVs) were identified in the five paired samples, ranging from 7-14 nsSNVs per sample. The median number of nsSNVs per Megabase was 0.26 in adults and 0.20 in children. After filtering for rare mutations, each sample showed 3-12 nsSNVs. Interestingly, the mean number of rare nsSNVs in children was smaller than that in adults, although the difference was not statistically significant (Table SIV).

In four samples with both *WES* and RNA sequencing data available, the SNVs were checked to determine whether they were transcribed or edited in the context of RNA sequencing.

Table II. Fusion genes identified using RNA sequencing.

Patient ID	Gene name 1	Gene name 2	Split read count	Span read count	Gene 1 chromosome	Gene 2 chromosome	Genomic break position 1	Genomic break position 2
P3	PDE4DIP	AL592284.1	10	6	1	1	144952201	144508284
	PTMS	PCBD2	5	8	12	5	6879325	134260382
P5	HNRNPA2B1	RGPD2	6	5	7	2	26236085	88124575
	ZFP36L1	RGPD2	4	5	14	2	69257549	88124733
	EEF1G	PCBD2	6	5	11	5	62339358	134260418
P7	RAB6C	OR4Q3	7	5	2	14	130737953	20134468
	GFAP	PCBD2	15	15	17	5	42991130	134260432
	IRAK2	NDFIP1	1	5	3	5	10282302	141532120
P9	YAPI	MAMLD1	13	6	11	X	102076805	149638017

PDE4DIP, phosphodiesterase 4D interacting protein; PTMS, parathymosin; PCBD2, pterin-4- $\alpha$ -carbinolamine dehydratase 2; HNRNPA2B1, heterogeneous nuclear ribonucleoprotein A2/B1; RGPD2, RAN binding protein 2-like and GRIP domain containing 2; ZFP36L1, zinc finger protein 36 homolog like 1; EEF1G, eukaryotic translation elongation factor 1 gamma; RAB6C, *Rab* protein family 6C; OR4Q3, olfactory receptor family 4 subfamily Q; GFAP, glial fibrillary acidic protein; IRAK2, interleukin-1 receptor-associated kinase 2; NDFIP1, Nedd4 family interacting protein 1; YAPI, yes-associated protein 1; MAMLD1, mastermind-like domain containing 1; P, patient.

SNVs identified in seven genes (*HARS2*, *SPSB3*, *BAZ2B*, *TRIO*, *SNX13*, *PLXNA4*, and *PARP12*) in WES were indeed transcribed, as per the RNA sequencing results (Table SV). In contrast, no case of editing was identified.

In the copy number variation (CNV) analysis, patient 3 (53-year-old female; LG and PF) showed insertions in chromosomes 1, 4, 5, 7, 8, 9, 12, 14, 15, 18, and 19. Patient 5 (25-year-old female; HG and PF) showed gains in chromosomes 12, 19 and 20, and losses in chromosomes 3, 6, 13, 16, 17, 21 and 22. Patient 6 (41-year-old male; HG and PF) showed an increased copy number in chromosome 1q and losses in chromosomes 3, 5, 17, 21 and 22. Patient 9 (6-year-old female; LG and ST) showed copy loss in chromosome 11, and patient 12 (3-year-old female; HG and PF) showed copy gain in chromosome 19. In summary, three of four PF cases showed a copy gain in chromosome 19, which is a rare finding. Moreover, children showed fewer CNVs than adults, as expected. Interestingly, trisomy 19 ependymomas, classified as ST WHO grade-III tumors in children (21), are usually associated with a clear cell morphology, which was not evident in our cases.

**Comprehensive cancer panel.** To identify the mutation status of cancer-related genes, we used the Ion AmpliSeq CCP. Among the 409 oncogenes included in the panel, 249 SNVs and indels in 156 genes were identified. A few mutations were observed in all seven patients. The *FGFR3*, *KIT*, and *TAF1L* genes harbored nonsynonymous SNVs, whereas the *KMT2C* gene harbored a deletion-insertion mutation; however, none of the mutations were pathogenic. Of note, we excluded two indels that occurred in a repeat area, which is a common error of the Ion AmpliSeq platform.

Mutations in the *CASC5* gene were identified in both WES and CCP analyses; however, their positions were different. Despite filtering common variants according to the population MAF database, probable germline variants were difficult to filter out without the corresponding information on paired normal samples.

**RNA sequencing.** The RNA sequencing results for five patients revealed the presence of multiple fusion genes (Table II), including a *YAPI* fusion in patient 9 (child; ST and LG), common in cases of pediatric ST ependymomas. The other fusion genes identified in three patients were novel fusion genes.

We compared the results with respect to severity (LG, n=2 vs. HG, n=3), age/location (adults or PF area, n=4 vs. children or ST, n=1), and sex (female, n=4 vs. male, n=1). Differentially expressed genes were detected via the comparison of the mean of the original value for each group against the mean of the corresponding Z-score. Genes exhibiting the largest differences in expression were analyzed; validation was further achieved via a literature search: Their relevance to ependymoma was studied and they were included in the MalaCards human disease database.

The differences in the original values associated with the RNA-seq results indicated that upregulated genes were preferentially ranked. In fact, genes such as *GFAP* and *SI00B* exhibited high levels of expression in the context of ependymoma. Therefore, they could not be used to confirm the presence of group differences. Because differences in Z-scores reveal relative differences in expression, originally small values have no significance. Therefore, the possibility of finding group-specific genes is only high when using intersecting genes.

The differences in the expression levels were the greatest between females and males in all group comparisons based on correlations. Our analysis showed that 595 genes were differentially expressed between the LG and HG groups (P<0.05). Of these genes, four (*EZR*, *PDPN*, *SHC3*, and *PRG2*) were directly related to ependymoma according to the MalaCards analysis.

## Discussion

Our data support the recent WHO guidelines describing ependymoma prognosis parameters using a 'cell of origin' concept,

suggesting that distinct groups of stem cells are specific to anatomical sites, and that the different origins of the stem cells can explain the predominant locations of ependymomas detected in different age groups (6). Our methylation analysis and RNA sequencing results suggested that there may be differences in gene expression profiles between different age groups as well as anatomical locations. In addition, the hierarchical clustering of 500 randomly selected CpG associated with differentially expressed genes ( $P < 0.05$  and  $\Delta\text{mean}$ ) demonstrated prominent differences between the ST and PF groups in both children and adults. Although ependymoma histology cannot reveal the clinical differences corresponding to patients with ST or PF tumors, the genetic profiles implied the presence of distinct phenotypes related to tumor location.

Drugs that target CpG methylation or histone demethylase inhibitors are considered potential therapeutic agents for the treatment of pediatric PFA ependymomas (22). Based on our results, targeting certain genes of the *HOX* family in adults (*HOXA7*, *HOXA9*, *HOXB6*, *HOXC4*, *HOXC5*, *HOXC6*, and *HOXC8*), or in ST tumors (*HOXC4*, *HOXD4*), may indeed be a useful treatment strategy. Of note, the *HOX* gene family plays a key role in development, providing the anterior and posterior axial coordinates for vertebrate embryos (23); e.g., the expression of *HOXA1-2* was associated with the hindbrain, whereas that of *HOXA9-13* was predominantly seen in the extremities (24).

A few *HOX* family genes have been implicated in spinal cord ependymomas; for instance, *HOXA9* (6), and *HOXB5* (25) were highly expressed in spinal tumors. In fact, some *HOX* family members, especially *HOXA9*, are selectively expressed in spinal ependymomas (26). However, in our study, *HOXA9* was hypermethylated in intracranial ependymomas, in adults. Koch and Wagner (20) reported that *HOXA9* was an age-related gene in different tissues such as the dermis or cervical smear cells. Further studies may be necessary to discover the function of *HOXA9* in intracranial ependymomas associated with aging.

In our epigenetics analysis using a methylation array, large differences in the methylation levels of many genes were detected between age groups. In adults, several more genes were associated with hypermethylation than with hypomethylation; these were largely related to neurogenesis and neuron differentiation (as per the GO analysis), representing a meaningful association with respect to tumor properties. Genes associated with hypomethylation in the PF group mainly included those in the *HOX* family, which Taylor *et al.* (6) suggested as a representative gene family that maintains the cancer stem cell phenotype in spinal ependymomas.

Molecular alterations are common in ependymoma. Therefore, accurate molecular subgrouping is necessary to predict patient outcomes and determine the best therapeutic approach. In the revised fourth edition of the WHO guidelines for CNS diseases, nine molecular groups were suggested based on a single study conducted by Pajtler *et al.* (8). This analysis focused on the use of DNA-methylation profiling to determine ependymoma subgroups and identified two ST subgroups characterized by the fusion genes *RELA* and *YAPI* (8). Their results also indicated molecular subgroups showing distinct copy number profiles, consistent with our findings concerning CNVs, as per WES analysis.

We compared our WES data with those reported by Korshunov *et al.* (27), who proposed three distinct molecular stages for intracranial ependymoma, based on patterns of cytogenetic alterations in 122 primary ependymoma samples from adults and children. Group 1 ependymomas were characterized by numerous aberrations affecting whole chromosomes or chromosome arms, including gains in chromosomes 9, 15q, and/or 18, and/or a loss in chromosome 6 without a 1q gain, and/or *CDKN2A* deletion. These alterations were observed in 34% of all patients and were associated with a 100% 5-year overall survival rate. Of note, patients 3 and 5 in our study exhibited characteristics that would place them into this group, and these patients were disease-free for 2 years and 1 year, respectively. Group 2 ependymomas, were characterized by a largely balanced genomic profile, and were observed in 42% of patients, with 5-year overall survival rates of 78% (27). In our study, patient 9 and patient 12 could be classified into this group. Patient 9, who was diagnosed with ST/LG ependymoma showed no recurrence for 49 months. However, patient 12, a child with PF/HG ependymoma, showed recurrence for five times, despite repeated gamma knife radiosurgery and radiotherapy. Finally, group 3 ependymomas showed copy number gains in chromosome 1q and/or homozygous *CDKN2A* deletion; 25% of patients were classified in this group, exhibiting 5-year survival rates of 32%, constituting the worst overall prognosis. Patient 6 in our study was included in this group and experienced no recurrence or death over a 16-month follow-up period. Overall, these results indicate that the CNV data alone cannot be used for an accurate molecular classification, due to the potential poor reproducibility.

Compared to the methylation microarray analysis, several molecular analysis tools have failed to find sufficient evidence to support genetic features associated with a particular disease or subtype. Before filtering the WES data, the absolute number of somatic mutations per Megabase was larger in adults, possibly resulting from the presence of age-related mutations. On the other hand, after filtering, few nsSNVs were detected via WES, especially in children, compared to those reported in other malignancies, in line with a previous analysis of PF ependymoma in children (7). In addition, a few mutations were identified in the CCP analysis, and no mutations were common between the WES and CCP datasets.

We detected a few fusion genes via RNA sequencing, including a *YAPI* fusion in a pediatric patient who had a tumor in the ST area and showed a good prognosis. This result is in line with the Pajtler's molecular classification (8). The other novel fusion genes identified in this study are not listed in the COSMIC database, possibly indicating that they are not functional. Another well-known fusion gene in addition to *YAPI* is *RELA*, which has been identified in about 70% of ST ependymoma cases in children (28). The C11orf95-*RELA* fusion is the most common structural variant found in ependymomas (8). In our RNA fusion study, only one available sample was from the ST; remarkably a *YAPI* fusion was detected in that sample. However, we did not find any *RELA* fusion in the five ST samples, possibly due to the small sample size.

Among the genes displaying a significant difference between the adult/PF and children/ST groups with respect to both the original genetic value and Z-scores in RNA sequencing, only *TIMP3* was listed in the human disease



database MalaCards. *TIMP3*, which plays a role in the development of the CNS and in the proteolysis pathway, was identified as one of the candidate methylated genes re-expressed in short-term cell cultures of ependymoma samples from pediatric patients following treatment with the demethylating agent 5-aza-2'-deoxycytidine (29). This suggests that the methylation of *TIMP3* plays a major role in ependymoma. However, *TIMP3* was not differentially methylated in our analysis with respect to patient age and tumor location subgroups.

Our experimental design had some limitations, mainly related to the small number of tumor samples (CNS tumors are relatively rare). As per the power analysis with respect to the methylation study, the actual power was 0.821 and the standard sample number calculated was 15. Consequently, the number of samples in our article is slightly insufficient to warrant significance. Moreover, the ST group only included one child and all patients in the PF group were adults. Because posterior ependymomas are more common among children, our patient cohort might not represent the major features present in ependymoma groups. In addition, there was no germline control used for RNA sequencing and CCP because of the shortage of samples. However, this limitation was overcome to a certain degree by comparing the results obtained using three independent molecular analysis methods.

In summary, we analyzed ependymomas from patients, focusing on different tumor grades and locations, as well as on patient age, using next-generation sequencing methods, including methylation array, WES, CCP, and RNA-seq analyses. Although the number of specimens was limited, different combinations of the various conditions allowed sufficient diversity within the cohort to obtain reliable data. This constitutes the first report of next-generation sequencing analysis of ependymomas in Korean patients. Importantly, our findings highlight the need for the application of more accurate diagnostic parameters, other than tumor grade. Furthermore, our study provides major insights into the genetic and epigenetic profiles of intracranial ependymomas, with the potential to impact the development of approaches to improve their prognostic accuracy.

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### Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

### Authors' contributions

HJC and HYP have verified the authenticity of all raw data. HJC wrote manuscript drafts and revised the final version

of the manuscript. HJC acquired the pathologic and clinical data, and provided data interpretation of the whole-exome sequencing, comprehensive cancer panel, RNA sequencing and DNA methylation. HYP analyzed the data of the whole-exome sequencing, comprehensive cancer panel and RNA sequencing, and KK and HC analyzed the DNA methylation data. SHPaek, SKK, CKP and SHC acquired patients' clinical data, tumor specimens and radiologic data, and provided data interpretation. SHPark conceived the concept, and designed and supervised the study. All authors read and approved the final manuscript.

### Ethics approval and consent to participate

The Institutional Review Board of Seoul National University, College of Medicine and Hospital approved the current retrospective study (using patients' paraffin blocks; approval no. H-1412-137-636). The requirement for written informed consent from patients was waived as the patients donated their cancer tissues to the tissue bank of Seoul National University Hospital (Seoul, Republic of Korea). The current research complied with the recommendations of the Declaration of Helsinki.

### Patient consent for publication

Not applicable.

### Competing interests

The authors declare that they have no competing interests.

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