Detecting the long non-coding RNA signature related to spinal cord ependymal tumor subtype using a genome-wide methylome analysis approach

LI WANG, CHI ZHANG, YUJIE XIE, WEI JIANG, JUAN HUANG, SHENGMIN GUO, FANGYUAN XU and JIANXIONG WANG

Rehabilitation Department, The Affiliated Hospital of Southwest Medical University, Luzhou, Sichuan 646000, P.R. China

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Abstract. Ependymoma is a type of intramedullary tumor that tends to occur in the adult spinal cord. Ependymoma affects the nervous system and has significant impacts on the quality of life, and it may lead to mortality. Previous studies have performed molecular classification of spinal cord ependymal tumors at the DNA methylation level. However, the DNA methylation status of non-coding regions in spinal cord ependymal tumors remains unclear. In the present study, a genome-wide methylome method was used to characterize the DNA methylation landscape of long non-coding RNAs (lncRNAs) in spinal cord ependymal tumor samples. The present study identified lncRNA signatures associated with tumor subtypes based on the methylation status of lncRNA promoters. The present results suggested that the identified lncRNA signatures were associated with cancer- or nervous system-related protein-coding genes. The majority of the identified lncRNAs was hypomethylated, and may have a role in spinal cord development. The present findings suggested that detection of tumor subtype-specific lncRNAs may facilitate the identification of novel diagnostic and therapeutic strategies to treat patients with spinal cord ependymal tumor.

Correspondence to: Professor Jianxiong Wang, Rehabilitation Department, The Affiliated Hospital of Southwest Medical University, 25 Taiping Road, Luzhou, Sichuan 646000, P.R. China
E-mail: wjx_swmu@163.com

Abbreviations: lncRNA, long non-coding RNA; PCGs, protein coding genes; SP-EPN, spinal ependymoma; SP-SE, spinal subependymoma; SP-MPE, spinal myxopapillary; TSS, transcript start site; GREAT, Genomic Regions Enrichment of Annotations Tool; HM450, Human Methylation 450; CSPGs, chondroitin sulfate proteoglycans; SNP, single nucleotide polymorphism; TEC, To be Experimentally Confirmed

Key words: spinal cord, tumor, DNA methylation, lncRNA, signature

Introduction

Spinal tumors are rare and, based on their location, can be classified into various subtypes, such as intramedullary and extramedullary tumors (1). Ependymoma is a type of intramedullary tumor that tends to occur in the spinal cord in adults (2). Ependymoma is the most common type of spinal cord tumor and has significant impacts on the quality of life of patients, and may cause mortality (3). Classifying ependymal tumors has represented a challenge, as the current grading systems do not accurately describe their clinical characteristics (4,5). However, previous studies have found that DNA methylation patterns are reliable biomarkers for the classification of different molecular subtypes of spinal ependymal tumors (6).

Improved detection methods and the identification of disease-associated biomarkers have been shown to facilitate the diagnosis and treatment of patients (7-11). DNA methylation is one of the most studied epigenetic modifications in mammals (12,13). Notably, aberrant methylation is associated with cancer and aging (14,15). Some DNA methylation markers have been used in commercially available clinical tests, and most of the sites of methylation are located in the promoters of genes (16). Despite several previous studies suggesting that DNA methylation is a reliable molecular biomarker in ependymal tumors, to the best of our knowledge, the role of methylation in long non-coding RNA (lncRNA) genes has not been investigated (4,6). lncRNAs are >200 nucleotides in length and do not encode proteins. Similarly to mRNAs, lncRNAs have their own promoters (17), are transcribed by RNA polymerase II and have a polyadenylated tail (18). An increasing number of studies have identified various roles of lncRNAs in multiple biological processes and diseases through various mechanisms (19,20). Previous studies have demonstrated that lncRNAs can serve as biomarkers for various cancer features (21). Recent studies have provided insight on the mechanism underlying DNA methylation of lncRNA genes in carcinogenesis (22,23). However, to the best of our knowledge, a systematic investigation of the DNA methylation status and the function of lncRNA genes in spinal cord ependymal tumor has yet to be reported.

In the present study, the DNA methylation landscape of lncRNAs in spinal cord ependymal tumors was investigated

and certain lncRNAs were identified to have distinct DNA methylation states among various tumor subtypes. Additionally, various tumor subtype-specific lncRNAs were identified to be involved in spinal cord development. In the present study, a functional characterization of lncRNAs was performed using lncRNA-protein interaction data, and a random forest algorithm was used to identify 30 lncRNAs with high classification efficiency. Notably, the majority of subtype-specific lncRNAs was identified to be hypomethylated. A subset of the identified lncRNAs was found to have potential roles in cancer and nervous system development. Furthermore, a functional analysis identified the role of subtype-specific lncRNAs in spinal cord ependymal tumors. To the best of our knowledge, the present study is the first to investigate the DNA methylation status of lncRNA genes in order to identify their clinical significance in various molecular subtypes of spinal cord ependymal tumors.

Materials and methods

Methylation and lncRNA data collection. Whole-genome DNA methylation data from spinal cord ependymal tumors were downloaded from Gene Expression Omnibus version 1 (24) [accession no. GSE65362 (4)]. Specifically, these samples were classified into three classes: i) Spinal ependymoma (SP-EPN); ii) spinal myxopapillary EPN (SP-MPE); and iii) spinal subependymoma (SP-SE). lncRNA annotation data were downloaded from GENCODE (version 28) (25). These lncRNAs were classified into nine groups based on their location and features (http://vega.archive.ensembl.org/info/about/gene_and_transcript_types.html) (Fig. 1A). Notably, the To be Experimentally Confirmed (TEC) category has been specifically created for the ENCODE project to indicate regions that could indicate the presence of protein coding genes (PCGs) that require experimental validation (25). Importantly, Ensembl (https://www.ensembl.org/info/genome/genebuild/biotypes.html) and Vega (http://vega.archive.ensembl.org/info/about/gene_and_transcript_types.html) databases classify TEC genes as lncRNAs.

Mapping methylation probes to lncRNA promoters. The genomic locations of the Infinium Human Methylation 450 (HM450) BeadChip (Illumina, Inc.) probes based on GRCh37 were converted to the genomic locations in GRCh38 (The Genome Reference Consortium; https://www.ncbi.nlm.nih.gov/grc) using the University of California, Santa Cruz (UCSC) Batch Coordinate Conversion liftOver tool version 2 (26). Probes exhibiting single nucleotide polymorphisms (SNPs) >5 bp from their 3'-end and probes with non-unique 3'-subsequences of 30 bases were excluded, as previously described (27). lncRNA promoter regions were defined as 3-kb windows from either side of the transcription start site (TSS), as previously described (22). Subsequently, the methylation probes were mapped to the lncRNA promoter regions using BEDtools version 2.24.0 (28). The same approach was used to map the PCG probes. In addition, methylation probes that simultaneously mapped to lncRNAs and PCGs were excluded.

lncRNA DNA methylation in spinal cord ependymal tumors. lncRNA promoter methylation values were calculated as the mean values of all probes located in the corresponding promoter. DNA methylation patterns around TSSs were calculated in 100 bp windows based on the median methylation value across samples. The lncRNA methylation value was used to cluster samples using the pheatmap package (version 1.0.12; https://cran.r-project.org/web/packages/pheatmap/index.html), using the default parameters, on R (version 3.3.0; https://cran.r-project.org/). The similarity between tumor samples was measured by Pearson correlation coefficients using R to determine if tumors within a subtype were more similar to each other than those from other subtypes. In addition, principal component analysis was used to investigate the methylation patterns of different tumor subgroups. Tumor subtype-specific lncRNAs were identified by ANOVA using R, as previously described (29). The RCircos package (version 1.2.1; https://cran.r-project.org/web/packages/RCircos/index.html) was used with R to display the distribution of methylation levels and the locations of tumor subtype-specific lncRNAs in the genome.

Tumor subtype-specific lncRNA-protein interaction network construction. RNA-protein interaction data were downloaded from the RAID database (version 2.0) (30), which integrates experimental and computational prediction interactions from the literature and other database resources under one common framework. lncRNA-protein interaction associations were calculated by mapping subtype-specific lncRNAs to mRNAs.

Tumor subtype-specific lncRNA signature identification. A random forest algorithm (31) was performed using the DNA methylation value of the lncRNAs identified in the constructed network. The lncRNAs with a feature importance value >0, calculated using the random forest algorithm, were considered potential tumor subtype-specific signatures (32). All statistical analyses were performed using R (version 3.3.0; R project).

Functional enrichment analysis of lncRNAs and PCGs. Tumor subtype-specific lncRNAs were annotated using their promoter regions by the Genomic Regions Enrichment of Annotations Tool (GREAT; version 3.3.0) using the default parameters (33); in addition, the lncRNA promoter regions were used as 'background regions'. Specifically, the genomic coordinates of the lncRNAs used in GREAT were first converted into GRCh37 coordinates using the UCSC liftover tool, as aforementioned. Kyoto Encyclopedia of Genes and Genomes (KEGG; version 73.0) (34) enrichment analysis was performed on the PCGs using the Enrichr online tool (version 2.0), using the default parameters (35), and the significantly enriched KEGG pathways were identified ($P<0.01$).

Results

Global DNA methylation patterns in lncRNA promoters in spinal cord ependymal tumors. To characterize lncRNA methylation patterns, a computational pipeline was used to annotate HM450 probes to lncRNA promoters. In total, 485,506 HM450 probes were successfully converted into GRCh38 coordinates, and 433,532 probes were obtained after filtering for SNPs and copy number-associated probes. The present analysis resulted in a set of 29,402 probes annotated in 6,967 lncRNA promoter regions (Fig. 1A). In total, 12,668 and 11,711 methylation probes were found in the promoters of long intergenic noncoding RNAs (lincRNAs) and antisense

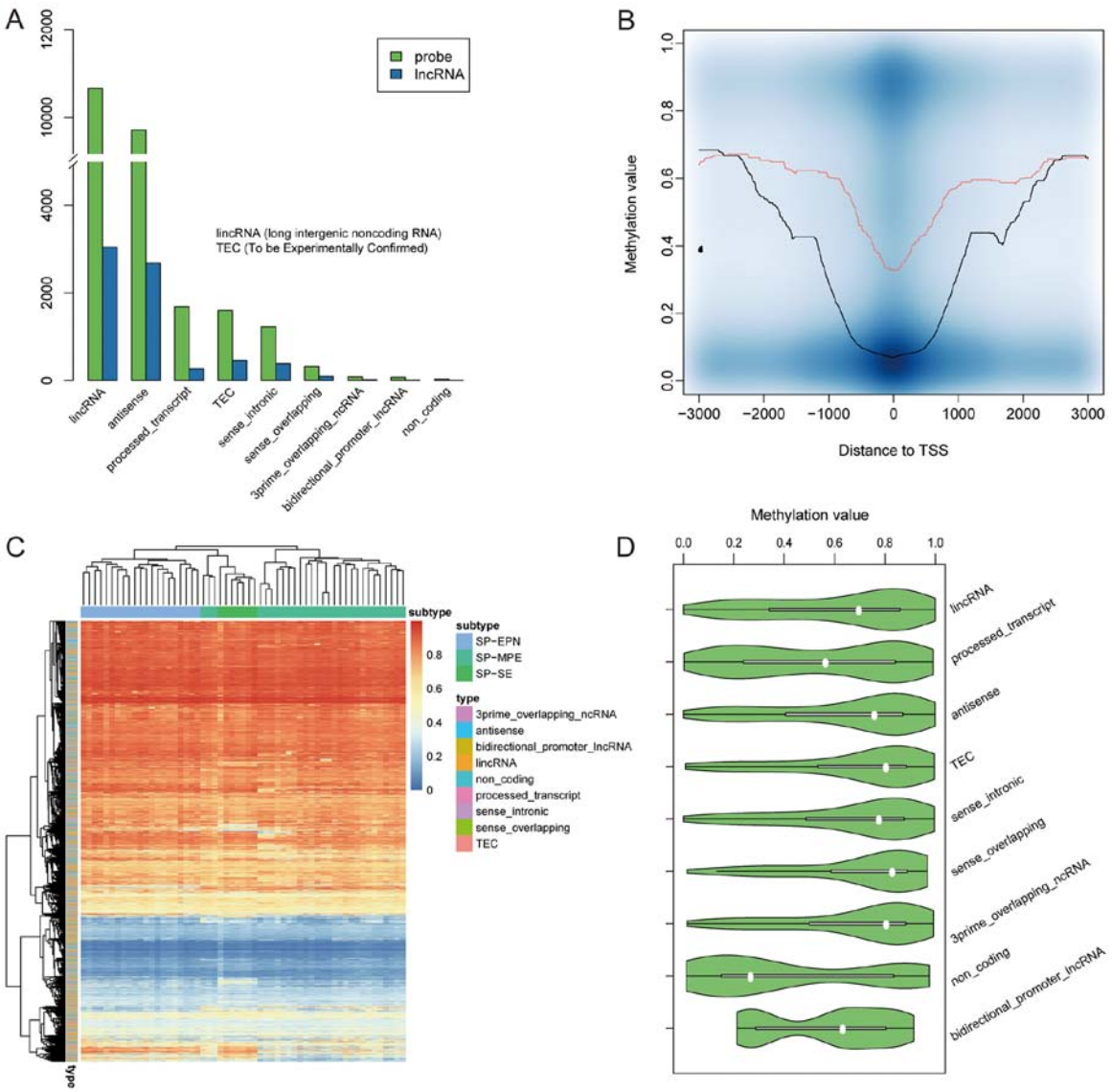

Figure 1. Probe annotation and methylation features of lncRNA promoters. (A) Number of probes and lncRNAs for each lncRNA category. (B) DNA methylation level around the TSS of lncRNAs (red line) and protein coding genes (black line). (C) Unsupervised hierarchical clustering of average methylation profiles of lncRNAs in spinal cord ependymal tumors. (D) Global methylation value distribution of each lncRNA category across all tumor samples, as visualized by violin plots. lncRNA, long non-coding RNA; lincRNA, long intergenic non-coding RNA; TEC, To be Experimentally Confirmed; SP-EPN, spinal ependymoma; SP-SE, spinal subependymoma; SP-MPE, spinal myxopapillary; TSS, transcription start site; ncRNA, non-coding RNA.

lncRNAs, respectively. Additionally, lncRNAs exhibited methylation states similar to those of PCGs around TSSs; both had low methylation values in proximity to the TSS and had higher methylation values as the distance from the TSS increased (Fig. 1B). However, the overall methylation level in lncRNAs was higher compared with PCGs (Fig. 1B). Cluster analysis suggested that tumor samples from patients with the same molecular subtype had similar methylation patterns in lncRNAs and that the methylation levels of various lncRNAs in the same class were not similar (Fig. 1C). Additionally, the majority of lncRNAs identified in the present study had a methylation value >0.4 (Fig. 1C). Certain lncRNAs, including those classified as 'processed_transcript' and 'bidirectional_promoter_lncRNA' lncRNAs, exhibited bimodally distributed methylation levels in tumor samples, whereas other lncRNAs, including 'antisense' and 'sense_intronic' lncRNAs, were hypermethylated in the majority of the patients (Fig. 1D).

DNA methylation in lncRNAs is correlated with histological characteristics of spinal cord ependymal tumors. The patterns of DNA methylation in the lncRNA genes were investigated in different tumor histopathological subtypes. In total, there were 57 patients with spinal cord tumors, 29 (50.9%) patients were in the SP-MPE group, whereas 21 (36.8%) and 7 (12.3%) were in the SP-EPN and SP-SE group, respectively. DNA methylation correlation values were calculated between each pair of samples, and tumor samples were clustered using their methylation correlation values. lncRNA methylation levels were found to be more similar within groups than between groups (Fig. 2A). In particular, patients in the SP-SE group exhibited the most consistent methylation status, with a mean methylation correlation value of 0.96. The mean methylation correlation values in the SP-EPN and SP-MPE were 0.93 and 0.92, respectively. This effect may be due to the little tumor heterogeneity among SE samples. According to the World Health Organization

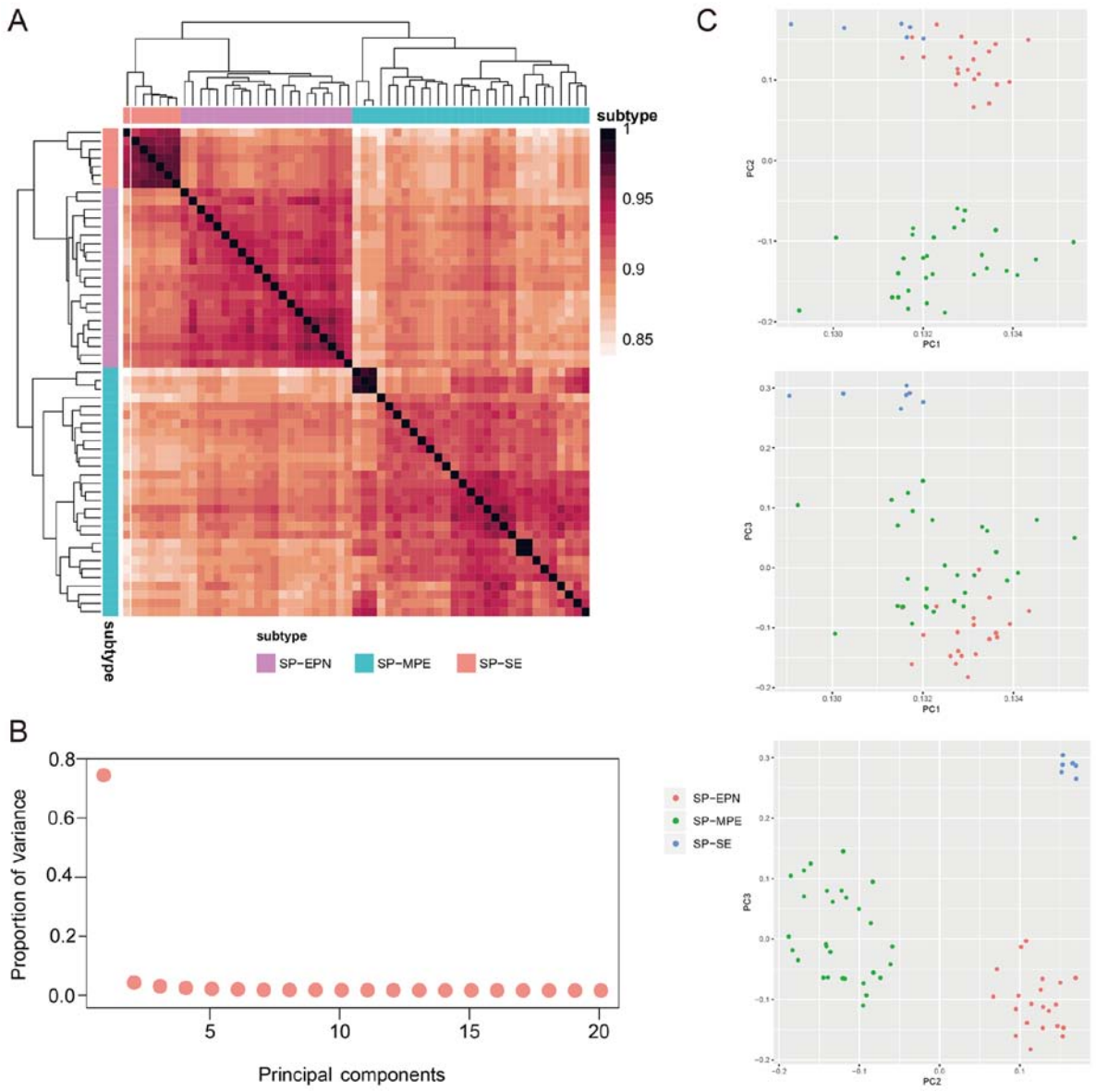

Figure 2. DNA methylation in long non-coding RNA genes exhibits tumor subtype-specific characteristics. (A) Unsupervised hierarchical clustering of Pearson correlation coefficients between each pair of samples. (B) Proportion of variance for the top 20 genotype principal components. (C) Top three genotype principal components stratified by tumor subtype. SP-EPN, spinal ependymoma; SP-SE, spinal subependymoma; SP-MPE, spinal myxopapillary.

classification (36,37), SP-SE is considered a grade I tumor and its prognosis is more favorable compared with the majority of ependymal tumors (36). In addition, principal component analysis was performed to analyze the tumor samples based on the DNA methylation of the lncRNAs. The first three principal components had the highest proportion of variance values (Fig. 2B). These three components were used to characterize the DNA methylation features in these tumor subtypes, and the results suggested that patients with the same tumor subtype were likely to have similar lncRNA methylation levels (Fig. 2C). The present results suggested that the DNA methylation in lncRNA genes was correlated with histological characteristics of spinal cord ependymal tumors.

DNA methylation-based detection of tumor subtypes-specific lncRNAs. Tumor subtype-specific lncRNAs were identified based on their DNA methylation values using ANOVA. In total, 1,046 subtype-specific lncRNAs were identified [false discovery rate (FDR) <0.05; Figs. 3 and S1]. These lncRNAs tended to have higher methylation values (Fig. 3A), and the majority had methylation values ~0.9. Furthermore, the patients in the SP-EPN group had the highest methylation levels, whereas the patients in the SP-SE had the lowest methylation levels (Fig. 3B and C). GREAT was used to investigate the potential mechanisms of lncRNAs in spinal cord ependymal tumors. Among the significant Gene Ontology (38) terms (FDR q-value <0.01), three of them were associated with spinal cord development (Fig. 3D). 'Ventral spinal cord development' and 'spinal cord development' processes can affect cell differentiation of spinal cord cells, and a previous study demonstrated that cell differentiation is associated with tumorigenesis (39). Therefore, the distinct DNA methylation levels of various lncRNAs may affect the expression levels of lncRNAs in each tumor subtype, thus affecting genes associated with the nervous system and spinal cord development. The present results indicated the role of lncRNAs in human spinal

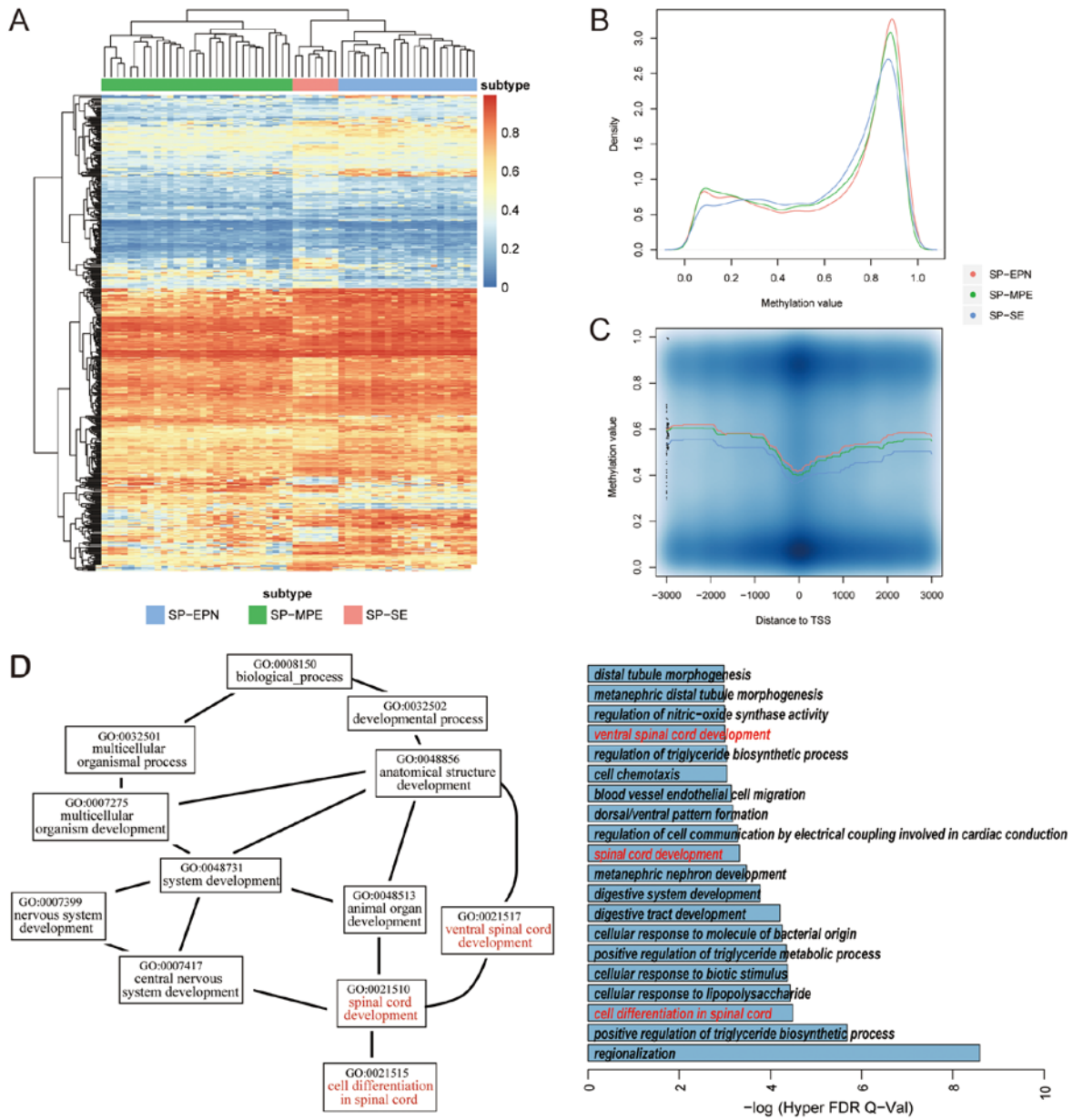

Figure 3. Tumor subtype-specific lncRNA methylation characteristics and their potential role in spinal cord development. (A) Unsupervised hierarchical clustering of average methylation profiles for tumor subtype-specific lncRNAs. (B) Density plot of DNA methylation levels in tumor subtype-specific lncRNAs stratified by tumor subtype. (C) DNA methylation levels around the TSS of tumor subtype-specific lncRNAs stratified by tumor subtype. (D) Schematic diagram of GO terms associated with spinal cord development and the enrichment results for the tumor subtype-specific lncRNAs sorted by FDR q-values. Red indicates terms associated with spinal cord development. SP-EPN, spinal ependymoma; SP-SE, spinal subependymoma; SP-MPE, spinal myxopapillary; lncRNA, long non-coding RNA; TSS, transcription start site; FDR, false discovery rate; GO, Gene Ontology.

cord development and suggested that they could be lncRNA signatures in spinal cord ependymal tumors.

Tumor subtype-specific lncRNA signature discovery. Biological networks represent the interactions between molecules *in vivo*, and can be used to identify regulatory pathways and processes (40,41). lncRNAs may contribute to cancer by interacting with proteins (42), and an increasing number of studies have investigated lncRNA-protein interactions (43). lncRNA-protein interaction data in humans have been downloaded from the RAID database (30). In total, 32 subtype-specific lncRNAs exhibited associations with proteins (Fig. 4A). The network degrees of lncRNAs and proteins were separately analyzed (Fig. 4B and C). lncRNAs exhibited more associations compared with proteins. The present results indicated the important role of lncRNAs in interacting with proteins and suggested that these 32 lncRNAs may affect the disease status by affecting protein function in spinal cord ependymal tumors. Furthermore, in order to identify the lncRNA signatures in ependymal tumors, a random forest classification method was used. In total, 30 lncRNAs with importance values >0, ranging between 0.48 and 2.98, were identified (Fig. 4D). Using the methylation values of these 30 lncRNAs, the three tumor subtypes could be reliably distinguished with sensitivity and specificity values of 1 (Fig. 4E). These lncRNAs may be potential biomarkers associated with tumor subtypes, and may facilitate the diagnosis and treatment of patients with spinal cord ependymal tumors.

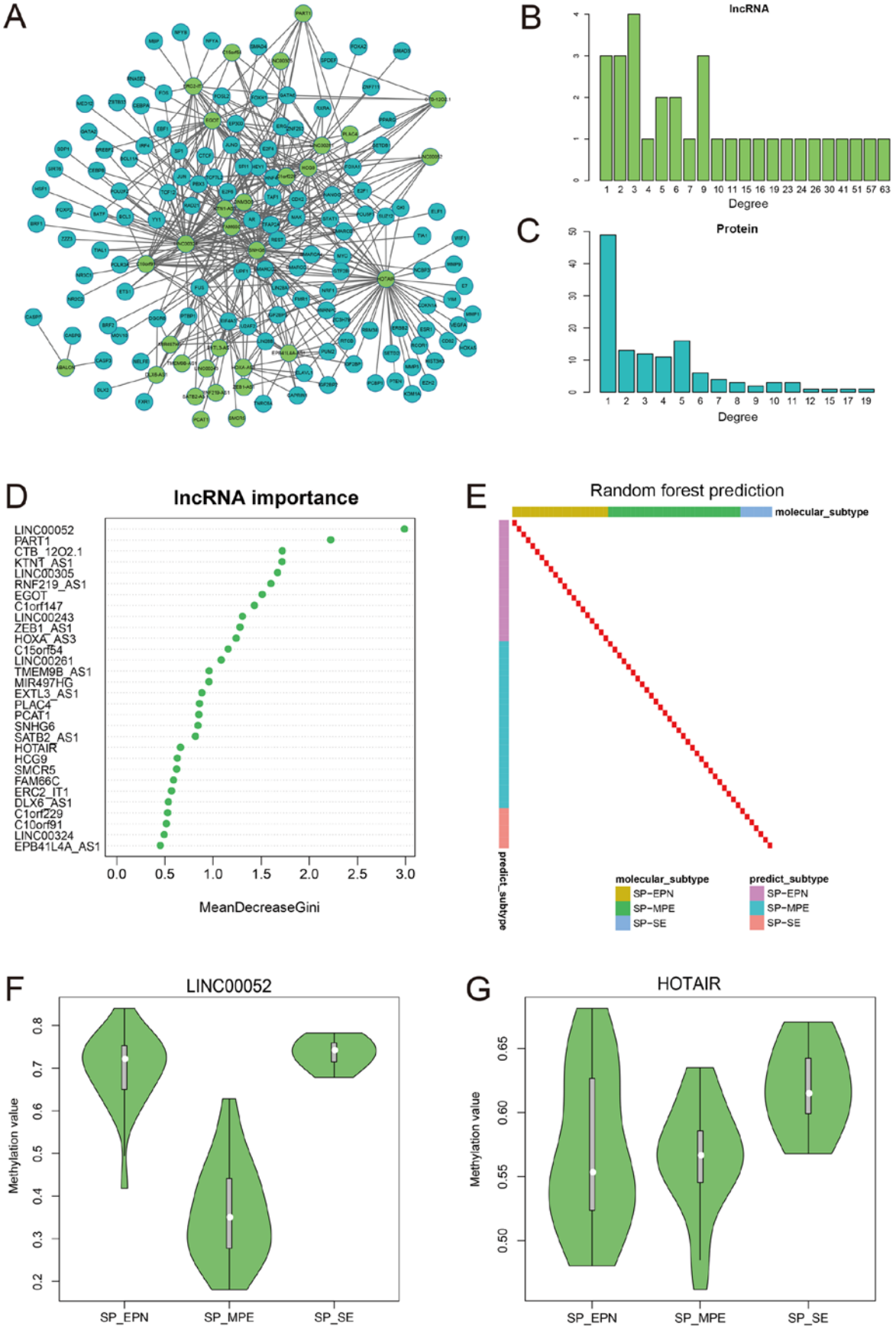

Figure 4. Identification of lncRNA signatures associated with tumor subtypes. (A) Visualization of lncRNA-protein interaction networks. Green nodes represent lncRNAs and blue nodes represent proteins. (B) Degree of distribution of lncRNAs in the network. (C) Degree of distribution of protein-coding genes in the network. (D) Importance value for each lncRNA evaluated by the random forest algorithm. (E) Classification result of each sample using the random forest classifier. Each sample was classified to its corresponding subtype. (F) Violin plot of methylation values of LINC00052 in each tumor subtype. (G) Violin plot of methylation values of HOTAIR in each tumor subtype. SP-EPN, spinal ependymoma; SP-SE, spinal subependymoma; SP-MPE, spinal myxopapillary; lncRNA, long non-coding RNA; HOTAIR, HOX transcript antisense RNA; LINC00052, long intergenic non-coding RNA 52.

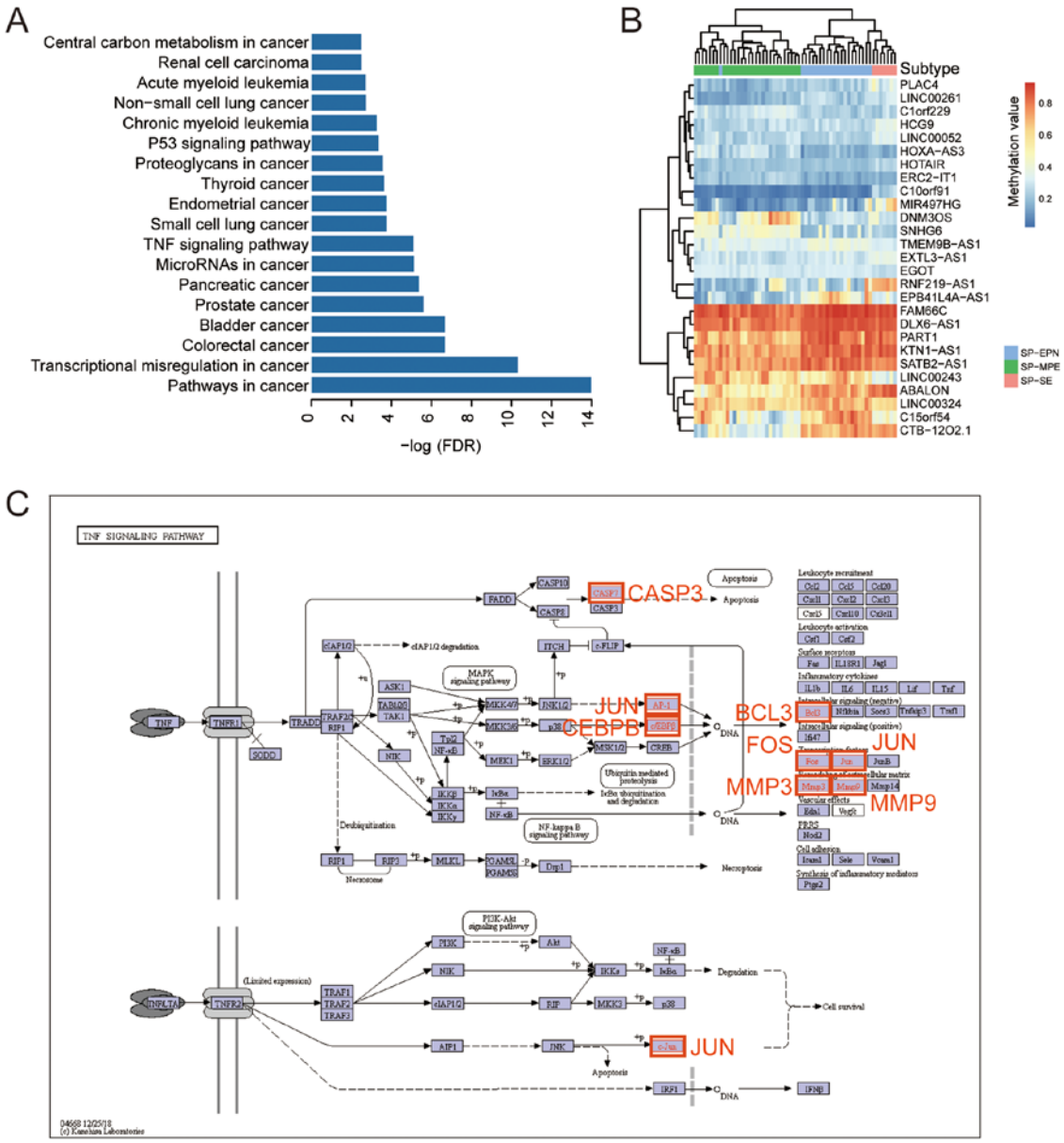

Figure 5. KEGG enrichment analysis results and methylation characteristics of lncRNA signatures. (A) KEGG enrichment pathways of the proteins that interact with the lncRNAs of the lncRNA signature sorted by adjusted P-value. (B) Unsupervised hierarchical clustering of DNA methylation levels of the lncRNA signature. (C) TNF signaling pathway and TNF-associated genes. Genes with red rectangles represent the genes in the network. SP-EPN, spinal ependymoma; SP-SE, spinal subependymoma; SP-MPE, spinal myxopapillary; lncRNA, long non-coding RNA; KEGG, Kyoto Encyclopedia of Genes and Genomes.

Long intergenic non-coding RNA 52 (LINC00052) is a lincRNA, and exhibited the highest importance value in the classification of the three tumor subtypes (Fig. 4D and F). LINC00052 has been previously shown to promote breast cancer and hepatocarcinoma development (44,45). HOX transcript antisense RNA (HOTAIR) is an oncogenic lncRNA in multiple types of cancer, including breast, gastric, colorectal and cervical cancer (46). Additionally, its DNA methylation status can serve as a biomarker in primary ovarian cancer (47). HOTAIR has been investigated as a prognostic factor in mesenchymal glioma, another type of tumor affecting the nervous system (48). In the network constructed in the present study, HOTAIR was identified to exhibit the features of a hub. HOTAIR interacted with 51 proteins and displayed tumor subtype-specific DNA methylation characteristics (Fig. 4A and G). The lncRNAs listed in Fig. 4D may be potential novel biomarkers for treating and diagnosing spinal cord ependymal tumors. Analyzing the DNA methylation values of these lncRNAs in patients with ependymal tumors may facilitate the classification of the tumors and the development of personalized therapies.

Functional and epigenomic characteristics suggest the roles of lncRNAs in spinal cord ependymal tumors. KEGG pathway enrichment analysis was performed in order to analyze the PCGs interacting with the identified lncRNA signatures. In total, 128 PCGs were analyzed. A total of 39 significantly enriched pathways (adjusted P<0.01; Fig. 5A) were identified. Out of 39 pathways, 17 were associated with cancer, including 'transcriptional misregulation in cancer', 'p53 signalling pathway', 'colorectal cancer' and 'bladder cancer'. Furthermore, the pathway 'proteoglycans in cancer' was identified, which is involved in central nervous system-associated functions and

diseases (49). Chondroitin sulphate proteoglycans (CSPGs) are enriched in the nervous system and contribute to neural cell migration and axon extension (50). A previous study demonstrated that CSPGs are upregulated after spinal cord injury (51). Additionally, the tumor necrosis factor (TNF) signaling pathway was identified, which is involved in various diseases (Fig. 5C). Interestingly, most of the lncRNAs identified to exhibit subtype-specific expression were hypomethylated compared with the total amount of lncRNAs identified (Figs. 1C and 5B).

Discussion

Ependymal tumor is a rare type of malignant tumor (52). Ependymal tumors can arise from both the brain and spinal cord (53). Notably, depending on its origin, there is a large genetic difference between these two types of ependymal tumor (54). Previous studies have investigated ependymal tumor arising from the brain (6,55), and the current knowledge of the molecular characteristics of spinal cord-derived ependymal tumor remains limited.

By investigating the DNA methylation status of lncRNAs, the present study provided novel insight into the understanding of ependymal tumors and the possible treatments of patients with this disease. The present study investigated the DNA methylation profile of multiple lncRNA promoters and identified that the majority of lncRNAs in ependymal tumors presented high methylation levels. The present study suggested that the DNA methylation level in lncRNA promoters are consistent among samples belonging to the same tumor subtype. The regions with differential methylation levels corresponded to lncRNA signatures that may be involved in spinal cord development.

The present study suggested that tumor subtype-specific lncRNAs may be involved in spinal cord development. By integrating lncRNA-PCG interaction data, a total of 30 lncRNAs were identified, and their DNA methylation levels may be a signature in spinal cord ependymal tumors. Some of these lncRNAs were identified to be associated with cancer and nervous system diseases. The PCGs regulated by the lncRNA signatures were identified to be enriched in many cancer- and nervous system-associated pathways. Interestingly, most of the lncRNAs interacting with cancer- and nervous system-associated proteins were found to be hypomethylated. In addition, the TNF signaling pathway was identified as being involved in spinal cord ependymal tumorigenesis. In previous studies, TNF-α was shown to be involved in cell survival, apoptosis, inflammation and immunity (56-58), and its role has been investigated in various diseases, particularly in cancer (59). A previous study investigating the function of TNF in the nervous system demonstrated that TNF affects the nervous system, and it is involved in neurodegenerative diseases (60). Although previous studies have examined the function of the TNF signalling pathway in the nervous system, the present study may provide insight for future studies aimed to investigate its function in spinal cord tumors. In the present study, lncRNAs with target genes associated with cancer and the nervous system were identified, and their methylation patterns in tumor samples were investigated. Therefore, DNA methylation may be a principal factor in regulating the biological features of lncRNAs, thus influencing the function of proteins downstream of these lncRNAs.

Collectively, spinal cord ependymal tumors were investigated using a novel approach, and the methylation status of lncRNAs in ependymal tumors was characterized. The present study may lay the foundations for future studies aimed to investigate spinal cord tumors. However, the present results require validation using a high number of tumor samples. The present findings may contribute to the development of novel strategies for diagnosing and treating spinal cord ependymal tumors.

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Availability of data and materials

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

Authors' contributions

JW conceived and designed the experiments. LW performed most of the experiments. CZ and YX performed certain experiments. JH and SG identified tumor-subtype specific lncRNA signatures. FX and WJ performed functional enrichment analysis. JH, SG, FX and WJ aided in the interpretation of the results and wrote the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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