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## Gene expression profiling identifies two chordoma subtypes associated with distinct molecular mechanisms and clinical outcomes

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### Abstract

**Purpose:** Chordoma is a rare bone tumor with a high recurrence rate and limited treatment options. The aim of this study was to identify molecular subtypes of chordoma that may improve clinical management.

**Experimental Design:** We conducted RNA sequencing in 48 tumors from Chinese skull-base chordoma patients and identified two major molecular subtypes. We then replicated the classification using a NanoString panel in 48 chordoma patients from North America.

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**Authorship:** All authors of this research paper have directly participated in the planning, execution, or analysis of the study. Specifically, JB, JS, CL, XRY, and YZ contributed to the study conception and design. JB, CL, YX, BH, AH, EK, MAT, ML, YS, TM, SW, SG, MM, DMP, AMG, YZ, and XRY contributed to patient recruitment, data and specimen collection, tissue processing, and experimental assays. JW and XL helped with the pathologic review. DW, TZ, LS, LW, and BZ performed bioinformatics analyses. JS and HK conducted all the statistical analyses. BH, DMP, AMG, YZ, and XRY provided administrative support. JB, JS, and XRY were responsible for data interpretation. YZ secured the funding for the sequencing analysis. XRY provided supervision in all components of the study from study design to data interpretation and drafting the manuscript.

**Conflict of Interest:** The authors declare no conflict of interests.

**Results:** Tumors in one subtype were more likely to have somatic mutations and reduced expression in chromatin remodeling genes, such as *PBRM1* and *SETD2*, while the other subtype was characterized by the upregulation of genes in epithelial-mesenchymal transition and Sonic Hedgehog pathways. Immunohistochemical staining of top differentially expressed genes between the two subtypes in 312 Chinese chordoma patients with long-term follow-up data showed that the expression of some markers such as *PTCH1* was significantly associated with survival outcomes.

**Conclusions:** Our findings may improve the understanding of subtype-specific tumorigenesis of chordoma and inform clinical prognostication and targeted options.

### Keywords

chordoma; molecular subtype; RNA sequencing; NanoString gene expression profiling; recurrence and survival

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### Introduction

Chordoma is a rare bone tumor, which is believed to originate from notochordal remnants (1) and occurs in the bones of the skull base and spine (2). Approximately one third of chordomas arise in the clivus (skull base) and patients with skull-base chordoma have the earliest average age-onset (usually less than 50 years) compared to chordomas occurring at other sites (>55 years) (3,4). Although chordomas are slow-growing, local recurrence is common especially among skull-base chordoma patients, largely due to incomplete tumor resection and difficulty in normal margin resection because of the closeness of the tumor to vital structures. Surgery with or without neoadjuvant or adjuvant radiation therapy is the first-line treatment for chordoma. Proton radiation therapy is often considered the best radiation treatment, but its availability is still limited (5). Currently, there is no clear clinical guidance on patient stratification regarding treatment such as post-surgery radiation therapy. In addition, treatment options for chordoma patients, particularly those with advanced disease, are still limited. The median survival is about 7 years; however, the clinical progression is extremely variable (6) and is likely determined by both surgical factors and tumor biology.

By morphology, chordomas are divided into classical (conventional), chondroid, poorly differentiated, and dedifferentiated. Expression of brachyury, which is a transcription factor encoded by the *TBXT* gene and plays a key role in notochord development, is considered as a specific diagnostic marker for chordoma (7,8). Dedifferentiated and poorly differentiated chordoma are rare subtypes and usually present with aggressive clinical behaviors (9,10), other histological subtypes did not show variation in clinical outcomes (11). A recent study identified prognostic chordoma subtypes based on DNA methylation profiles (12), suggesting that chordomas are molecularly heterogeneous. A better understanding of the molecular processes in chordoma is critically needed to develop prognostic prediction tools and better-tailored treatments. To address this question, we conducted a transcriptome analysis using RNA sequencing (RNA-Seq) in 48 chordoma tumors collected from skull-base chordoma patients in China and replicated the major findings in an independent chordoma patient population (n=48) from North America. Further, we identified several

key markers defining molecular subtypes that were associated with clinical outcomes in a large set of skull-base chordoma patients (n=312) with long-term follow-up data available.

## Methods

### Study Populations:

We included three chordoma patient cohorts in this study. The discovery analysis (RNA-Seq cohort) included 48 patients who were diagnosed with skull-base chordoma and underwent endoscopic endonasal surgeries at the Neurosurgery Department of Beijing Tiantan Hospital, Capital Medical University, between June 2016 and May 2018. Fresh-frozen tumor samples and clinicopathological characteristics including age, tumor histologic type, tumor volume, Ki67 status, gross resection rate, pre-surgery radiation therapy (RT), post-surgery RT, recurrence, and death status were collected. The chordoma diagnosis was confirmed with brachyury staining for the majority of patients and was confirmed by morphology in combination with cytokeratin (CK) and epithelial membrane antigen (EMA) markers for the remaining patients without brachyury staining data. The survival cohort was derived from a retrospective cohort of 507 skull-base chordoma patients who were also treated at the Beijing Tiantan Hospital between January 2008 and September 2014. The current analysis included 312 patients with chordoma tumors constructed on tissue microarrays (TMAs) and clinical follow-up data collected. The study protocols were conducted in accordance with the Declaration of Helsinki and approved by the ethics committee of the Beijing Tiantan Hospital, and written informed consent was obtained for all study participants.

The replication cohort (NanoString cohort) included chordoma patients, unselected for tumor site, identified from the United States (US) and Canada who participated in an ongoing sporadic chordoma study conducted at the National Cancer Institute, which has been previously described (13). All diagnoses of chordoma were confirmed by reviewing pathologic slides or reports, medical records, or death certificates, and all study subjects were of European ancestry. The current analysis included 48 patients whose RNA was extracted from formalin-fixed paraffin-embedded (FFPE) tumors and passed all quality control measures in the expression profiling analysis using a Nanostring custom-made panel. The average age at diagnosis among these 48 patients was 50 years; the vast majority (98%) had classic chordoma histology; and 48.9% had skull-base chordoma. The study was approved by the institutional review boards at the National Institutes of Health and all participants provided written informed consent.

### RNA-Seq and whole-genome sequencing (WGS) analysis:

Biospecimen collection, quality control, and tissue processing steps were described previously (14). For total RNA extraction, fresh frozen tissue sections were processed with TRIzol (Thermo, US) according to the manufacturer's instructions. RNA was run on 1% agarose gels to check for degradation and contamination. RNA quality and quantity were assessed using the RNA Nano 6000 Assay Kit of the Bioanalyzer 2100 system (Agilent Technologies, CA, USA). RNA samples with an integrity number (RIN) of over 6.8 were included for transcriptome library preparation and sequencing. RNA-Seq was carried out by the Novogene Corporation (Beijing, China) using Illumina HiSeq as previously

described (14). Raw data in fastq format were processed by removing reads containing adapters or ploy-N and low-quality reads. Fastqc was used for quality control process (<http://www.bioinformatics.babraham.ac.uk/projects/fastqc/>). Short reads were then mapped to hg19 genome and gene expression was further quantified as TPM (transcripts per million) using RSEM with default parameters (<https://github.com/deweylab/RSEM>), and log2TPM was used for statistical analyses.

WGS data based on paired tumor and blood samples was available for a subset of these patients (n=34). Sequencing and bioinformatic analyses were previously described in detail (14). Tumor purity was estimated from ESTIMATE (Estimation of STromal and Immune cells in MAlignant Tumor tissues using Expression data) (15) based on RNA expression data, and mutations and copy number alterations in the WGS analysis (14), respectively. Since purity estimated based on RNA-Seq and WGS analyses showed high correlation (Pearson Correlation Coefficient = 0.69, p=0.0004), we used RNA-based purity estimate in the downstream analysis.

### **Molecular classification:**

Unsupervised hierarchical clustering was conducted using ConsensusClusterPlus (16), based on the 2,000 genes showing the most variable expression levels in the dataset with Euclidean distance calculated from z-statistics using the R package “Complex Heatmap” (v2.10.0). For each of the 500 resampling of subjects, we sampled 80% of the subjects and clustered them using agglomerative hierarchical clustering with Pearson correlation as the distance metric. We evaluated up to 6 clusters and chose 3 consensus clusters (k=3; CC1, CC2, and CC3) as it best fit the data. Since tumors in CC3 had lower *TBXT* gene expression and lower tumor purity estimated by both gene expression and WGS analyses, it is likely that this subtype is enriched with low-purity tumors. We therefore focused the subsequent analyses on CC1 and CC2 and performed differential gene expression analyses between the two subtypes. We selected 21 top-ranked differentially expressed genes (DEGs) based on p values ( $p < 10^{-6}$ ) and fold changes (FC,  $\log_2FC > 1$ ), which were included in the panel for NanoString profiling analysis of FFPE tumors in the replication dataset. Gene Set Enrichment Analysis (GSEA) was performed using DEGs ranked by scores that incorporate p values and  $\log_2FC$  using the GSEA package developed by the Broad Institute with the use of the Molecular Signatures Database (MSigDB v7) (17,18).

### **NanoString RNA profiling and DNA panel sequencing:**

Genomic DNA and total RNA were extracted from FFPE tissue blocks or sections from North American chordoma patients (replication cohort) that were processed to enrich for tumor cells through macro-dissecting the tumor region. Targeted panel sequencing (gene list see Supplementary Table 1) was conducted on paired tumor and germline DNA, with an average coverage of 1,158x. Torrent Variant Caller (TVC) was used for variant calling. Variants that were QC flagged, with allele frequency  $>0.001$  in the ExAC database, variant allele fraction  $<10\%$  in tumors, and  $<50$  total reads were removed from the analysis. Variant calls for targeted genes were checked manually through visual assessment using the Integrative Genomics Viewer (IGV).

RNA was quantitated using a ThermoScientific NanoDrop 2000 Spectrophotometer (cat. # ND-2000) and Agilent 4200 TapeStation. After assessing for low concentration or low percentages of RNA molecules > 300 nucleotides long (3%), the remaining samples were processed by the University of North Carolina Translational Genomics Laboratory using the Nanostring nCounter Platform. Samples were run on a custom codeset that included gene sets for the 21 top DEGs between CC1 and CC2 in the RNA-Seq analysis (*AHR, ANGPTL4, GLI1, GULP1, HHIP, LEF1, LRRC32, ODZ2, ODZ4, OMD, PCOLCE, PDZRN4, PEG10, PKDCC, PTCH1, SLC6A20, SPON2, TMTC1, TNNC1, TP63, and TSPAN11*). 48 samples passed quality check and were randomized to two batches and Stratagene Universal Human References were included to assess batch variability. Batch variability was low, with correlations between reference standards exceeding 0.99.

### **Tissue Microarray (TMA) and immunohistochemical staining:**

TMAAs were constructed from FFPE skull-base chordoma tumor donor blocks using a Tissue Array MiniCore 3 (ALPHELYS, Plaisir, France) with two 2-milimeter cores per donor block. A 4- $\mu$ m thick section of each TMA was acquired using the Leica RM 2135 Rotary Microtome and sections were baked for 1 h at 65 °C followed by deparaffinization with dimethylbenzene. After being hydrated in a graded ethanol series and subjected to antigen retrieval, the sections were treated with 3% H<sub>2</sub>O<sub>2</sub> and blocked using normal goat serum. The sections were then incubated with primary antibodies overnight at 4°C and followed by second antibody and Diaminobenzidine (DAB) treatment. Finally, the sections were dehydrated, cleared, and mounted. The following antibodies were used for IHC: anti-LEF1 (1:500, ab137872, abcam, Cambridge, Massachusetts), anti-PTCH1 (1:200, ab53715, abcam), anti-GLI1 (1:100, BS90575, bioworld, Bloomington, Illinois), anti-CLDN11 (1:100, ab53041, abcam), anti-EPHA3 (1:100, ab126261, abcam). Marker expression levels were independently assessed by two pathologists (JW and XL) and consensus calls were achieved through discussion in the case of disagreement between the two pathologists. Slightly different criteria were used for different markers to accommodate distinct patterns in staining across markers. PTCH1 expression was categorized into three groups: 0=negative staining in both nucleus and plasma, 1=positive staining in nucleus but negative or weak staining in plasma, 2=positive staining in both nucleus and plasma (strong). EPHA3 expression was assessed based on nuclear and membrane staining and was characterized into four categories: 0=negative staining, 1=overall weak staining or staining with intermediate intensity in <10% tumor cells, 2=overall intermediate staining or strong intensity in <10% tumor cells, 3=strong staining in 10% tumor cells. Nuclear staining was assessed for CLDN11, CLII, and LEF1. For CLDN11, we dichotomized the staining levels (0=negative and 1=positive) because of less variable staining intensity among positive cells. For CLII, on the other hand, the proportion of positive stained cells showed a wide range, we therefore used the quartile to categorize the staining. LEF1 was categorized into three groups: 0=negative, 1= 10% tumor cells, and 2= >10% tumor cells. Average values were taken for the two cores from same donor blocks.

### **Statistical Analysis:**

Wilcoxon rank or Kruskal-Wallis test was used to compare media differences in gene expression or signature contribution across tumor subtypes. Logistic regression models were

used to assess the associations between risk factors/tumor features and tumor subtypes. The Kaplan–Meier curve was used to assess survival among patients, stratified by the different levels of protein expression defined by immunohistochemical staining intensity. Hazard ratios (HRs), 95% confidence intervals (CIs), and p values were obtained using multivariate Cox proportional hazards model with the adjustment of age, sex, pre- and post-surgery radiation therapy. All statistical tests in the present study were two-sided and performed using SAS version 9.4 (SAS Institute, Cary, NC, USA) or R version 3.6.3 (R Foundation for Statistical Computing, Vienna, Austria).

### Data availability

The WGS and RNA-Seq data generated in this study have been deposited in the Database of Genotypes and Phenotypes (dbGaP) under Accession Code phs002301.v1.p1. The targeted sequencing and NanoString data for the European ancestry replication dataset will be released to dbGaP under accession # phs001280.v1.p1.

## Results

### Patient cohorts

The discovery analysis (RNA-Seq cohort) included 48 patients with skull-base chordoma who were diagnosed and treated at Beijing Tiantan Hospital, China. The detailed clinical characteristics of these patients are shown in Table 1. In brief, the mean age at initial diagnosis of chordoma among these patients was 43.6 years (range: 6–72); 64.6% were males, 54% had conventional/classical chordoma and 46% had chondroid chordoma. After an average follow-up period of 52 months, there were 30 recurrences and 9 deaths, all from chordoma (Table 1).

The molecular replication dataset (NanoString cohort) included 48 patients collected from chordoma patients residing in North America (the United States and Canada) unselected for tumor sites (13) with RNA extracted from FFPE tumors. The average age at diagnosis was 50 years (range 6–78) and the site distribution was 48.9% skull-base, 25.6% spinal, and 25.6% sacral (Table 1). The vast majority of patients (98%) had classic chordoma histology.

Since follow-up time was short in the Chinese RNA-Seq cohort and clinical outcome data were not collected in the North America NanoString cohort, we used another Chinese patient cohort with a large number of chordoma tumors (N=312) constructed on TMAs and long-term follow-up data to assess the clinical outcome in relation to molecular subtypes (survival cohort). All patients in the survival cohort had skull base chordoma and the distribution of patient characteristics is shown in Table 1.

### Molecular classification of chordoma based on whole-transcriptome RNA-Seq analysis

We conducted unsupervised consensus clustering analysis of 48 skull-base chordoma tumors using expression of 2,000 most variable genes. The best separation was achieved by dividing the tumors into three subtypes (CC1: n = 23; CC2: n = 11; CC3; n = 14; Figure 1A). Compared with CC1 and CC2 tumors, tumors in CC3 had lower tumor purity estimated using ESTIMATE (see Method, Figure 1B) as well as lower *TBXT* gene expression (Figure

1C, 1D), which is commonly considered as a hallmark of chordoma, suggesting that this subtype might be enriched with low-purity tumors. We therefore focused the subsequent analyses on CC1 and CC2. The sensitivity analysis of restricting to 34 CC1 and CC2 tumors caused minimal change in classification, with only two patients moved from CC1 to CC2. Therefore, we used the original classification in all downstream analyses.

CC1 and CC2 did not differ significantly in age, tumor size, or histologic subtype in the multivariable logistic regression analysis with the mutual adjustment of these variables (Supplementary Table 2), although CC2 tumors were more likely to have a higher frequency of chondroid subtype (73%) as compared to CC1 (52%). Among 22 CC1 and CC2 tumors with whole-genome sequencing (WGS) data (14), CC2 tumors were less likely to have mutations or structural variants involving *PBRM1* (0 in CC2 vs. 4 in CC1) or *SETD2* (0 in CC2 vs. 1 in CC1) (Supplementary Table 3), and had higher expression levels of *PBRM1* ( $p=0.0009$ ) and *SETD2* ( $p=0.0012$ ) (Supplementary Figure 1). The overall tumor mutational burden (0.43 mutations/Mb for CC1 and 0.59 mutations/Mb for CC2,  $p=0.33$ ) or copy number alteration patterns (14) did not vary significantly between the two subtypes. Mutational signatures based on single-base substitution (SBS) did not show significant differences between CC1 and CC2, whereas small indel (ID) signatures demonstrated suggestive differences by subtype (Supplementary Figure 2). CC1 tumors appeared to have a higher contribution of signature A ( $p=0.005$ ), which was not mapped to any COSMIC ID signatures and is composed predominantly of 2bp insertions (mainly AT and TA) at long ( 5) repeats (14). On the other hand, compared to CC1 tumors, CC2 tumors were more likely to have a higher fraction of signature E ( $p=0.021$ ), which reflected combinations of COSMIC ID3, 4, 5, and 9 (14). Results based on number of mutations in these signatures showed similar patterns (Supplementary Figure 2).

Top differentially expressed genes (DEGs) between CC1 and CC2 are shown in Figure 2A, with the majority showing higher expression in CC2 than in CC1 (complete list of DEGs shown in Supplementary Table 4). Top DEGs included those that play roles in embryonic development (*PEG10*(19), *HHIP*(20), *PTCH1*(21), *EPHA3*(22), *LEF1*(23)), neural development (*ODZ2*(24), *SPON2*(25), *NGFR*(26), *DPYSL3*(27), *PPP1R9A*(28), *NOTCH3*(29)), and bone development (*OMD*(30), *PKDCC*(31), *MATN3*(32), *TMEM119*(33), *COL10A1*(34)), which are biologically relevant in chordoma development. Gene set enrichment analysis (GSEA) revealed that the most significant pathways (nominal  $p<0.0001$ ) these DEGs were enriched for included epithelial-mesenchymal transition (EMT), which was upregulated in CC2, and interferon alpha response and TNF alpha signaling via NFkB, which were downregulated in CC2 (Figure 2B, 2C, Supplementary Table 5).

### Replication of the molecular classification in an independent chordoma cohort

To replicate the molecular classification, we designed a NanoString panel including 21 of the top DEGs (*AHR*, *ANGPTL4*, *GLI1*, *GULP1*, *HHIP*, *LEF1*, *LRRC32*, *ODZ2*, *ODZ4*, *OMD*, *PCOLCE*, *PDZRN4*, *PEG10*, *PKDCC*, *PTCH1*, *SLC6A20*, *SPON2*, *TMTCl*, *TNNC1*, *TP63*, and *TSPAN11*) from RNA-Seq and measured expression of these genes in 48 FFPE tumors collected from chordoma patients in North America (the NanoString



cohort). The expression of these genes separated tumors into two main groups (NCC1 and NCC2), with tumors in NCC2 having upregulation of most genes (Figure 3), a pattern that is similar to CC2 in the RNA-Seq cohort. The distribution of sex and tumor site did not vary significantly between the two groups, although NCC2 appeared to be enriched with younger (<50 years,  $p=0.160$ ) patients. Similar classifications were found in the 23 skull-base and 25 non-skull-base patients, respectively (Supplementary Figure 3).

We then searched for mutations in potential chordoma driver genes in this set of tumors by designing a targeted DNA sequencing panel including genes reported in two chordoma landscape studies (Supplementary Table 1) (14,35). Consistent with results from the RNA-Seq cohort, we found that NCC2 tumors were less likely to have mutations involving *PBRM1* (0 in NCC2 vs. 2 in NCC1) and *SETD2* (0 in NCC2 vs. 2 in NCC1) and were more likely to have higher expression levels of these genes (*PBRM1*:  $p=0.0005$ , *SETD2*:  $p=0.0002$ , Supplementary Figure 1). In addition, among three patients carrying mutations in *LYST*, a potential chordoma driver gene reported by Tarpey *et al.*(35), all had NCC1 tumors. In contrast, among six patients carrying mutations in *PIK3CA*, three had NCC1 and three had NCC2 tumors, and the single *TP53* mutation carrier had NCC2 tumor. These results suggest that the two major molecular subtypes may be associated with distinct sets of chordoma driver genes.

### Clinical relevance of the molecular classification

Since follow-up time was short in the RNA-Seq cohort and clinical outcome data were not collected in the NanoString cohort, we examined expression levels of top DEGs in relation to clinical outcomes in the survival cohort of 312 skull base chordoma patients with an average of 5 years of follow-up time to determine the clinical relevance of the molecular subtypes. We selected five genes (*PTCH1*, *LEF1*, *EPHA3*, *GLII*, *CLDN11*) that showed differential expression between the two subtypes (CC1/NCC1 and CC2/NCC2) in both RNA-Seq and NanoString analyses and had commercial antibodies available. We conducted immunohistochemical (IHC) staining of these markers on 312 tumors constructed on TMAs. Among the five markers evaluated, *PTCH1* protein expression showed significant associations with both chordoma-specific survival (CSS: hazard ratio [HR] = 2.74, 95% confidence interval [CI] = 1.51 – 4.99,  $p=0.001$ , comparing strong to negative staining) and recurrence-free survival (RFS: HR = 2.52, 95% CI = 1.49 – 4.26,  $p=0.001$ , comparing strong to negative staining) (Figure 4). *CLDN11* was associated with RFS (HR = 1.48, 95% CI = 1.09 – 2.01,  $p=0.013$ , comparing positive to negative staining) but not with CSS (HR = 1.26, 95% CI = 0.88 – 1.82,  $p=0.21$ , comparing positive to negative staining) (Supplementary Figure 4). The protein expression of the other three markers examined did not show significant associations with either CSS or RFS (Supplementary Figure 4).

### Discussion

Using whole-transcriptomic analysis, we identified two major molecular subtypes of skull-base chordoma, which were distinguished by expression levels of genes involved in embryonic development, neural development, and bone development. Significant pathways enriched in the differentially expressed genes between the two subtypes included EMT,

inflammatory response, angiogenesis, interferon gamma, apical junction, p53, and allograft rejection, with EMT showing the strongest enrichment. These two major subtypes were replicated in an independent chordoma cohort containing patients of different ethnic ancestries and unselected tumor locations, suggesting the robustness of the classification. DNA sequencing data from both datasets suggest that the two subtypes may be associated with distinct sets of driver genes. Further, some DEGs showed significant associations with clinical outcomes in a large chordoma cohort with long-term follow-up data, demonstrating the prognostic relevance of the molecular classification.

EMT is a process characterized by epithelial cells losing contact with their neighbors and gaining mesenchymal properties, which plays an important role in embryonic development and cancer progression (36). *TBX1*, which encodes a transcription factor (Brachyury) in notochord and plays an essential role in chordoma development (8,37), has been shown to promote EMT in a variety of cancers including chordoma (38). Although the two subtypes showed similar *TBX1* expression levels, CC2 was associated with the upregulation of EMT genes, suggesting other factors may drive the different levels of EMT between CC1 and CC2. For example, *TWIST1*, a transcription factor and master regulator of embryonic morphogenesis that plays an essential role in metastasis through promoting EMT (39), showed higher expression in CC2 than CC1 ( $p=0.046$ , Supplementary Figure 5). Similarly, most of the Snail family members (*SNAIL* [ $p=0.017$ ] and *SNAIL3* [ $p=0.098$ ]), which are also prominent EMT inducers and are implicated in important developmental processes including neural differentiation (40), were also upregulated in CC2 tumors (Supplementary Figure 5). Since CC2 also showed significant enrichment of genes in inflammation, angiogenesis, and p53 pathways, it is possible that these processes may contribute to inducing and sustaining EMT in CC2. Since EMT is the major mechanism by which cancer cells become metastatic, targeting molecules in this pathway may present potential therapeutic applications for chordoma patients with CC2 tumors. To support this hypothesis, a recent study suggested that *Twist*-silenced MUG-Chordoma cells were less migratory and invasive compared with negative controls (41).

Chordoma is derived from the remnants of the embryonic notochord, which plays an important role in directing vertebral column formation and segmentation (42). Sonic Hedgehog (*SHH*), a morphogen secreted by the embryonic notochord, directs the development of the vertebral axis when it binds to the *PTCH1* receptor. *SHH* signaling is inactive in later embryonic stages and its persistent activation may lead to neoplastic transformation of notochordal remnants (43). Somatic mutations in *PTCH1* have been identified in more than 90% of sporadic basal cell carcinoma patients but are infrequent in other tumors (44). In our patients with WGS data available, *PTCH1* mutations, either germline or somatic, were not observed. However, we observed that *PTCH1* and *GLI1* expression was upregulated in the CC2 subtype in both discovery RNA-Seq and replication NanoString datasets, suggesting the activation of *SHH* signaling in CC2 tumors. In addition, we also observed the upregulation of *HHIP*, a member of the hedgehog-interacting protein, in CC2. *HHIP* acts as a vertebrate-specific inhibitor of HH signaling (45) and like *PTCH1*, *HHIP* transcription is directly induced by *SHH* signaling and then negatively regulates the signaling pathway(46). Thus, the upregulation of this molecule may also suggest the activation of *SHH* signaling in the CC2 subtype. These findings are consistent with

results from a recent study by Yang *et al.* showing the extensive expression of *SHH* molecules including *PTCH1* in chordoma patients and the therapeutic potential of targeting the *SHH* signaling pathway in treating chordoma (43). Consistent with this, we showed that increasing *PTCH1* protein expression was associated with worse clinical outcomes in our survival cohort, similar to previous studies that reported higher *PTCH1* expression associated with worse prognosis in breast cancer patients (47,48). On the other hand, unlike what was reported by Yang *et al.* (43), *GLI1* protein expression was not associated with clinical outcomes in our TMA analysis (Supplementary Figure 4).

While CC2 tumors demonstrated the activation of EMT and *SHH* pathways, the CC1 subtype seemed to be enriched with tumors showing mutations and reduced expression in chromatin remodeling pathways, such as *PBRM1* and *SETD2*. Although the association is based on small numbers of tumors with both molecular subtyping and DNA sequencing data, we observed similar associations in the replication dataset. *SWI/SNF* genes (such as *PBRM1* and *SMARCB1*) and *SETD2* have been described as potential driver genes in both skull-base and sacral chordomas (14,35). Mutations and loss of function in these genes may predict and contribute to responses to immune checkpoint inhibitors (49–51). Immune checkpoint inhibitors are a potential line of therapy in chordomas and clinical trials of agents that block the PD-1/PD-L1 pathway are ongoing in a range of cancers, some of which included chordoma patients (52).

Clinical outcomes of chordoma patients are highly variable and disease progression is likely determined by both surgical factors and tumor biology. Currently, there is no clear clinical guidance on patient stratification regarding treatment such as post-surgery RT. Previous studies have associated copy number alterations such as deletions of chromosomal regions 1p36, 9p21, 9q, and 22q (14,53) and methylation profiles (12) with clinical outcomes of chordoma patients. Our findings here further demonstrate the potential of developing molecular prognostic tools to predict patient outcomes by integrating genomic and gene expression markers. Together, these studies highlight the need and feasibility of developing molecular marker panels to predict clinical courses of chordoma patients. For patients who are predicted to have a poor prognosis, more proactive treatments should be considered such as aiming towards maximum tumor resection, application of high-dose RT and shortening the interval between RT and surgery, and early enrollment for molecular therapy trials.

The major limitations of our study include the small sample size of the discovery dataset and lack of clinical outcome data in both discovery and replication datasets, which may have limited the discriminative power and direct assessment of clinical implications. This is particularly true for analyses involving DNA mutation data, which was based on a smaller number of patients. Future studies are needed to validate our findings and develop a set of markers to accurately discriminate subtypes regarding prognosis. In addition, in our analysis we focused on the two major subtypes with higher tumor purity, however, the third subtype we observed may not be completely driven by the low tumor purity due to sampling. Some of the tumors in this subtype may be enriched with immune and other cells in the tumor microenvironment, which may reflect a biologically distinct entity. Indeed, a recent study of epigenetic classification of chordoma tumors found that one of the subtypes they observed showed higher abundance of tumor infiltrating immune

cells (12). By further examining H&E and Brachyury stained images of CC3 patients, we confirmed that samples in this subtype contained lower tumor cells, which fell into different scenarios. For example, some tumors indeed had high stromal content primarily driven by fibroblasts, endothelial cells, and inflammatory cells, while other samples were contaminated by normal adjacent tissue, such as dura mater and bone or bone marrow components. Future studies using spatial profiling analyses such as single-cell RNA-Seq are warranted to follow up this group of patients. Another potential limitation is that the Nanostring cohort had tumors from various anatomic locations and the vast majority had classic histology, while the other two datasets were exclusively skull-base and had variable histology. However, the validation of the classification across datasets indicates the robustness of the classification that is not restricted to certain ethnic groups, histology, and tumor locations. Despite the limitations, our study included several clinically well-annotated patient cohorts and replicated the molecular classification in tumors of different ancestries and profiled using different platforms. Further, using a large chordoma cohort with clinical follow-up data and tissue samples available, we demonstrated the clinical relevance of this molecular classification scheme, indicating the potential of developing key molecular markers to improve patient prognostication.

In summary, we defined at least two major molecular subtypes of chordoma using gene expression profiling data. Our findings shed light on subtype-specific molecular mechanisms underlying chordoma development and progression and may have important clinical implications in patient stratification and targeted treatments.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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## Data availability

The WGS and RNA-Seq data generated in this study have been deposited in the Database of Genotypes and Phenotypes (dbGaP) under Accession Code phs002301.v1.p1. The targeted sequencing and NanoString data for the European ancestry replication dataset have been deposited in dbGaP under accession # phs001280.v1.p1.

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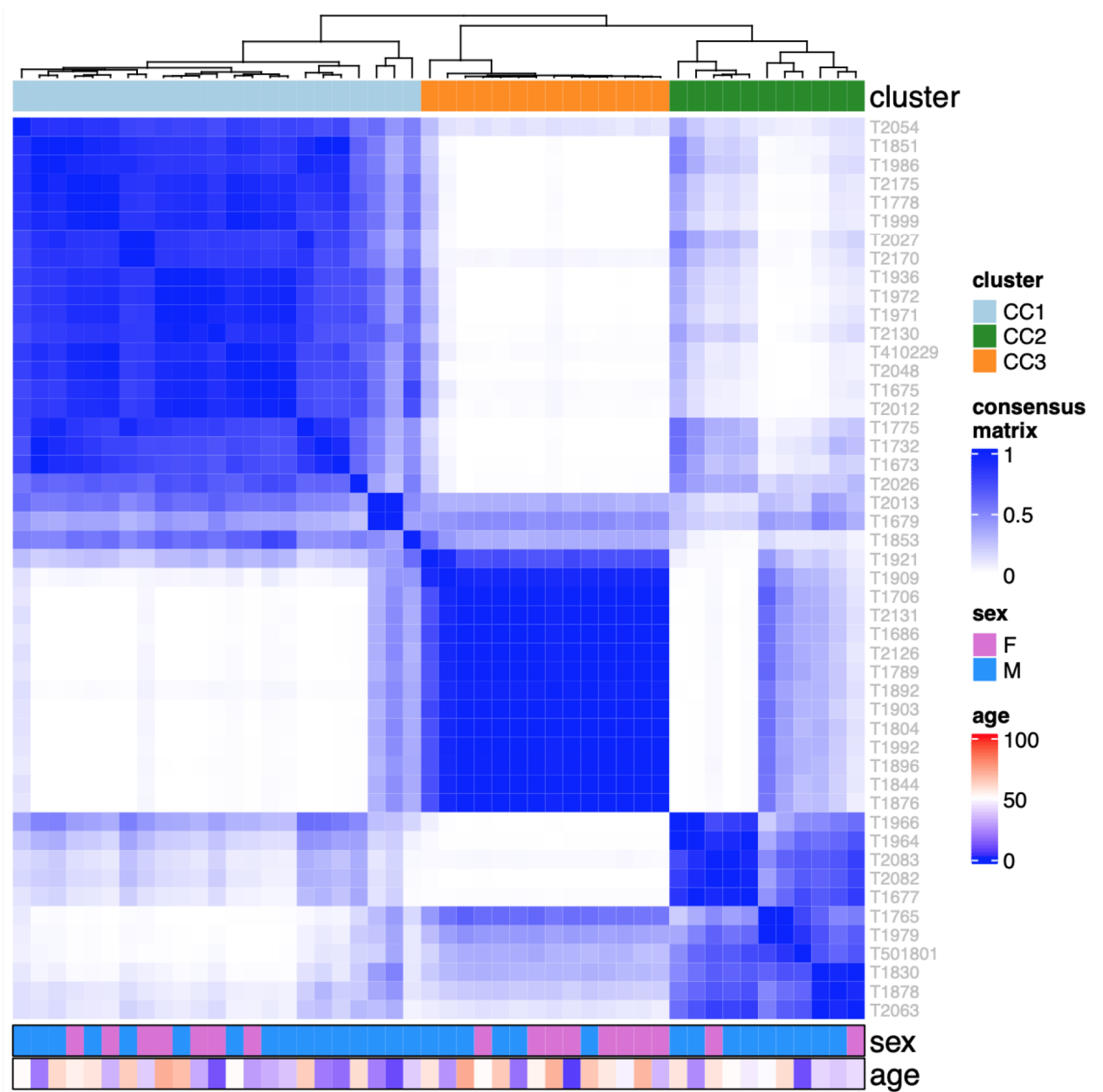
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### Translational relevance

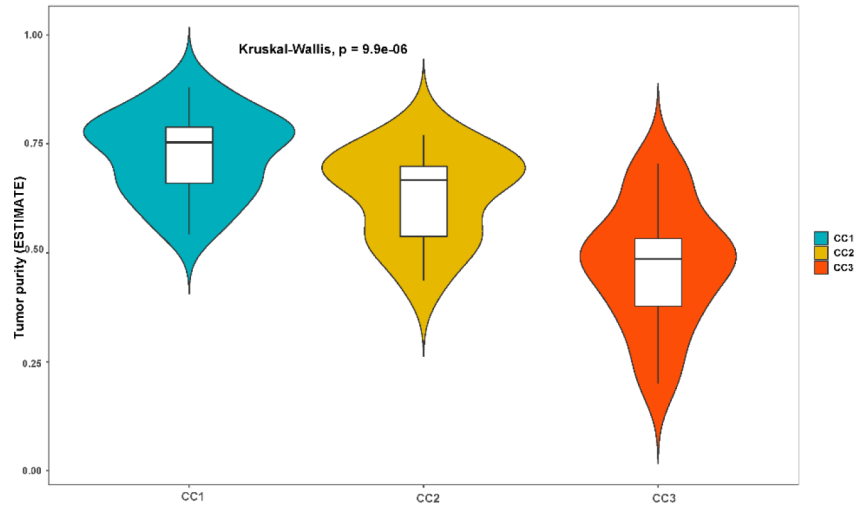
Chordoma is a rare bone tumor with a largely unclear etiology and high recurrence rate. Currently, there are no clear guidelines on patient stratification for targeted therapies. In addition, treatment options are limited for patients with advanced disease. Here, using gene expression data obtained from two independent datasets comprising of ethnically different patients, we identified at least two major molecular subtypes of chordoma that were characterized by different genomic features and biological pathways. Our findings provide a first glimpse into the subtype-specific tumorigenesis of chordoma, a tumor type with limited information on etiology and biology. Further, in a large cohort with long-term follow-up and tumor samples available, we demonstrated the association of the molecular subtypes with clinical outcomes, supporting the feasibility of developing molecular marker panels to improve patient stratification regarding prognostication and targeted treatment.



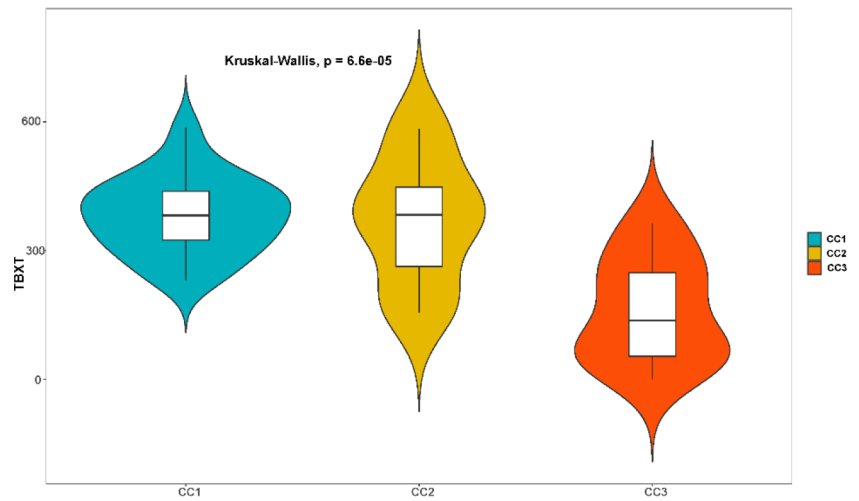
**A. Consensus clustering analysis identified three groups (CC1, CC2, CC3).**



**B. Tumor purity inferred by ESTIMATE in the three groups.**

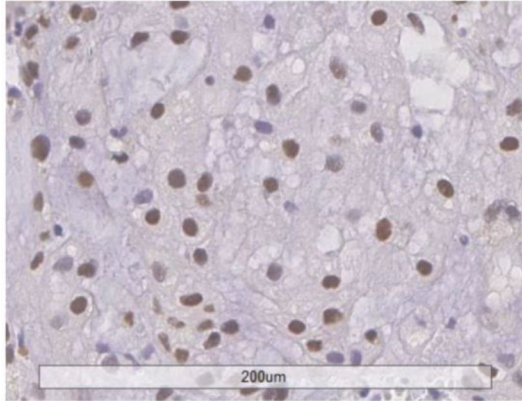


**C. TBXT gene expression in the three groups.**

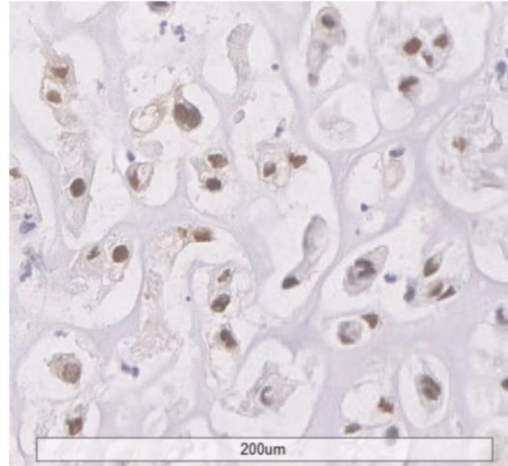


**D. Representative images of Brachyury immunohistochemical staining in the three groups.**

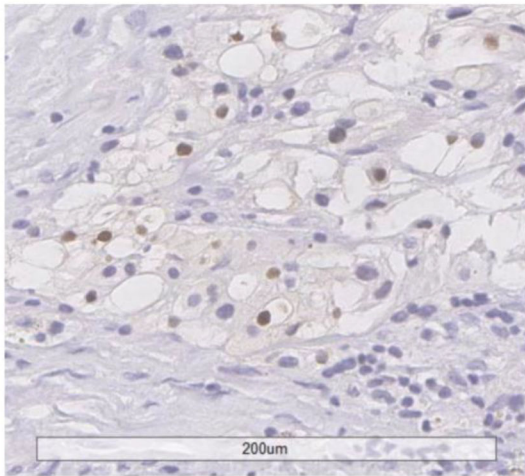
CC1



CC2

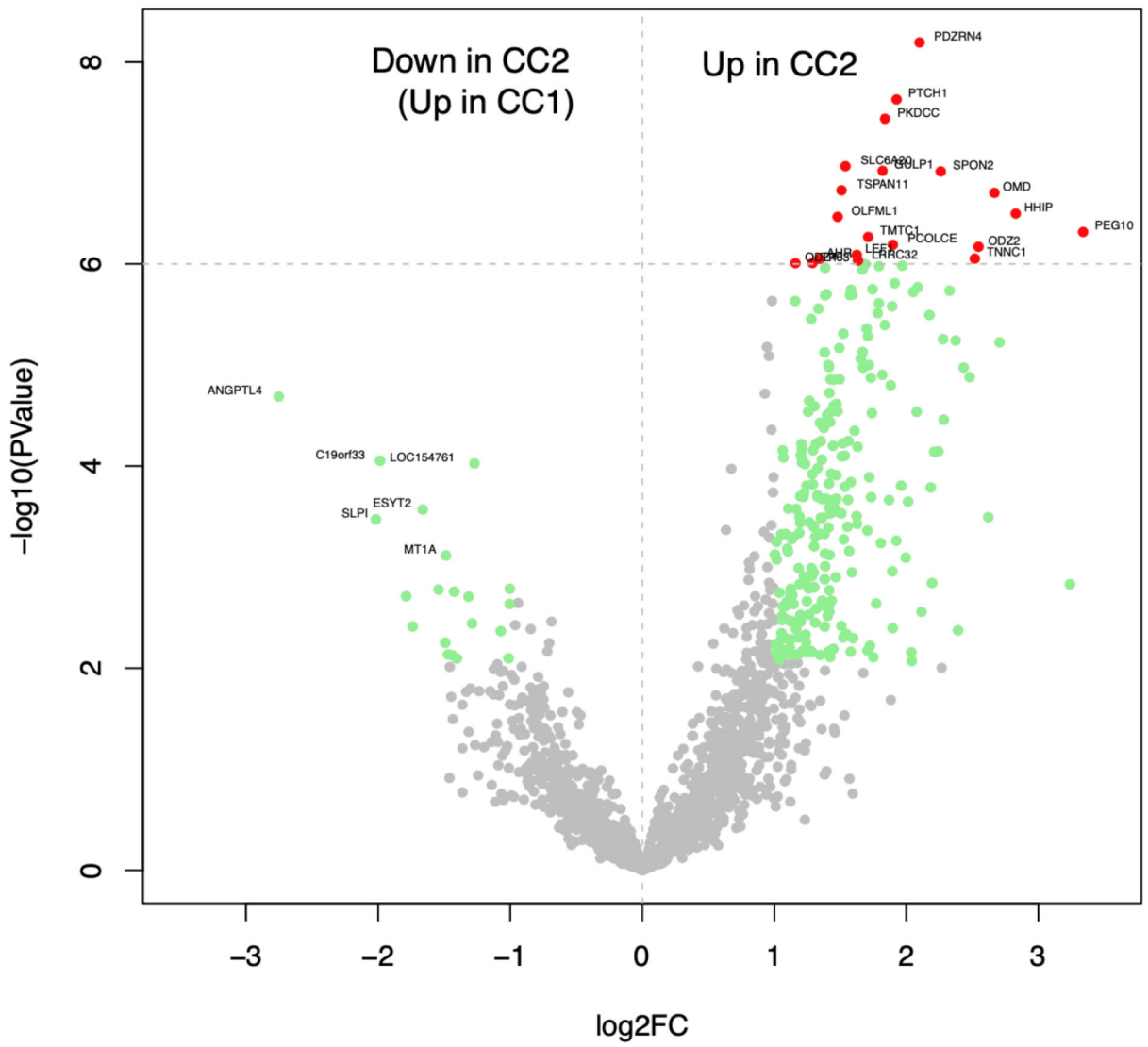


CC3

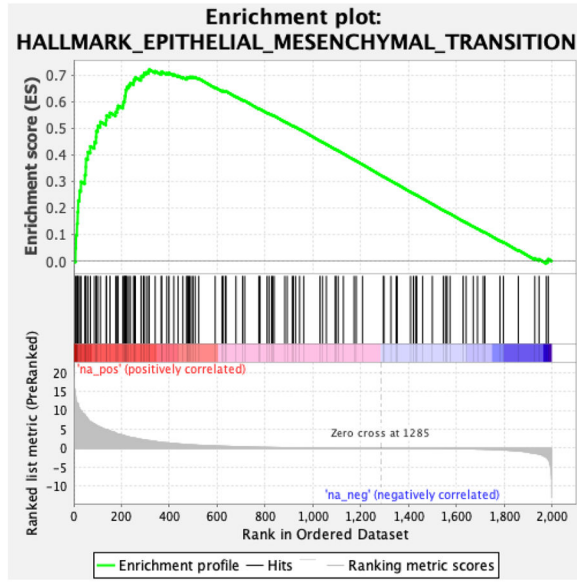


**Figure 1.** Unsupervised consensus clustering of tumors based on RNASeq data from 48 Chinese skull-base chordoma patients

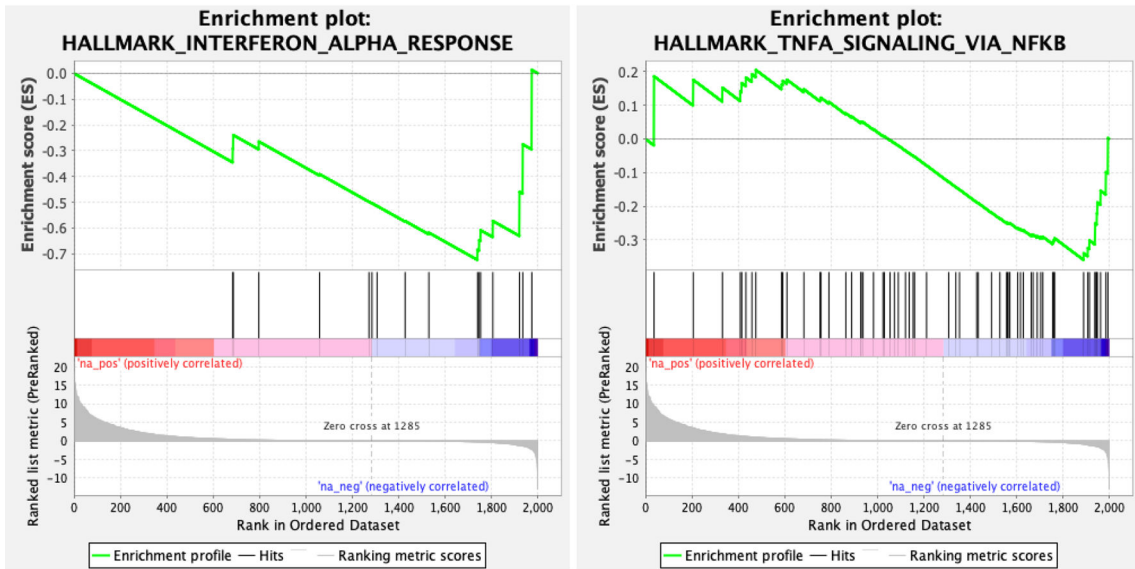
A. Volcano plot showing differentially expressed genes between CC1 and CC2



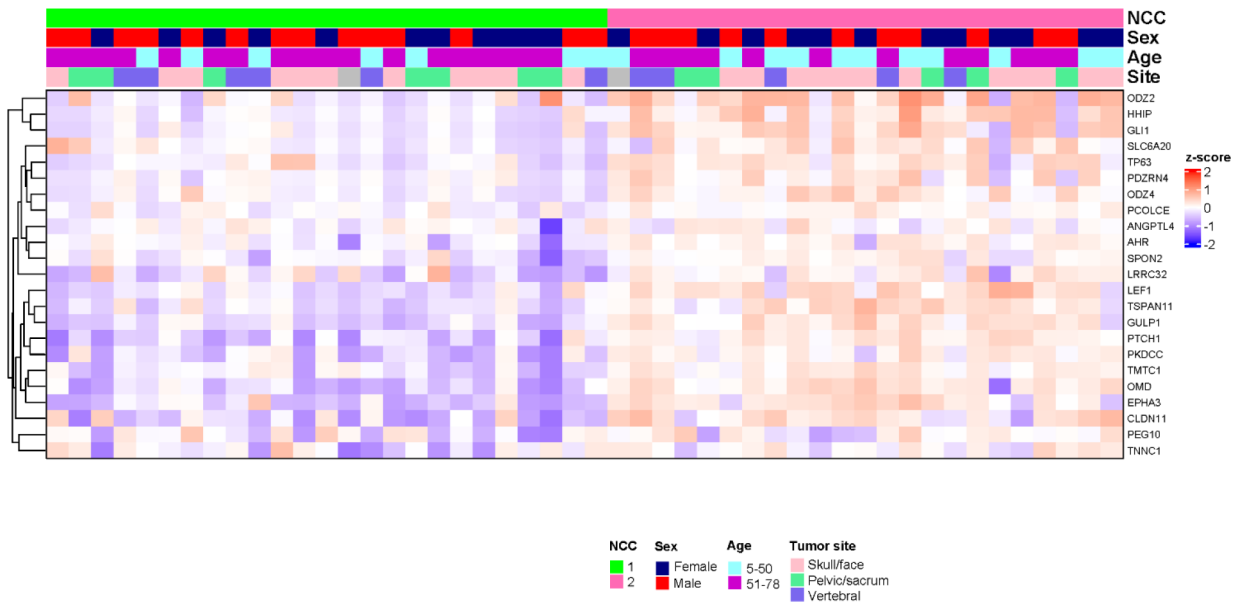
**B. Enrichment plot showing the top upregulated gene set (Epithelial Mesenchymal Transition) in CC2 vs. CC1**



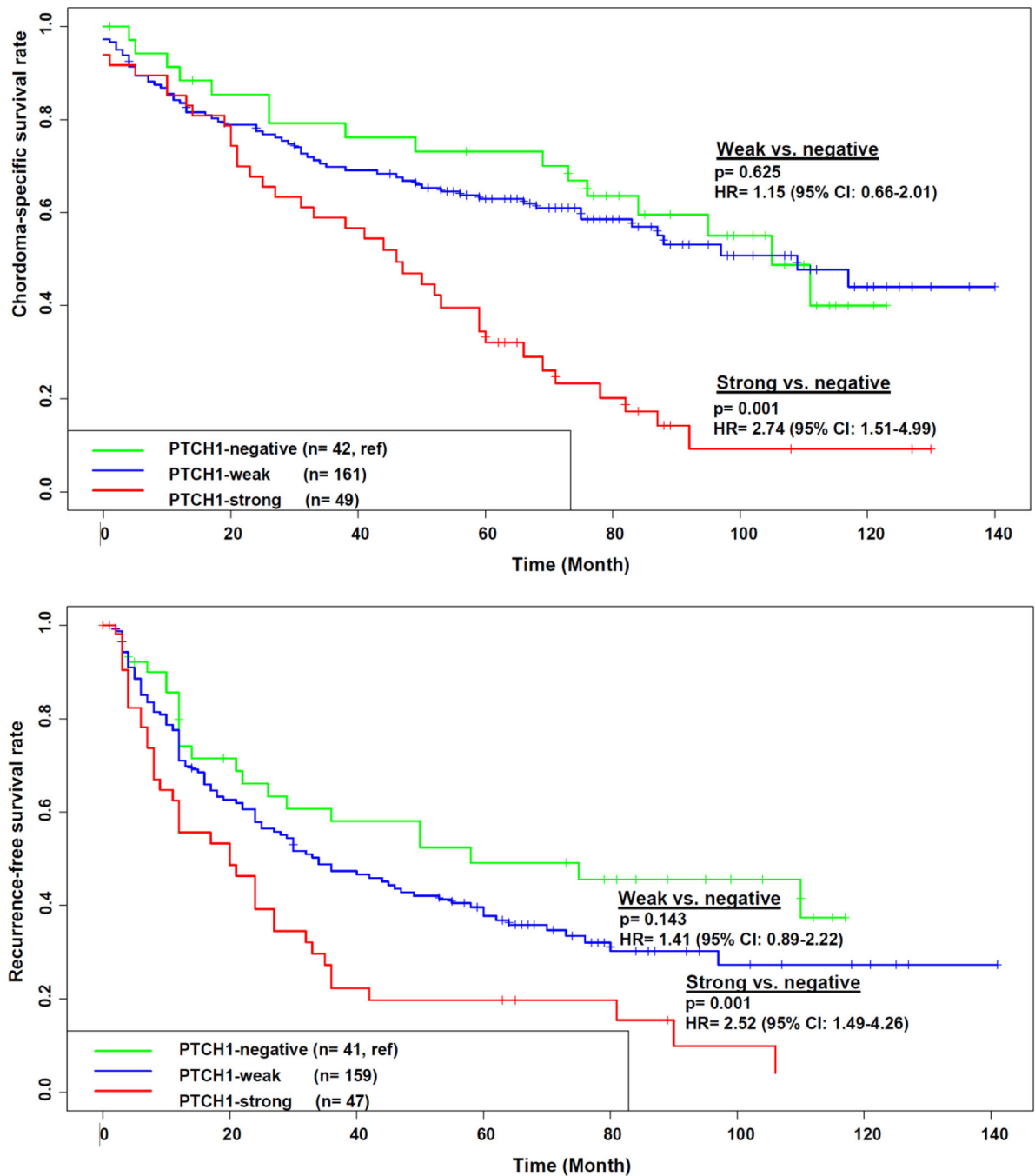
**C. Enrichment plot showing the top downregulated gene set (Interferon-alpha-response and TNFA-signaling-via-NFKB) in CC2 vs. CC1**



**Figure 2.**  
Differentially expressed genes between CC1 and CC2



**Figure 3.**  
Gene expression profiling based on the NanoString panel in chordoma patients from North America



**Figure 4.** Expression of PTCH1 in relation to overall survival (upper panel) and recurrence-free survival (lower panel) in 312 Chinese chordoma patients with long-term follow-up data

**Table 1:**

Description of the three chordoma patient cohorts included in this study

|  | RNAseq cohort (N= 48) | Nanostring cohort (N= 48) | Survival analysis cohort (N= 312) |                      |
|--|-----------------------|---------------------------|-----------------------------------|----------------------|
| Variables  | N (%) Mean (SD)       | N (%) Mean (SD)           | N (%) Mean (SD)                   | P-value <sup>a</sup> |
| <b>Age (year)</b>                                | 43.6 (18.7)           | 50.0 (16.4)               | 40.7 (14.5)                       | <b>0.0004</b>        |
| <25  | 10 (20.8)             | 5 (10.4)                  | 51 (16.3)                         | <b>&lt;.0001</b>     |
| 25–50  | 15 (31.3)             | 13 (27.1)                 | 172 (55.1)                        |                      |
| 50   | 23 (49.9)             | 30 (62.5)                 | 89 (28.6)                         |                      |
| Missing  |                       |                           |                                   |                      |
| <b>Sex</b>                                       |                       |                           |                                   | 0.4323               |
| F  | 17 (35.4)             | 23 (47.9)                 | 137 (43.9)                        |                      |
| M  | 31 (64.6)             | 25 (52.1)                 | 175 (56.1)                        |                      |
| Missing  |                       |                           |                                   |                      |
| <b>Tumor site</b>                                |                       |                           |                                   | <b>&lt;.0001</b>     |
| Skull/face                                       | 48 (100)              | 23 (48.9)                 | 312 (100)                         |                      |
| Pelvic/sacrum                                    | 0 (0)                 | 12 (25.5)                 | 0 (0)                             |                      |
| Vertebral  | 0 (0)                 | 12 (25.6)                 | 0 (0)                             |                      |
| Missing  |                       | 1                         |                                   |                      |
| <b>Histological type</b>                         |                       |                           |                                   | <b>&lt;.0001</b>     |
| Classic  | 26 (54.2)             | 47 (97.9)                 | 227 (72.7)                        |                      |
| Chondroid  | 22 (45.8)             | 1 (2.1)                   | 82 (26.3)                         |                      |
| Poorly differentiated                            | 0 (0)                 | 0 (0)                     | 3 (1.0)                           |                      |
| Dedifferentiated                                 | 0 (0)                 | 0 (0)                     | 0 (0)                             |                      |
| Missing  |                       |                           |                                   |                      |
| <b>Pre-surgery RT<sup>b</sup>treatment</b>       |                       |                           |                                   | <b>0.0419</b>        |
| No   | 46 (95.8)             |                           | 266 (85.3)                        |                      |
| Yes  | 2 (4.2)               |                           | 46 (14.7)                         |                      |
| Missing  |                       |                           |                                   |                      |
| <b>Post-surgery RT<sup>b</sup>treatment</b>      |                       |                           |                                   | 0.8736               |
| No   | 27 (56.3)             |                           | 151 (58.5)                        |                      |
| Yes  | 21 (43.7)             |                           | 107 (41.5)                        |                      |
| Missing  |                       |                           | 54                                |                      |
| <b>Post-surgery RT<sup>b</sup>treatment type</b> |                       |                           |                                   | <b>&lt;.0001</b>     |
| Proton   | 11 (45.8)             |                           | 16 (20.0)                         |                      |
| Radiosurgery                                     | 2 (8.4)               |                           | 47 (58.8)                         |                      |
| Photon/Carbon                                    | 11 (45.8)             |                           | 0 (0)                             |                      |
| Intensity-modulated radiation therapy            | 0 (0)                 |                           | 17 (21.2)                         |                      |
| Missing  |                       |                           | 27                                |                      |
| <b>Recurrence status</b>                         |                       |                           |                                   | 0.1944               |
| No   | 18 (37.5)             |                           | 83 (28.9)                         |                      |



|   | RNAseq cohort (N= 48) | Nanostring cohort (N= 48) | Survival analysis cohort (N= 312) |                      |
|---|-----------------------|---------------------------|-----------------------------------|----------------------|
| Variables                               | N (%) Mean (SD)       | N (%) Mean (SD)           | N (%) Mean (SD)                   | P-value <sup>a</sup> |
| Yes                                     | 30 (62.5)             |                           | 204 (71.1)                        |                      |
| Missing                                 |                       |                           | 25                                |                      |
| <b>Recurrence-free survival (month)</b> | 22 (2–53)             |                           | 33 (0–141)                        | <b>0.0378</b>        |
| <b>Death status</b>                     |                       |                           |                                   |                      |
| No                                      | 39 (81.2)             |                           | 158 (50.7)                        | <b>&lt;.0001</b>     |
| Yes                                     | 9 (18.8)              |                           | 154 (49.3)                        |                      |
| Missing                                 |                       |                           |                                   |                      |
| <b>Overall survival (month)</b>         | 52 (10–174)           |                           | 61 (0–140)                        | 0.0916               |

<sup>a</sup>Results from Chi-square, Fisher's Exact, or ANOVA tests.

<sup>b</sup>Radiation therapy

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