Experimental Study on Differences in Clivus Chordoma Bone Invasion: An iTRAQ-Based Quantitative Proteomic Analysis

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Abstract

Although a bone tumor, significant differences in the extent of bone invasion exist in skull base chordoma, which directly affect the extent of surgical resection, and have an impact on its prognosis. However, the underlying mechanism of the phenomenon is not clearly understood. Therefore, we used an iTRAQ-based quantitative proteomics strategy to identify potential molecular signatures, and to find predictive markers of discrepancy in bone invasion of clivus chordoma. According to bone invasive classification criteria, 35 specimens of clivus chordoma were classified to be either endophytic type (Type I) or exophytic type (Type II). An initial screening of six specimens of endophytic type and six of exophytic was performed, and 250 differentially expressed proteins were identified. Through the GO and IPA analysis, we found evidence that the expression of inflammatory activity-associated proteins up-regulated in endophytic type, whereas the expression of cell motility-associated proteins up-regulated in exophytic ones. Moreover, TGFβ1 and mTOR signal pathway seemed to be related with bone invasion. Thus, TGFβ1, PI3K, Akt, mTOR, and PTEN were validated in the following 23 samples by immune histochemistry and Western blot. The expression levels of TGFβ1 and PTEN were significantly lower in the endophytic type than in the exophytic ones. It was found that TGFβ1 may play an important role in its bone invasion. The mechanisms may be related with conducting an increased inflammatory cell response and a decline in cytoskeletal protein expression. PTEN is confirmed to be associated with the degree of bone invasion. The PI3K/AKT/mTOR signaling pathway might be associated with the bone invasion, but still needs a larger sample size to be verified. These results, for the first time, not only demonstrate the biological changes that occur in different growth patterns from the perspective of proteomics, but also provide novel markers that may help to reveal the mechanisms behind clivus chordomas.
Introduction

Chordoma is a type of bone tumor originating from notochordal remnants. It often occurs in the body axis, including the skull base and sacral, and skull base chordoma accounts for about 32% of cases [1]. Radical surgery is the most effective treatment choice [2,3]. However, due to the depth of skull base chordoma and its proximity to complex structures, as well as tumor infiltration into adjacent bone, skull base chordoma resection is very difficult, and relapses after surgery are frequent occurrences. A retrospective study of our research group found that the skull base chordoma recurrence rates after 5 and 10 years are 52.9% and 88.3%, respectively [2].

The extent of skull base bone invasion in this kind of tumors is quite different. Bone invasion and destruction in some cases were quite heavy, which in some others were relatively light. Based on results of previous studies [2–4], as well as clinical practice, our research group discovered that the degree of bone invasion and the integrity of skull base dural barrier are independent risk factors affecting the clinical prognosis of skull base chordoma patients. In addition, Therefore, it is necessary to explore the causes and mechanisms of the differences in bone invasion.

The protein expression levels of Cadherins, Catenins, MMPs, Cathepsin B and uPA are related to the invasion of skull base chordoma [5,6], and these levels may affect treatment effect and prognosis. However, due to limitations of the experimental method, it is not yet possible to integrate and systematically analyze the proteins associated with chordoma bone invasion.

Integrated tumor proteomics research, especially differential proteomics and functional proteomics research, is a new tool of protein research [7]. Currently, there is only one report on chordoma proteomics research. The study analyzed the different proteins in chordomas and adjacent muscle tissues, but it failed to find specific protein associated with its prognosis [8]. Isobaric tags for relative and absolute quantitation (iTRAQ) is an isobaric labeling method used in quantitative proteomics by tandem mass spectrometry to determine the amount of proteins from different sources in a single experiment. It was a high-throughput quantification method which were more and more wildly used for quantitative proteomics.

This study grouped differences of clivus chordoma based on different bone infiltration imagings preoperatively, and used iTRAQ-based quantitative proteomic technology to analyze and compare the differentially expressed proteins in the corresponding subgroups. Furthermore, protein expression was confirmed by immune histochemical staining and Western blot.

Materials and Methods

1. Case Selection criteria

The Institutional Review Board (IRB) of Beijing Tiantan Hospital, Capital Medical University approved the study. From January 2009 to January 2013, patients admitted to the Skull-base Ward, Neurosurgery Department of Beijing TianTan Hospital, Capital Medical University were included. All the patients were signed the Ethnic statements when they were enrolled. The documents were scanned and stored in the hospital. The written informed consent was obtained from the participants prior to their participation. In addition, the included patients should be primary untreated, and had lesions in the region of clivus, rather than in the foramen magnum, jugular foramen or spine. They all received tumor resection and were pathologically diagnosed as classical chordoma cases.
2. Bone invasive classification criteria

Enrolled patients were classified according to preoperative images (including plain and enhanced head MRI, thin layer skull base CT scanning and 3-D reconstruction). The maximum diameter at eyeball level of the T2 axial MR images was set as the baseline level, and the area of the bilateral carotid cavernous lateral walls connected to the bilateral petrous apex at the baseline level was set as the standard region. If at the baseline level, 50% or more of the tumor, which can invade the bone through all directions, was located within the standard region, and the clivus bone transformed like a “bubble” or a “dumbbell”, this kind of lesions was termed as endophytic type (Type I). If, on the baseline level, 50% or more of the tumor was located outside of the standard region, which had limited bone invasiveness, they may show “bulge-like” image from the clivus into the intracranial areas on the MR and CT scans, and this subgroup of tumors was termed exophytic type (Type II) (see Fig. 1A-C). The selected cases were classified according to these criteria.

3. Specimen collection

Fresh tumor specimens were surgically resected immediately, divided into blocks, stored in liquid nitrogen, and fixed in 10% neutral formalin solution. They were paraffin fixed within one week and then stored in a 4°C refrigerator for future use. All specimens underwent Hematoxylin and eosin (HE) staining before use to determine the percentage of tumor cells; specimens with fewer than 70% of the cells classified as tumor cells were excluded.

4. Proteomics methods

1. Protein extraction and digestion: Eighty-milligram samples from each of the 12 frozen tissue samples selected for proteomics screening were rinsed with PBS, and each sample was then mixed with lysis buffer (50 mM Tris-HCl, 2.5 M thiourea, 8 M urea, 4% CHAPS, 65 mM DTT) to extract total protein. Cell debris was removed by centrifugation at 20,000 g for 45 min at 4°C. The total protein concentration of each sample was determined using the Bio-Rad RC DC Protein Assay. The proteins from each sample were pooled equally according to the total amount of protein and digested by filter-aided sample preparation combined with
a microwave-assisted protein preparation method as previously described[9,10]. After digestion, peptides from the Type I and Type II samples were desalted on C18 columns (3 cc, 60 mg, Oasis) according to the manufacturer’s instructions, washed seven times with 500 μL 0.1% formic acid and eluted with 500 μL 100% ACN. Elutions were dried by vacuum centrifugation and stored at −80°C.

2. iTRAQ labeling: The digested chordoma samples were mixed at the same amount as internal standard. The chordoma samples and internal standard were labeled by 116,117, and 118 iTRAQ. Labeling was performed according to the manufacturer’s protocol (ABSciex). The chordoma samples were mixed into one sample at the same amount and lyophilized.

3. 2D-LC/MS/MS: The pooled mixture from labeled samples was first fractioned by high-pH RPLC column from Waters (4.6mm×250mm, C18, 3μm). The samples were loaded onto the column in buffer A2 (pH = 10). The eluted gradient was 5–90% buffer B2 (90%ACN; pH = 10, flow rate, 1mL/min) for 60 min. The eluted peptides were collected as a fraction per minute, and the 60 fractions were pooled into 20 samples. Each sample was analyzed by RP C18 self-packing capillary LC column (75μm×100mm, 3μm). The eluted gradient was 5–30% buffer B1 (0.1% formic acid, 99.9% ACN; flow rate, 0.5 μL/min) for 100 min. Triple-TOF 5600 were used to analyze the sample. The MS data were acquired with high sensitivity mode using the following parameters: 30 data-dependent MS/MS scans per every full scan; full scans was acquired at resolution 40,000 and MS/MS scans at 20,000; 35% normalized collision energy, charge state screening (including precursors with +2 to +4 charge state) and dynamic exclusion (exclusion duration 15 s); MS/MS scan range was 100–1800 m/z and scan time was 100 ms.

4. Database search: The MS/MS spectra were respectively searched against the SwissProt human database from Uniprot website (http://www.uniprot.org) using Mascot software version 2.3.02 (Matrix Science, UK). Trypsin was chosen as cleavage specificity with a maximum number of allowed missed cleavages of two. Carbamidomethylation (C) and iTRAQ 4-plex label was set as a fixed modification. The searches were performed using a peptide and product ion tolerance of 0.05 Da. Scaffold was used to further filter the database search results by decoy database method. The following filter was used in this study, 1% false positive rate at protein level and each protein with 2 unique peptides. After filtering the results by above filter, the peptide abundances in different reporter ion channels of MS/MS scan were normalized. The protein abundance ratio was based on unique peptide results.

5. GO functional analysis: All differential proteins identified by two approaches were assigned their gene symbol via the Panther database (http://www.pantherdb.org/). Protein classification was performed based on their functional annotations using Gene Ontology (GO) for biological process, and molecular function. When more than one assignment was available, all of the functional annotations were considered in the results.

6. IPA network analysis: All differential proteins were used for pathway analysis.

For this purpose, the SwissProt accession numbers were inserted into the Ingenuity Pathway Analysis (IPA) software (Ingenuity Systems, Mountain View, CA). This software categorizes gene products based on the location of the protein within cellular components and suggests possible biochemical, biological and molecular functions. Furthermore, proteins were mapped to genetic networks available in the Ingenuity and other databases and ranked by score. These genetic networks describe functional relationships between gene products based on known interactions in literature. Through the IPA software, the newly formed networks were associated with known biological pathways.
5. Immunohistochemical methods and analysis

Paraffin-embedded tumor tissue sections were immunohistochemically stained by the streptavidin peroxidase conjugated method (SP method). Antibodies tested on the IHC included: rabbit anti-actin and anti-TGFβ1 (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA), anti-AKT, anti-mTOR, anti-PI3K, and anti-PTEN (Cell Signaling Technology, Inc., Danvers, MA, USA). Two independent pathologists viewed the sections under a microscope under good tissue structure and clear background conditions; they were unaware of the clinical data and prognoses of the selected patients. Positive signals of translational growth factor β1 (TGFβ1), PI3K, Akt, mTOR, and PTEN are yellowish brown particles appearing in the cytoplasm and/or nuclei. Using the staining intensity and the percentage of positive cells, we developed the following criteria: 1) 0 points for no stain, 1 point for light yellow, 2 points for yellowish-brown, 3 points for dark brown; 2) percentage of positive cells: 0 points for (0%), 1 point for (< 20%), 2 points for (20 to 50%), 3 points for (> 50%). A total score of less than 2 was denoted as negative, 3–4 was denoted as weakly positive, and 5–6 was denoted as strongly positive.

6. Western Blot Analysis

WBs of the additional 23 samples were performed to validate the proteomic quantitation of four selected candidate proteins (PI3K, AKt, mTOR, and PTEN). Electrophoresis and immunoblotting was performed on the protein extracts using the standard protocol, using 20 μg of protein per sample. Antibodies tested on the immunoblots included: rabbit anti-actin (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA), anti-AKT, anti-mTOR, anti-PI3K, and anti-PTEN (Cell Signaling Technology, Inc., Danvers, MA, USA). Following hybridization with the secondary antibody, the blots were incubated with Immun-Star horseradish peroxidase luminal/enhancer (Bio-Rad) and exposed onto Kodak Biomax MR Film (Eastman Kodak Company, Rochester, NY, USA).

7. Statistical Methods

Immunohistochemistry, western blot and clinical data were analyzed using the SPSS11.5 software package for statistical analysis. The differences in expression of TGFβ1, PI3K, AKt, mTOR, and PTEN, tumor volume, texture and degree of adhesion with TGFβ1, PI3K, AKt, mTOR and PTEN were analyzed by a chi-square test, and p < 0.05 was considered different.

Results

1. Bone invasiveness classification and clinical outcomes

This study enrolled 35 patients, of which 12 were subjected to proteomic experiments (experimental group) and 23 were subjected to IHC and Western blot (confirmation group). Classifications were made in accordance with the aforementioned bone invasion criteria; there were six cases of Type I and II in the experimental group. There were 10 and 13 cases classified as Types I and II, respectively, in the confirmation group. There were no differences between the Type I and II patients in terms of sex, age, lesion size, tumor texture, or degree of adhesion in the validation group, as shown in Table 1. The workflow of the iTRAQ proteomic strategy is demonstrated in Fig. 2.
2. Identification of differentially expressed proteins in different growth pattern

This iTRAQ-labeling proteomic study compared the total proteomes of tissue from Type I patients (n = 6) with the proteomes of tissues from Type II patients (n = 6). Each individual sample in the two groups was separately analyzed. By querying the human IPI database with the Mascot algorithm, 2251 proteins were quantified.

Before performing comparative analysis between groups, the coefficient of variation was employed to filter out data with poor linearity among the biological replicates within each group.

**Table 1. The basic information of included patients.**

| Group | Type | Age(year) | Gender | Volume(ml) | Time for Chief complain (m) |
|-------|------|-----------|--------|------------|-----------------------------|
| 1     | I    | 16        | 1      | 68         | 12                          |
| 1     | I    | 46        | 2      | 58         | 12                          |
| 1     | I    | 15        | 1      | 36         | 3                           |
| 1     | I    | 32        | 1      | 36         | 24                          |
| 1     | I    | 44        | 1      | 10         | 4                           |
| 1     | I    | 58        | 2      | 55         | 48                          |
| 1     | II   | 21        | 1      | 50         | 3                           |
| 1     | II   | 47        | 1      | 6          | 6                           |
| 1     | II   | 36        | 1      | 45         | 2                           |
| 1     | II   | 18        | 1      | 24         | 24                          |
| 1     | II   | 17        | 1      | 40         | 1                           |
| 1     | II   | 60        | 2      | 25         | 7                           |
| 2     | I    | 40        | 2      | 11         | 1                           |
| 2     | I    | 40        | 2      | 21         | 6                           |
| 2     | I    | 28        | 1      | 14         | 9                           |
| 2     | I    | 50        | 1      | 36         | 12                          |
| 2     | I    | 30        | 1      | 16         | 6                           |
| 2     | I    | 13        | 1      | 18         | 0                           |
| 2     | I    | 46        | 2      | 40         | 24                          |
| 2     | I    | 48        | 2      | 30         | 6                           |
| 2     | I    | 23        | 2      | 12         | 5                           |
| 2     | I    | 51        | 1      | 24         | 3                           |
| 2     | I    | 44        | 2      | 5          | 1                           |
| 2     | I    | 42        | 1      | 12         | 12                          |
| 2     | I    | 22        | 1      | 14         | 4                           |
| 2     | II   | 50        | 2      | 168        | 12                          |
| 2     | II   | 39        | 2      | 14         | 3                           |
| 2     | II   | 12        | 2      | 60         | 6                           |
| 2     | II   | 47        | 1      | 60         | 7                           |
| 2     | II   | 56        | 1      | 15         | 24                          |
| 2     | II   | 15        | 1      | 70         | 1                           |
| 2     | II   | 25        | 2      | 25         | 36                          |
| 2     | II   | 57        | 1      | 11         | 3                           |
| 2     | II   | 16        | 2      | 80         | 12                          |
| 2     | II   | 38        | 1      | 27         | 24                          |

Note: Group: 1, experimental group; 2, confirmation group. Type: I, endophytic type (type I); II, exophytic type (type II). Gender: 1, male; 2, female.

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group. To maintain a low false-positive rate of comparative analysis between the groups, an average coefficient of variation of 0.2 (CV = 0.2) was accepted to filter out data with poor linearity.

Next, we applied a threshold of >1.5-fold and \( p < 0.01 \) to identify proteins that were differentially expressed. A total of 250 proteins meeting the criteria were classified as differentially expressed. Among these proteins, 59 proteins were up-regulated and 191 proteins were down-regulated in endophytic type (Type I) compared with exophytic type (Type II), as demonstrated in Table 2.

3. Interaction Networks and Functional Pathway Analysis

Functional pathway analysis was performed for the selected differentially expressed proteins to better understand their biological changes post-treatment. Panther analysis allowed us to elucidate the different functions and processes in which the 250 proteins are putatively involved compared with whole genome data. The cellular compartment, molecular function and biological process of the differentially expressed proteins are presented in Fig. 3. Organelles, extracellular complexes are significantly increased in chordomas, while metabolic process and cellular processes in biological processes significantly reduced, which may be related with the invasive growth of the tumor.

Thereafter, we analyzed the 250 selected proteins using IPA and found that all of the proteins were eligible for network analysis (focus molecule) based on the IPA knowledgebase criteria. By employing the dataset of proteins that are differentially expressed between endophytic type (Type I) and exophytic type (Type II), the role of these proteins in canonical pathways and
Table 2. The list of differentially expressed proteins.

| Accession Number | Protein Names                                      | Change Folds | Molecular Weight |
|------------------|----------------------------------------------------|--------------|------------------|
| P08729           | Keratin, type II cytoskeletal 7                    | 1.8          | 51 kDa           |
| P35555           | Fibrillin-1                                         | 0.6          | 312 kDa          |
| P0CG38           | POTE ankyrin domain family member I                 | 0.6          | 121 kDa          |
| P17661           | Desmin                                             | 0.6          | 54 kDa           |
| P51888           | Prolargin                                           | 2            | 44 kDa           |
| P16112           | Aggrecan core protein                              | 1.65         | 250 kDa          |
| P21333           | Filamin-A                                           | 0.6          | 281 kDa          |
| Q15582           | Transforming growth factor-beta-induced protein ig-h3 | 1.6          | 75 kDa           |
| P02545           | Prelamin-A/C                                        | 0.6          | 74 kDa           |
| P24821           | Tenascin                                            | 0.6          | 241 kDa          |
| Q15063           | Periostin                                           | 0.45         | 93 kDa           |
| Q05707           | Collagen alpha-1(XIV) chain                        | 0.4          | 194 kDa          |
| P13611           | Versican core protein                              | 0.4          | 373 kDa          |
| P69905           | Hemoglobin subunit alpha                            | 0.65         | 15 kDa           |
| Q12805           | EGF-containing fibulin-like extracellular matrix protein 1 | 0.55         | 55 kDa           |
| P07237           | Protein disulfide-isomerase                         | 1.8          | 57 kDa           |
| P68371           | Tubulin beta-4B chain                              | 0.65         | 50 kDa           |
| P21810           | Biglycan                                            | 2.4          | 42 kDa           |
| P10915           | Hyaluronan and proteoglycan link protein 1          | 2.6          | 40 kDa           |
| Q06828           | Fibromodulin                                        | 1.95         | 43 kDa           |
| P11047           | Laminin subunit gamma-1                             | 0.6          | 178 kDa          |
| Q99879           | Histone H2B type 1-M                                | 0.6          | 14 kDa           |
| Q8N257           | Histone H2B type 3-B                                | 0.55         | 14 kDa           |
| P55268           | Laminin subunit beta-2                              | 0.6          | 196 kDa          |
| Q9Y6C2           | EMILIN-1                                            | 0.65         | 107 kDa          |
| P02458           | Collagen alpha-1(II) chain                         | 1.6          | 142 kDa          |
| Q9Y240           | C-type lectin domain family 11 member A             | 2.75         | 36 kDa           |
| P07451           | Carbonic anhydrase 3                                | 0.6          | 30 kDa           |
| P02788           | Lactotransferrin                                    | 1.7          | 78 kDa           |
| Q7Z7G0           | Target of Nesh-SH3                                  | 0.6          | 119 kDa          |
| Q8N2S1           | Latent-transforming growth factor beta-binding protein 4 | 0.4         | 173 kDa          |
| P04179           | Superoxide dismutase [Mn], mitochondrial            | 0.4          | 25 kDa           |
| P12821           | Angiotensin-converting enzyme                       | 1.95         | 150 kDa          |
| Q14314           | Fibroleukin                                         | 1.75         | 50 kDa           |
| Q16363           | Laminin subunit alpha-4                             | 0.6          | 203 kDa          |
| O43852           | Calumenin                                           | 1.55         | 37 kDa           |
| P62979           | Ubiquitin-40S ribosomal protein S27a                | 0.5          | 18 kDa           |
| P50454           | Serpin H1                                           | 0.6          | 46 kDa           |
| P23142           | Fibulin-1                                           | 0.4          | 77 kDa           |
| Q7Z406           | Myosin-14                                           | 1.6          | 228 kDa          |
| Q8UX7            | Adipocyte enhancer-binding protein 1                | 1.55         | 131 kDa          |
| P24844           | Myosin regulatory light polypeptide 9               | 0.6          | 20 kDa           |
| Q14112           | Nidogen-2                                           | 0.6          | 151 kDa          |
| P10412           | Histone H1.4                                        | 0.6          | 22 kDa           |
| P16403           | Histone H1.2                                        | 0.65         | 21 kDa           |
| Q8VF2            | Protein AHNAK2                                      | 1.95         | 617 kDa          |

(Continued)
| Accession Number | Protein Names                                      | Change Folds | Molecular Weight |
|------------------|----------------------------------------------------|--------------|------------------|
| P01876           | Ig alpha-1 chain C region                          | 1.65         | 38 kDa           |
| P12429           | Annexin A3                                         | 2.2          | 36 kDa           |
| P02649           | Apolipoprotein E                                   | 1.6          | 36 kDa           |
| Q15113           | Procollagen C-endopeptidase enhancer 1             | 0.5          | 48 kDa           |
| P02461           | Collagen alpha-1(III) chain                        | 0.35         | 139 kDa          |
| Q14573           | Inositol 1,4,5-trisphosphate receptor type 3       | 1.55         | 304 kDa          |
| P35442           | Thrombospondin-2                                   | 0.6          | 130 kDa          |
| P07942           | Laminin subunit beta-1                            | 0.65         | 198 kDa          |
| P22626           | Heterogeneous nuclear ribonucleoproteins A2/B1     | 0.55         | 37 kDa           |
| Q06682           | Caldesmon                                          | 0.6          | 93 kDa           |
| P09936           | Ubiquitin carboxyl-terminal hydrolase isozyme L1   | 2            | 25 kDa           |
| P61978           | Heterogeneous nuclear ribonucleoprotein K          | 0.6          | 51 kDa           |
| O43491           | Band 4.1-like protein 2                            | 0.6          | 113 kDa          |
| O60687           | Sushi repeat-containing protein SRPX2              | 2.4          | 53 kDa           |
| P14543           | Nidogen-1                                          | 0.6          | 136 kDa          |
| Q13740           | CD166 antigen                                      | 1.9          | 65 kDa           |
| P06702           | Protein S100-A9                                    | 1.75         | 13 kDa           |
| P98095           | Fibulin-2                                          | 0.6          | 127 kDa          |
| P06748           | Nucleophosmin                                      | 0.45         | 33 kDa           |
| P01911           | HLA class II histocompatibility antigen, DRB1–15 beta chain | 2.2   | 30 kDa          |
| Q30154           | HLA class II histocompatibility antigen, DR beta 5 chain | 1.75 | 30 kDa          |
| Q00839           | Heterogeneous nuclear ribonucleoprotein U          | 0.55         | 91 kDa           |
| P37837           | Transaldolase                                      | 0.65         | 38 kDa           |
| Q9BXN1           | Asporin                                            | 0.2          | 43 kDa           |
| P07339           | Cathepsin D                                       | 1.55         | 45 kDa           |
| O00339           | Matrilin-2                                         | 0.45         | 107 kDa          |
| P02511           | Alpha-crystallin B chain                           | 1.95         | 20 kDa           |
| Q8WX93           | Palladin                                           | 0.6          | 151 kDa          |
| Q9UBX5           | Fibulin-5                                          | 0.5          | 50 kDa           |
| P39060           | Collagen alpha-1(XVIII) chain                      | 0.6          | 178 kDa          |
| P07099           | Epoxide hydrolase 1                                | 1.9          | 53 kDa           |
| P07737           | Profilin-1                                         | 0.65         | 15 kDa           |
| P20908           | Collagen alpha-1(V) chain                          | 0.55         | 184 kDa          |
| Q9UKU9           | Angiopoietin-related protein 2                     | 1.8          | 57 kDa           |
| O43405           | Cochlin                                            | 3.35         | 59 kDa           |
| Q31610           | HLA class I histocompatibility antigen, B-81 alpha chain   | 1.6  | 40 kDa           |
| P05109           | Protein S100-A8                                    | 1.55         | 11 kDa           |
| Q01995           | Transgelin                                         | 0.3          | 23 kDa           |
| P78539           | Sushi repeat-containing protein SRPX                | 2.05         | 52 kDa           |
| P31943           | Heterogeneous nuclear ribonucleoprotein H          | 0.6          | 49 kDa           |
| P55795           | Heterogeneous nuclear ribonucleoprotein H2         | 0.5          | 49 kDa           |
| O94832           | Unconventional myosin-Id                           | 0.6          | 116 kDa          |
| P12107           | Collagen alpha-1(XI) chain                         | 1.55         | 181 kDa          |
| Q14766           | Latent-transforming growth factor beta-binding protein 1 | 0.5  | 187 kDa          |
| P30043           | Flavin reductase (NADPH)                           | 0.65         | 22 kDa           |
| P51812           | Ribosomal protein S6 kinase alpha-3                | 0.65         | 84 kDa           |
| P07910           | Heterogeneous nuclear ribonucleoproteins C1/C2     | 0.6          | 34 kDa           |
| Accession Number | Protein Names                          | Change Folds | Molecular Weight |
|------------------|----------------------------------------|--------------|------------------|
| Q9NR99           | Matrix-remodeling-associated protein 5 | 0.45         | 312 kDa          |
| Q14195           | Dihydropyrimidinase-related protein 3 | 0.55         | 62 kDa           |
| P37802           | Transgelin-2                            | 0.4          | 22 kDa           |
| P38159           | RNA-binding motif protein, X chromosome | 0.65        | 42 kDa           |
| P49747           | Cartilage oligomeric matrix protein     | 0.4          | 83 kDa           |
| P43243           | Matrin-3                                | 0.6          | 95 kDa           |
| Q9BXJ4           | Complement C1q tumor necrosis factor-related protein 3 | 2.65 | 27 kDa |
| Q13361           | Microfibrillar-associated protein 5     | 0.4          | 20 kDa           |
| Q94769           | Extracellular matrix protein 2          | 1.65         | 80 kDa           |
| P14866           | Heterogeneous nuclear ribonucleoprotein L | 0.6      | 64 kDa           |
| P01859           | Ig gamma-2 chain C region               | 1.85         | 36 kDa           |
| O75367           | Core histone macro-H2A.1                | 0.65         | 40 kDa           |
| Q13263           | Transcription intermediary factor 1-beta | 0.65  | 89 kDa           |
| O6UVY6           | DBH-like monoxygenase protein 1         | 1.55         | 70 kDa           |
| P26447           | Protein S100-A4                         | 0.45         | 12 kDa           |
| P60981           | Destrin                                 | 0.5          | 19 kDa           |
| P13797           | Plastin-3                               | 0.5          | 71 kDa           |
| Q07955           | Serine/arginine-rich splicing factor 1   | 0.65         | 28 kDa           |
| Q14192           | Four and a half LIM domains protein 2    | 0.65         | 32 kDa           |
| P01137           | Transforming growth factor beta-1        | 0.65         | 44 kDa           |
| P16070           | CD44 antigen                            | 0.6          | 82 kDa           |
| P22352           | Glutathione peroxidase 3                | 0.6          | 26 kDa           |
| P46063           | ATP-dependent DNA helicase Q1           | 0.6          | 73 kDa           |
| Q9Y3Z3           | SAM domain and HD domain-containing protein 1 | 0.65   | 72 kDa           |
| P09429           | High mobility group protein B1          | 0.55         | 25 kDa           |
| O15232           | Matrilin-3                              | 0.45         | 53 kDa           |
| P02763           | Alpha-1-acid glycoprotein 1             | 1.65         | 24 kDa           |
| Q02878           | 60S ribosomal protein L                 | 0.65         | 33 kDa           |
| Q9GZM7           | Tubulointerstitial nephritis antigen-like | 0.6    | 52 kDa           |
| Q14019           | Coactosin-like protein                  | 0.5          | 16 kDa           |
| P21291           | Cysteine and glycine-rich protein 1     | 0.65         | 21 kDa           |
| P07305           | Histone H1.0                            | 0.65         | 21 kDa           |
| Q9BXJ0           | Complement C1q tumor necrosis factor-related protein 5 | 1.8 | 25 kDa |
| P62829           | 60S ribosomal protein L23               | 0.65         | 15 kDa           |
| P62424           | 60S ribosomal protein L7a               | 0.65         | 30 kDa           |
| O95865           | N(G),N(G)-dimethylarginine dimethylaminohydrolase 2 | 0.5 | 30 kDa |
| P55060           | Exportin-2                              | 0.65         | 110 kDa          |
| Q8WXF7           | Atlastin-1                              | 1.7          | 64 kDa           |
| P39023           | 60S ribosomal protein L3                | 0.65         | 46 kDa           |
| P16401           | Histone H1.5                            | 0.6          | 23 kDa           |
| Q99969           | Retinoic acid receptor responser protein 2 | 2.2     | 19 kDa           |
| P84103           | Serine/arginine-rich splicing factor 3   | 0.6          | 19 kDa           |
| P12268           | Inosine-5’-monophosphate dehydrogenase 2 | 0.65   | 56 kDa           |
| P13861           | cAMP-dependent protein kinase type II-alpha regulatory subunit | 0.55 | 46 kDa |
| Q15165           | Serum paraoxonase/arylesterase 2        | 1.65         | 39 kDa           |
| Q16853           | Membrane primary amine oxidase          | 0.6          | 85 kDa           |
| Q6UX06           | Olfactomedin-4                          | 1.8          | 57 kDa           |

(Continued)
Table 2.  (Continued)

| Accession Number | Protein Names                                              | Change Folds | Molecular Weight |
|-------------------|------------------------------------------------------------|--------------|------------------|
| P31942            | Heterogeneous nuclear ribonucleoprotein H3                 | 0.6          | 37 kDa           |
| P62136            | Serine/threonine-protein phosphatase PP1-alpha catalytic subunit | 0.65         | 38 kDa           |
| Q12905            | Interleukin enhancer-binding factor 2                      | 0.65         | 43 kDa           |
| Q8UKV3            | Apoptotic chromatin condensation inducer in the nucleus     | 0.65         | 152 kDa          |
| P35268            | 60S ribosomal protein L22                                   | 0.5          | 15 kDa           |
| Q9UEY8            | Gamma-adducin                                              | 0.6          | 79 kDa           |
| O14979            | Heterogeneous nuclear ribonucleoprotein D-like              | 0.55         | 46 kDa           |
| P26599            | Polypyrimidine tract-binding protein 1                      | 0.55         | 57 kDa           |
| Q92522            | Histone H1x                                                | 0.65         | 22 kDa           |
| P60866            | 40S ribosomal protein S20                                   | 0.65         | 13 kDa           |
| Q07507            | Dermatopontin                                              | 0.6          | 24 kDa           |
| P51858            | Hepatoma-derived growth factor                              | 0.6          | 27 kDa           |
| Q15417            | Calponin-3                                                 | 0.6          | 36 kDa           |
| Q07092            | Collagen alpha-1(XVI) chain                                | 0.65         | 158 kDa          |
| Q99983            | Osteomodulin                                               | 0.4          | 49 kDa           |
| P51991            | Heterogeneous nuclear ribonucleoprotein A3                  | 0.55         | 40 kDa           |
| Q0ZGT2            | Nexilin                                                    | 0.65         | 81 kDa           |
| Q53TN4            | Cytochrome b reductase 1                                   | 0.6          | 32 kDa           |
| P46776            | 60S ribosomal protein L27a                                  | 0.6          | 17 kDa           |
| P17612            | cAMP-dependent protein kinase catalytic subunit alpha       | 0.65         | 41 kDa           |
| Q86D15            | Reticulocalbin-3                                           | 0.6          | 37 kDa           |
| Q99733            | Nucleosome assembly protein 1-like 4                        | 0.65         | 43 kDa           |
| P61254            | 60S ribosomal protein L26                                   | 0.6          | 17 kDa           |
| Q15818            | Neuronal pentraxin-1                                       | 2.4          | 47 kDa           |
| O43854            | EGF-like repeat and discoidin I-like domain-containing protein 3 | 0.5          | 54 kDa           |
| P52566            | Rho GDP-dissociation inhibitor 2                            | 0.55         | 23 kDa           |
| P17302            | Gap junction alpha-1 protein                               | 1.6          | 43 kDa           |
| P08138            | Tumor necrosis factor receptor superfamily member 16       | 0.6          | 45 kDa           |
| P35443            | Thrombospondin-4                                           | 0.5          | 106 kDa          |
| Q13185            | Chromobox protein homolog 3                                | 0.6          | 21 kDa           |
| Q9UHB6            | LIM domain and actin-binding protein 1                      | 0.65         | 85 kDa           |
| Q9BX66            | Sorbin and SH3 domain-containing protein 1                  | 0.65         | 143 kDa          |
| P62266            | 40S ribosomal protein S23                                   | 0.65         | 16 kDa           |
| Q16629            | Serine/arginine-rich splicing factor 7                      | 0.6          | 27 kDa           |
| P08574            | Cytochrome c1, heme protein, mitochondrial                  | 0.6          | 35 kDa           |
| P19013            | Keratin, type II cytoskeletal 4                             | 0.65         | 57 kDa           |
| Q16576            | Histone-binding protein RBBP7                               | 0.55         | 48 kDa           |
| P05186            | Alkaline phosphatase, tissue-nonspecific isozyme            | 0.55         | 57 kDa           |
| Q06033            | Inter-alpha-trypsin inhibitor heavy chain H3                | 0.65         | 100 kDa          |
| Q5JT6            | Placenta-specific protein 9                                | 0.65         | 10 kDa           |
| O75368            | SH3 domain-binding glutamic acid-rich-like protein           | 0.65         | 13 kDa           |
| P10620            | Microsomal glutathione S-transferase 1                      | 1.65         | 18 kDa           |
| Q14956            | Transmembrane glycoprotein NMB                              | 2.15         | 64 kDa           |
| P19652            | Alpha-1-acid glycoprotein 2                                 | 1.8          | 24 kDa           |
| O00231            | 26S proteasome non-ATPase regulatory subunit 11             | 0.65         | 47 kDa           |
| P52597            | Heterogeneous nuclear ribonucleoprotein F                   | 0.5          | 46 kDa           |
| Q13363            | C-terminal-binding protein 1                                | 0.65         | 48 kDa           |

(Continued)
| Accession Number | Protein Names                          | Change Folds | Molecular Weight |
|------------------|----------------------------------------|--------------|------------------|
| Q75N90           | Fibrillin-3                             | 0.65         | 300 kDa          |
| O60701           | UDP-glucose 6-dehydrogenase             | 0.65         | 55 kDa           |
| P50225           | Sulfotransferase 1A1                    | 0.65         | 34 kDa           |
| O75821           | Eukaryotic translation initiation factor 3 subunit G | 0.6 | 36 kDa |
| Q9BUF5           | Tubulin beta-6 chain                    | 0.6          | 50 kDa           |
| P55769           | NHP2-like protein 1                     | 0.6          | 14 kDa           |
| Q92598           | Heat shock protein 105 kDa              | 0.65         | 97 kDa           |
| Q15717           | ELAV-like protein 1                     | 0.6          | 36 kDa           |
| Q9Y6U3           | Adseverin                               | 0.4          | 80 kDa           |
| Q08170           | Serine/arginine-rich splicing factor 4  | 0.5          | 57 kDa           |
| P63167           | Dynein light chain 1, cytoplasmic       | 0.6          | 10 kDa           |
| Q13595           | Transformer-2 protein homolog alpha     | 0.6          | 33 kDa           |
| P08294           | Extracellular superoxide dismutase [Cu-Zn] | 0.6 | 26 kDa |
| P67809           | Nuclease-sensitive element-binding protein 1 | 0.65 | 36 kDa |
| Q88BL7           | Olfactomedin-like protein 2A             | 1.6          | 73 kDa           |
| Q8N163           | DBIRD complex subunit KIAA1967          | 0.6          | 103 kDa          |
| P04208           | Ig lambda chain V-I region WAH          | 0.4          | 12 kDa           |
| Q99439           | Calponin-2                              | 0.5          | 34 kDa           |
| P61313           | 60S ribosomal protein L15               | 0.65         | 24 kDa           |
| Q9H8L6           | Multimerin-2                            | 0.65         | 104 kDa          |
| Q8UHX1           | Poly(U)-binding-splicing factor PUF60   | 0.5          | 60 kDa           |
| P51570           | Galactokinase                           | 0.65         | 42 kDa           |
| P29762           | Cellular retinoic acid-binding protein 1 | 0.4 | 16 kDa |
| P62241           | 40S ribosomal protein S8                | 0.6          | 24 kDa           |
| P51911           | Calponin-1                              | 0.45         | 33 kDa           |
| Q9HB0L0          | Tensin-1                                | 0.6          | 186 kDa          |
| P01861           | Ig gamma-4 chain C region               | 2            | 36 kDa           |
| Q53EL6           | Programmed cell death protein 4         | 0.6          | 52 kDa           |
| O43927           | C-X-C motif chemokine 13                | 2.15         | 13 kDa           |
| Q13247           | Serine/arginine-rich splicing factor 6  | 0.55         | 40 kDa           |
| P51674           | Neuronal membrane glycoprotein M6-a     | 1.6          | 31 kDa           |
| Q9Y625           | Glypican-6                              | 0.6          | 63 kDa           |
| Q13243           | Serine/arginine-rich splicing factor 5  | 0.55         | 31 kDa           |
| Q9H4G4           | Golgi-associated plant pathogenesis-related protein 1 | 0.55 | 17 kDa |
| Q9BRX8           | Redox-regulatory protein FAM213A        | 1.7          | 26 kDa           |
| Q92629           | Delta-sarcoglycan                       | 0.6          | 32 kDa           |
| P56377           | AP-1 complex subunit sigma-2            | 0.65         | 19 kDa           |
| P17252           | Protein kinase C alpha type             | 0.6          | 77 kDa           |
| Q9BU7T1          | 3-hydroxybutyrate dehydrogenase type 2  | 0.6          | 27 kDa           |
| Q9UBQ7           | Glyoxylate reductase/hydroxypyruvate reductase | 0.65 | 36 kDa |
| O14890           | Exportin-1                              | 0.65         | 123 kDa          |
| Q4V9L6           | Transmembrane protein 119               | 0.6          | 29 kDa           |
| Q6IBS0           | Twinfilin-2                             | 0.6          | 40 kDa           |
| P62851           | 40S ribosomal protein S25               | 0.6          | 14 kDa           |
| P24557           | Thromboxane-A synthase                  | 0.6          | 61 kDa           |
| P47914           | 60S ribosomal protein L29               | 0.5          | 18 kDa           |
| P55290           | Cadherin-13                             | 0.5          | 78 kDa           |

(Continued)
Among the top five canonical pathways returned, “EIF2 Signaling” and “Regulation of eIF4 and p70S6K signaling” were highly correlated with protein synthesis through the regulation of translation initiation, whereas “mTOR signaling” plays important roles in several cellular functions, particularly cell survival and proliferation.

According to the analysis, the main molecular functions of the differential proteins are primarily in the areas of cell motility, cell growth and proliferation, cellular organization and aggregation. The most important function of proteins is cell movement, and 91 types of proteins are related to cell movement. 34 proteins of these proteins are associated with tumor cell invasion, including VCAN, TGFB1, TGFβ1, FMOD and FLNA, which are common cell invasion-related proteins (Table 3).

Further classification analysis showed that the expression of inflammatory activity-associated proteins in endophytic type up-regulated, whereas the expression of cell motility-associated proteins. The table below shows the accession number, protein names, change folds, and molecular weight of these proteins.

| Accession Number | Protein Names                                      | Change Folds | Molecular Weight |
|------------------|----------------------------------------------------|--------------|------------------|
| P04433           | Ig kappa chain V-III region VG (Fragment)          | 1.6          | 13 kDa           |
| P26583           | High mobility group protein B2                     | 0.6          | 24 kDa           |
| O43809           | Cleavage and polyadenylation specificity factor subunit 5 | 0.65         | 26 kDa           |
| O95715           | C-X-C motif chemokine 14                           | 2.9          | 13 kDa           |
| Q9UH65           | Switch-associated protein 70                       | 0.6          | 69 kDa           |
| Q9NQ79           | Cartilage acidic protein 1                         | 0.6          | 71 kDa           |
| P52943           | Cysteine-rich protein 2                            | 0.6          | 22 kDa           |
| P30273           | High affinity immunoglobulin epsilon receptor subunit gamma | 0.65       | 10 kDa           |
| Q15185           | Prostaglandin E synthase 3                         | 0.55         | 19 kDa           |
| Q9BY50           | Signal peptidase complex catalytic subunit SEC11C  | 0.55         | 22 kDa           |
| Q8N3U4           | Cohesin subunit SA-2                               | 0.6          | 141 kDa          |

Fig 3. Panther analysis of endophytic clivus chordomas vs exophytic ones. Graph a shows cellular compartment analysis; Graph b shows molecular function analysis; and Graph c shows biological process analysis.

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Table 2. (Continued)
proteins in exophytic type up-regulated. In the endophytic chordoma tissues, inflammatory cells, especially phagocytic cells, had significantly increased motor function. For example, CXCL13, CXCL14 and CLEC11A promoted inflammatory cell movement; the expression of these molecules in the endophytic chordomas was significantly higher than that in exophytic cones. In the exophytic chordoma tissues, the expression of tumor cell motility-associated proteins such as TGFβ1, HGDF, THBS2 and FBLN5 were significantly higher than that of the endophytic type. These proteins have a significant promotion effect on tumor migration.

![Fig 4. Mapping of the 250 proteins differentially expressed between endophytic clivus chordomas and exophytomes by IPA analysis.](image)

It illustrates the top 7 canonical pathways, while the mTOR pathway ranked the fourth.

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Table 3. The list of molecules that are related to cellular movement.

| Category                     | Diseases or Functions Annotation | p-Value     | Molecules                                                                 | Number of Molecules |
|------------------------------|---------------------------------|-------------|---------------------------------------------------------------------------|---------------------|
| Cellular Movement            | cell movement                   | 2.53E-16    | ACAN, ALCAM, ANGPTL2, ANXA3, AOC3, APOE, ARHGDIB, BGN, CD44, CDH13, CLEC11A, CNN1, CNN2, COL18A1, COL2A1, COL3A1, COMP, CRYAB, CSE1L, CTBP1, CXCL13, CXCL14, DPT, DYSPL, DSTM, EDIL3, EFEMP1, FBLN2, FBLN5, FBN1, FCG, FGFR1, FGFR2, FLNA, FMOD, GJA1, GLIIPR2, GPM6A, HGDF, HMG1, HMG2, HNRNP2B1, HNRNPK, HNRNPPL, LAMB1, LAMC1, LAMA1, LMNA, LTF, MATN2, NEXN, NGFR, NPM1, NPTX1, OLFM4, ORM1, PALLD, PDCD4, PON2, POSTN, PRKACA, PRKCA, RARRES2, RPS6KA3, S100A4, S100A8, S100A9, SOD2, SOD3, SRPX2, SWAP70, TAGLN2, TBXAS1, TGFBI, THBS2, THBS4, TNC, TNS1, UGDH, VCAN, YBX1 | 83                  |
|                              | migration of cells              | 8.77E-13    | ACAN, ALCAM, ANGPTL2, ANXA3, AOC3, APOE, ARHGDIB, BGN, CD44, CDH13, CLEC11A, CNN2, COL18A1, COL3A1, COMP, CRYAB, CSE1L, CTBP1, CXCL13, CXCL14, DPT, DYSPL3, EDIL3, FBLN2, FBLN5, FCG, FGFR1, FGFR2, FLNA, FMOD, GJA1, GLIIPR2, GPM6A, HGDF, HMG1, HNRNP2B1, HNRNPK, HNRNPPL, LAMB1, LAMC1, LAMA1, LMNA, LTF, MATN2, NEXN, NGFR, NPM1, OLFM4, ORM1, PALLD, PDCD4, PON2, POSTN, PRKACA, PRKCA, RARRES2, RPS6KA3, S100A4, S100A8, S100A9, SOD2, SOD3, SRPX2, SWAP70, TGFBI, THBS2, THBS4, TNC, TNS1, UGDH, VCAN | 71                  |
|                              | cell movement of tumor cell lines | 1.70E-08    | ARHGDIB, CD44, CDH13, CNN1, COL18A1, CSE1L, CTBP1, CXCL13, CXCL14, EFEMP1, FBLN2, FBLN5, FBN1, FCG, FLNA, GJA1, HGDF, HNRNP2B1, HNRNPK, PALLD, POSTN, PRKCA, RARRES2, RPS6KA3, S100A4, S100A8, SOD2, SRPX2, TAGLN2, TBXAS1, TGFBI, THBS2, TNC, YBX1 | 35                  |
|                              | Invasion of cells               | 7.28E-08    | ALCAM, A3, CD44, COL18A1, CSE1L, CTBP1, CTSD, EFEMP1, FBLN1, FBLN2, FBLN5, FLNA, FOD, GJA1, HGDF, HMG1, LAMC1, LTF, NPM1, PALLD, PDCD4, POSTN, PRKCA, PTGES3, S100A4, S100A9, SOD2, TAGLN, TAGLN2, TGFBI, TGFBI, THBS2, VCAN | 34                  |

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From the IPA network analysis, twenty-one major overlapping interaction networks were identified, and the top six networks all had a score over twenty. By merging the "Cellular Movement, Cell Morphology, Connective Tissue, and Development and Function" and "Cellular Movement, Cellular Development, Skeletal and Muscular System Development and Function" networks, a molecular network was identified, as shown in Fig. 5. According to the score of the network and the result of the functional analysis, the most significantly related functions derived from these overlapping networks included protein metabolism and a series of cellular functions. The expression of many extracellular matrix proteins and cytoskeletal proteins, such as LAMA4, LAMB1, LAMB2, LAMBC1, NID1, NID2, NEXN, COTL1 and MYL9, changed significantly in endophytic chordomas, and TGFβ1, which was down-regulated, is the main protein upstream of these molecules. This molecular network shows that TGFβ1 can not only directly influence the migration of tumor cells, but also indirectly influence the movement of tumor cells by controlling the expression of cell matrix proteins and skeletal proteins. Based on these data, TGFβ1 may influence bone infiltration of clivus chordoma.

4. Validation of the Identified Differentially Expressed Proteins

Based on the result of the IPA analysis, 5 proteins (TGFβ1, PI3K, Akt, mTOR and PTEN) related to specific functions, such as proteins synthesis, cellular functions and cancer, were selected for verification. Selection of proteins for validation was also performed on the basis of fold

![Protein synthesis and cellular function networks from IPA analysis](https://example.com/fig5.png)
change of the proteins, the classification of proteins as secretory and the availability of antibodies. Validation of the five selected differentially expressed proteins was performed using IHC and WB in the additional 23 samples to confirm the results of proteomic analysis.

In the study of IHC, the TGFβ1 protein in positive cells are located intracellularly, and the positive expression rate in the confirmation group was 95.6% (22/23). Compared with its expression in endophytic clivus chordoma (Type I), TGFβ1 expression in exophytic clivus chordoma (Type II) was greater; the difference between the two values was significant ($p = 0.033$), as shown in Fig. 6, which is consistent with the results from the differential proteomic analysis.

The mTOR protein is located in the cytoplasm of chordoma cell. The positive expression rate in the confirmation group was 80.7% (20/23); there was no significant difference ($p = 0.092$) in the expression of mTOR between Type I and II, which is also consistent with the results from the differential proteomic analysis. The PTEN expression in the confirmation group was 16/23 (69.6%). Expression of the endophytic type (Type I) PTEN was significantly lower than that of the exophytic type, and the differences between the two types were significant ($p = 0.004$). There was no significant difference in the expression of PI3K ($p = 0.125$) and Akt ($p = 0.254$) between Type I and II. The results are shown in Table 4.

Furthermore, these four candidate proteins (PI3K, Akt, mTOR and PTEN) were validated using WB of the same additional 23 samples, and a quantitative analysis of the results was performed (Fig. 7). As shown in the graph, statistically significant differences were found in PTEN between endophytic type and exophytic type, which is consistent with the results from the differential proteomic analysis and IHC. The expression of mTOR level was higher in endophytic type and the level was higher in exophytic type, but there were no spastically significant differences for these two proteins. There were not any difference for PI3K level between endophytic type and the exophytic type.
Table 4. Immunohistochemical staining results of TGFβ, mTOR, and PTEN.

| proteins | staining intensity | exophytic type (1) | endophytic type (1) | P value |
|----------|--------------------|-------------------|-------------------|---------|
| TGFβ1    | 0                  | 1                 | 0                 | 0.033   |
|          | 1                  | 0                 | 3                 |         |
|          | 2                  | 7                 | 7                 |         |
|          | 3                  | 5                 | 0                 |         |
| mTOR     | 0                  | 0                 | 1                 | 0.092   |
|          | 1                  | 1                 | 0                 |         |
|          | 2                  | 5                 | 8                 |         |
|          | 3                  | 7                 | 1                 |         |
| PTEN     | 0                  | 0                 | 7                 | 0.004   |
|          | 1                  | 4                 | 1                 |         |
|          | 2                  | 7                 | 2                 |         |
|          | 3                  | 2                 | 0                 |         |

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Fig 7. Western blot analysis for PI3K, Akt, mTOR and PTEN in 23 additional tissue samples. Graph a shows that high levels of PI3K, Akt, mTOR were detected in both exophytic and endophytic clivus chordomas. In contrast, the expression levels of PTEN in both exophytic and endophytic clivus chordomas were relatively lower. Graph b shows the quantification of expression levels using densitometry. The mean values of each group are represented in the bar graph; * p<0.05.87x81mm (600 x 600 DPI).

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Discussion

Significant differences in the extent of skull base chordoma bone invasion exist, and they directly affect the extent of surgical resection that is possible[2]. For clivus chordoma with extensive infiltration of the skull base bone, surgical resection is difficult. In contrast, surgery more easily achieves subtotal or total resection of lesions with minor bone destruction. Based on the positional relationship between the tumor and bone clivus, this study classifies tumors as endophytic type (Type I) or exophytic type (Type II) to distinguish the degree of tumor invasion into the clivus bone. Endophytic tumors are typically within the clivus bone, and show expansive growth in the clivus with severe destruction in the clivus bone. In contrast, though the exophytic tumor is located at the clivus, the majority of it lies outside the clivus bone region, and the clivus bone destruction is less prominent. The classification of these two types of tumor can be used for research on the bone invasiveness of clivus chordoma, and the clinical prognosis of this two growth patterns was different, which will not be discussed in this paper.

This study is the first application of iTRAQ-based quantitative differential proteomic methods in determining the extent of bone invasion of clivus chordoma. By this high-throughput proteomic quantification method, 2251 proteins were quantified and 250 differential proteins were discovered. According to the GO and IPA analysis of differential proteins, we discovered that the inflammatory cells especially phagocytic cells in endophytic chordoma tissues exhibited significantly increased motor function; meanwhile, the expression levels of extracellular matrix proteins and cytoskeletal proteins generally decreased. It is speculated that increased inflammation and decreased expression of cytoskeletal proteins played a facilitating role in bone invasion of chordoma.

This study confirmed that the expression level of the upstream regulatory molecule TGFβ1 was significantly lower in the endophytic type than in the exophytic ones. TGFβ1 is widespread in the human leukocyte antigen system, and it can inhibit the inflammatory response; it can also play an important role in tumorigenesis by promoting tumor metastasis and angiogenesis, as well as changing the microenvironment and evading immune attack [11]. Accordingly, we hypothesized that due to the low level of TGFβ1 expression, the skull base chordoma on one hand promotes inflammation by negative feedback. On the other hand, it directly causes the downregulation of downstream extracellular matrix proteins and cytoskeletal proteins, thereby promoting the invasion and destruction of bone. Therefore, we imply that TGFβ1 plays an important role in skull base chordoma bone invasion.

PTEN, as a tumor suppressor gene, regulate multiple signal transduction pathways that function in cell growth, cell migration and apoptosis [12]. By immunohistochemical staining and Western blot analysis in this study, it was discovered that PTEN levels were lower in endophytic chordoma than in exophytic chordoma. Therefore, PTEN expression levels may be associated with the degree of bone invasion by chordoma and with tumor texture. PTEN is a known negative regulator of PI3K proteins, and it can inhibit PI3K-Akt-mTOR signaling [13–15]. Low expression of PTEN could lead to the upregulation of mTOR, which is related to the poor clinical prognosis of sacral chordoma[13].

Both TGFβ1 and PTEN regulate cell proliferation through a variety of signaling pathways, including mTOR signaling pathway[16,17]. The mTOR signaling channel has been regarded as an important channel for intracellular signal transduction that affects cell growth, tumor formation and cell invasion, including chordoma[18,19]. However, whether the mTOR signaling pathway is regulated by TGFβ1 to participate in bone invasion has not been reported. In this iTRAQ proteomic research, the mTOR signaling pathway has not been proved to be related with bone invasion as demonstrated in Fig. 4A. Moreover, IHC staining and Western blot confirmed that although the mTOR expression in chordoma cells was relatively high, there was no
statically significant difference in the expression levels of mTOR, PI3K, and AKt in different subtypes of clivus chordomas. The activation of the PI3K-Akt-mTOR might mainly through the phosphorylation status but not through the expression levels. Thus, that the role of PI3K-Akt-mTOR signaling in the pathological process of bone invasion by clivus chordoma and the mechanism of the PI3K-Akt-mTOR signaling in clivus chordoma needs to be further studied.

Several publications reported that the TGF-β1 could activate the PI3K/Akt/ mTOR pathway, and further activate the downstream proteins, p70S6K. Thus, the TGF-β1 and PTEN are two important tumor related proteins which all regulated PI3K/Akt/ mTOR pathways. However, the mTOR pathway was partially activated in endophytic chordoma than the exophytic chordoma, but the TGF-β1 was down regulated in endophytic chordoma. The role of TGF-β1 in PI3K/Akt/mTOR pathways in chordoma needs further studied.

This study is methodologically innovative, but there are still some limitations, including a limited number of patients, which limited the application of statistical methods. Although differential proteomics research methods can reveal a large number of differentially expressed proteins, due to the limitation of validation method, only a small amount of protein could be verified, the high-flux MRM will be used for verification in the future. The analysis method depended on using currently known protein functions, and therefore, useful information was likely overlooked.

Conclusion

Depending on the extent of bone invasion by clivus chordoma, the tumors can be divided intoendophytic and exophytic types by imagings. By integrating proteomic, IHC and Western blot’results, it was found that TGFβ1 may play an important role in bone invasion by clivus chordoma. The mechanisms may be related to mediating an increased inflammatory cell response and a decline in cytoskeletal protein expression. The expression level of PTEN may be associated with the degree of bone invasion by chordoma tumor. The exact signaling pathway through which TGFβ1 and PTEN play a role in clivus chordoma bone invasion remains to be confirmed by further studies.

Author Contributions

Conceived and designed the experiments: ZW LW WS CJY. Performed the experiments: LW ZGG KW YZ KBT. Analyzed the data: ZW LW KW WS. Contributed reagents/materials/analysis tools: JTZ ZW CJY. Wrote the paper: LW KW WS.

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