The Sonic Hedgehog Signaling and Its Components in Recurrent Chordoma of the Spine

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Objective

Chordomas are uncommon primary malignant tumours that have a high rate of recurrence. They are thought to form along the spine from remains of the embryonic notochord. Treatment for recurrent tumours is complicated and contentious.
They are unresponsive to conventional chemotherapy and radiotherapy. Chordomomas simply lack a viable chemotherapeutic standard. Throughout the fetus's development, the Sonic Hedgehog (SHH) pathways connecting a variety of processes involved in tissue and organ expansion and differentiation. To investigate the role of signalling the hedgehog in recurrent spinal chordomas, immunohistochemistry was used to identify SHH and GLI1 levels. In situ hybridization was also used to differentiate PTCH1 and GLI1 expressions.
Methods
From 1997 to 2020, we looked at 23 paraffin-embedded recurrent spinal chordoma samples from 23 patients (9 men, 14 women; median age: 63 years). All the patients were treated at the University Medical Center Goettingen in Germany and Azad University of Medical Sciences in Tehran, Iran. This study only included patients who had been diagnosed with conventional chordoma.
Results
SHH expression (+) and GLI1 expression were discovered in all 23 cases (+) immunohistochemically. GLI1 and SHH levels were markedly increased by recurrent spinal chordoma scores. In the recurrent spinal chordoma, in situ hybridization demonstrated positive responses for PTCH1 and GLI1.
Conclusion
The Shh sample that represents is believed to play a role in spinal chordoma recurrence.
The increased amounts of SHH and GLI1 activity in all chordoma samples, according to the study, indicate an autocrine ligand-dependent activation of the conventional HH signalling cacade. It's hard to rule out a non-canonical or paracrine pathway. Hedgehoginhibitors, such as SHH- and GLI-inhibitors, are believed to be associated in our findings, could be a promising approach for treating recurrent spinal chordomas.

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Introduction
Categories are extraordinary bone tumors of the spine that are believed to develop from the em-bryological notochord's remains. [1]. They're infrequent first malignant tumours with a high risk of recurrence. Treatment for recurrent tumours is complex and disputed. Although the efficacy of radiation therapy alone is disputed, surgery is still the gold standard for chordoma treatment. Previous treatments could have contaminated the area, and damaged local anatomical features could make reoperation more likely. It's a tumour that's both intrusive and damaging on a local level., despite its slow development pace. Chordomas are tumours that are difficult to treat. The therapy procedure at this time is a Illumination after a major surgical resection. The therapy of choice is still en block excision with poor margins. Although the efficacy of radiation therapy alone is disputed, surgery is still the gold standard for chordoma treatment. Because sacrum segments have a high incidence rates, surgical follow-up and associated exams are critical for identifying tumor growth in a timely manner. Furthermore, these tumours have a significant recurrence rate [2,3]; Contamination from previous procedures, as well as disrupted local anatomical features, can raise the risk of reoperation. As a result, chordoma treatment remains a difficulty. Only a few papers have documented recurring sacral chordomas: corresponding results, treatment, and outcome. [4].
Chordomoma formation starts from the notochord in the third embryonic week, according to previous research [5]. Understanding the embryonic notochord's biological functions and evolutionary process may shed light on the pathophysiology of chordoma. During early development, the notochord secretes a variety of signalling molecules, containing the Sonic-Hedgehog (SHH) protein, which controls developmental cell growth, differentiation, and viability [6-9]. Hedgehog transmission has been linked to tumour

emergence and expansion in a number of human cancers, particularly adamantine craniopharyngioma, glioblastoma, medulloblastoma, and rhabdomyosarcoma. [10,11].
It's unclear whether extracellular signalling pathways, such as the hedgehog signalling pathway, have a major part in the development and recurring of chordomas. There is no chemotherapeutic prescription for chordomas and relapses at the moment, and Through both regionally advanced stage chordomas, no treatment has been proven to be beneficial. [3].
A chemotherapeutic drug could be employed as a treatment technique if a signalling cascade is discovered.
Patched (PTCH) is an HH communication channel that is classified into two types: PTCH1 and PTCH2 [12,13]. A change with in ratio between the activating and repressive forms of the GLI family of zinc-finger signaling pathways transmits the HH signal. Hedge-hog signalling effectors GLI1, GLI2, and GLI3 are known, GLI 1 and GLI 2 are transcription stimulators, while GLI 3 is a transcription regulator. [14].
The activation of the HH warning has a significant impact on GLI 1 expression, hence it's used as a reading path activation marker. Since GLI1 stimulates PTCH transcription, both GLI1 and PTCH1 can be regarded target genes and valid markers for an active HH/PTCH signalling cascade. [12]. Dysfunctions of the SHH signalling cascade have already been linked to the development of different illnesses or neoplasia [11]. SMO is a proto-oncogene, whereas PTCH1 is a tumour suppressor gene. [15,16].
Non-canonical methods, on the other hand, can also induce GLI1 transcription. HH signalling can, however, work through signalling routes that aren't as well-known, such as the RAS (Rat Sarcoma) and Transforming Growth Factor Beta (TGFb) signalling cascades, rather than through GLI activation, according to a number of recent studies. [17,18].
The current study looked into whether the SHH signalling pathway is involved in recurrent spinal chordoma.

There is presently no typical pharmaceutical prescription for the prevention of chronic spinal chordoma if proof of an activated SHH signalling pathway is revealed, a chemotherapeutic approach for these individuals may be plausible.

Materials and methods

23 paraffin-embedded recurrent spinal chordoma samples from 23 patients (9 males, 14 females; median age: 63 years) from 1997 to 2020 were included in the research. The patients were all treated in Tehran, Iran, at Goettingen University Medical Center, Tuebingen University Medical Center, and Azad University of Medical Sciences. This study only included patients who had been diagnosed with conventional chordoma. Patients with recurring spine chordoma were on mean 63 years old., with the youngest being 21 years old and the oldest being 84 years old. All the patient had received radiotherapy after the initial operation. There was no further treatment prior biopsy. All recurrent spinal chordomas had Shh and GLI1 expression levels assessed immunohisto chemically. All of the recruited tumour samples (23 total) had recurrent spinal chordoma and were examined for PTCH1 and GLI1 expression.

Immunohistochemistry

Intraoperatively, samples were collected and dehydrated for 1 hour in a sequence of different doses of pure alcoholic solutions (one bath of 50% methanol, 70% methanol, 80% methanol, ninety percent methanol, plus four 100% methanol washes). The samples were then washed in xylene (three treatments, one hour each) before being embedded in paraffin. On all spinal chordoma tissues, anti-SHH (1:500, Rabbit Polyclonal Antibody, Dunn, Asbach, Germany) and anti-ti-Gli1 antibodies were utilised in immunohistochemistry (1:100 Rabbit Polyclonal Antibody, Dunn, Asbach, Germany). All of the slides were processed at the same time and under the same conditions using standard methods. For antigen retrieval, the tissue fragments were treated in the microwave at 80 degrees Celsius with citrate pH 6. 0. Following that, For staining, these tissues were well before including an antibody with either an Avidine-Biotin Peroxidase (Immunotech, Marseille, France) or an Alkaline Phosphatase Detection Kit (Vector, Burlingame, CA, USA) according to normal immunohistochemistry protocols. [19]. There were also negative control images, and all images were conducted at the same time and with the same parameters. To every antibody and image preparation, positively and negatively control portions were given. Negative samples to every protein extraction procedure were TMA images that were not treated with primary antibody. The samples taken were masked for 3 min in H2O2/TBS (Tris Buffered Saline) 3%, then incubated for ten min in TBS/0.1 percent Triton X-100 and 20 min in 0.2% casein to decrease nonspecific binding. The cultures were analyzed in the antiserum for 90 min at room temperature before being washed with TBS/0.1 % Triton X-100. The specimens were then incubated with the secondary antibody for 30min until being detected with 3,3'-diaminobenzidine and counterstained with hematoxylin.

Immunohistochemical Scoring for SHH and GlI1

Two physicians who have been blind to all health records and other histology detection techniques analysed reactivity. As previously stated, immunoreactivity was discovered. [20]. Every tumour was recognised based on the percentage of stained cells (0 = none, 1 = less) from 25% to 50%, and 3 = more than 50%) and the amount of marking in the nucleus or cytoplasm (no staining = 0, fragile staining = 1, moderate staining = 2, strong staining = 3). These two parameters were used to determine the amounts of GLI1 and SHH expression (between 0 and 6). No movement was assigned a value of 0, weak expression was assigned a rating of 1, moderate expression was assigned a rating of 2, and robust expression was assigned a rating of 3–6. Negative control slides from primary spinal chordoma were included, and all slides were performed at the same time under the same conditions.

In Situ Hybridization

As previously disclosed [21,22], the manufacture of digoxigenin-labeled probes and in situ hybridization on cryosections were carried out. A digoxigenin tagging kit (Roche, Mannheim, Germany) was used to tag the riboprobes, which were then exposed

using BCIP/NBT (Roche). Gli1 and Ptch1 ISH probes were generously given by the University Medical Center Göttingen's Department of Human Genetics. All of the portions were handled the same way, both positive and negative controls. In the negative control sections, incubation of the PTCH1 Sense and GLI1 Sense probes. In a nutshell, rehydrated sections were treated for 20 minutes with proteinase K (20 g/mL) before being re-fixed for 5 minutes in PBS containing 4% paraformaldehyde. Sections were washed in 50percent primary amines in 2 SSC for 2 hours at 37°C, then hybridised overnight at 42°C with 5 g/mL digoxigenin-labeled tag in the follows hy-bridization buffer: 4 SSC, 40% formamide, 10% dextran sulphate, 1 Denhardt's solution, 1 mg/mL yeast RNA, 10 mmol/L Dithiothreit, and 1 mg/mL yeast RNA 4 SSC, 2 SSC, 20 g/mL RNase A, 1 SSC, 0.1 SSC in buffer 1 (100 mmol/L Tris-Cl, 150 mmol/L NaCl, pH 7.5) for 3 15 minutes at 37°C, then in buffer 2 (100 mmol/L Tris-Cl, 150 mmol/L NaCl, pH 7.5) for 3 15 min. The samples were blocked for 30 minutes in buffer 1 (-2 percent goat serums, 0.1 percent Tri-tonX-100), The cells were then treated in blocking buffer overnight at room temperature using alkaline phosphatase-conjugated anti-digoxigenin antibody (Roche) at 1:500 dilutions. Rinses in buff-ers 2 (100 mmol/L Tris-Cl, 100 mmol/L NaCl, 50 mmol/L MgCl2, pH 7.5) and 3 (100 mmol/L Tris-Cl, 100 mmol/L NaCl, 50 mmol/L MgCl2, pH 9.5) for 2–10 minutes. The colors reactions were place in the darkness in an NBT/BCIP (Roche) solution that contains 10 mmol/L le-vamisole. Strong hybridization was demonstrated by blue staining. Sense probes were utilised as negative controls in all hybridizations, and no positive signals were seen. Finally, the tissue sections were preserved using an aqueous mounting media (Aquatex®, Sig-Ma-Aldrich, Temecula, CA, USA).

Permission for Participation and Recognition of Morality

All methods used in research participant's studies complied with institution and/or national research review panel ethical criteria, as well as the 1964 Helsinki Declaration and subsequent amendments or comparable professional ethics. The ethical guidelines of the University Medical Center Göttingen in Germany authorised the study process. and Azad University of Medical Sciences in Iran.

Results

Immunohistochemistry

When compared to SHH strong positive samples (19 samples; 82 percent) from 23 patients, GLI1 was slightly more sensitive to all patients, with 21 (91 percent) strong positive results. There were 18 (78%) samples with both positive expression of Shh (+) and GLI1 (+). Based on our data, the expression level of GLI1 and SHH were markedly increased compared to primary spinal chordoma (Table 1).In summary, all 23 recurrent spinal chordoma showed positive expression of SHH and GLI1 (Figure 1)

In Situ Hybridization

The expression of PTCH1 and GLI1 in 23 recurrent spinal chordomas from 23 patients was investigated using in situ hybridization. 18 (78%) of the patients tested positive for PTCH1 (+) and 21 (91%) tested positive for GLI1 (+). The tumour cell nucleus was the only place where PTCH1 and GLI1 were expressed. (See Illustration 2). In neighbouring stromal cells, PTCH1 and GLI1 expression was negative.

Discussion

Hedgehog signalling, popularly called as the Hedgehog-Gli1 system, is a vertebrate embryo development regulator that has evolved over time [23]. In the developing embryo, Hh signalling improves cell survival, boosts cell proliferation, and directs cell differentiation. Hedgehog pathway activation causes the transcriptional activator Gli1 to be expressed, which subsequently increases downstream target gene transcription, Hedgehog signalling receives positive feedback. [24]. The Shh-Gli1 pathway controls the fate of the notochord during normal embryonic development, but it is inert in adults. The sonic hedgehog molecule is one of the signal molecules expressed by the notochord, which controls embryonic cell proliferation, differentiation, and survival [6-9]. Sonic-Hedgehog is a member of the HH protein family of external signalling molecules that play an important role in many species' embryonic development. [25]. Basal cell, ovarian, prostate, breast, clear cell renal cell, and lung carcinomas, and also glioblastoma, medulloblastoma, Rhab-domyosarcoma, and adamantinösen craniopharyngeoma, have all been linked to active Hh signalling [7,26-29].

Chordomomas are currently treated with surgical excision followed by radiation [3]. In addition to surgical excision and radiation, using a Hh inhibitor in combination with chemotherapy to identify a signal cascade could be a viable option. Shh and GLI1 expression was studied immunohistochemistry on formalin-fixed paraffin-embedded tissues from 23 persons for this study. In order to determine an active Shh signal cascade, to investigate, in situ hybridization was used the levels of PTCH1 and GLI1 expression in these samples. Overexpression, mutation, or loss of function of signalling molecules can result in pathological activation of the Shh signalling cascade, this can result in the development of neoplasia. Three possible signaling pathways routes for the HH cascades in carcinogenesis were studied by Teglund and Toftgrd [30].

The first (non-canonical) model highlights intrinsic, ligand-independent SHH signal stimulation produced by polymorphisms that results in functional loss or gain, leading to greater activi-ty. [30,31]. Lower PTCH expression, for example, could signal a PTCH mutations or lost opportunity, as found in basal cell carcinoma, Gorlin-Goltz syndrome, medulloblastoma, and rhabdo-myosarcoma [32,27,33-34]. In this circumstance, PTCH is no more to block SMO, causing the signaling cascading to be activated [13,15,29].

The second kind is autocrine and Shh-dependent, and it is thought to play a role in tumour cells manufacturing their own HH ligand [30-31]. This mechanism has been discovered in glioblastoma, melanoma, breast, prostate, colon, pancreatic, and small cell lung cancer [35-40].

At last, in the paracrine classical model, cancer cells produce HH ligands that trigger the HH signalling pathway in nearby stromal cells [30]. Angiogenesis elements (IGF, VEGF), interleukin 6, and the Wnt signaling cascade for additional ligand synthesis promote tumour cells through with a paracrine feedback mechanism. [13]. Multiple myeloma and lymphoma have previously been linked to a paracrine pathway [31,41]. It's worth mentioning that lung, pancreatic, esophageal, colon, and prostate cancers all include pathways that are both autocrine and paracrine [13]. In summary, all three pathways promote tumour growth. [18,29].
Due to the rarity of chor-domas and subsequent recurrences, Simultaneous chemotherapy in the treatment of chor-domas and subsequent relapses has not been proven in an investigation. [3].
The goal of this study was to see if intracellular signalling pathways, the HH signalling pathway, for example, may play a role in the recurrence of spinal chromes. Our findings show that, all 23 recurrent spinal chordomas tested positive for SHH and GLI1. SHH and GLI1 are extensively expressed in tumour cells, therefore classical activation of the SHH pathway in recurrent spinal chordomas is thought to be likely. In our work, the results of in situ hybridization for PTCH1 and GLI1 in recurring spine chordoma are now only good alternative. Despite the fact that this studies demonstrated considerable expression of PTCH1 and GLI1, it is critical to ensure that tumour cell RNA is retained and accessible for hybridization. Because tumour cell RNA might be both localised and weak, false-negative results can occur. Decalcified tissues have a higher rate of false-negative results, which could be due to RNA degradation [42].
Scheil et al., on the other hand, The SHH gene was not found to be overexpressed in chordoma. In chordae, neither of the SHH pathway's elements were transcription factor active. There has been no other description of chordoma kinds except ours. This could explain why the results of Scheil et al's SHH expression study differed from ours. It's been speculated that this condition is caused by an autocrine/paracrine regulation system. SHH transcription in tumour cells has indeed been linked to increased expression of PTCH1 and GLI1 in surround stromal cells [25], that was not detected in these tests, indicating a potential paracrine activation. While in situ hybridization, GLI1 and PTCH1 higher expression were only discovered in the nuclei of tumour cells, with no expression found in the adjacent stromal cells, according to our findings. Based on the available data, an autocrine activation function has already been discovered. Due to the high amount of Shh transcription in the initial tumours as well as first recurrent, an intrinsic, ligand-independent SHH communication was not predicted [29]. SHH appears to serve as a ligand, initiating the cascade and causing GLI1 transcription, because GLI1 was positive in all the samples tested. Moreover, utilising targeted DNA sequencing, the chordoma samples needed to be further examined for recognized alterations in order to control out a probable PTCH dysfunction. [42]. Thus, in chordomas, an unknown HH signalling cascade pathway cannot be excluded out [43,44,45].
In a previous study [56], we found some good responses to PTCH1 and GLI1 in primary spinal chordoma (ranging from 25% to 40%). This could be due to radiotherapy and chemotherapy administered prior to the first surgery by patients who refused the initial procedure.
Several important points in the active process are possible for a successful blockade. The first option is to prevent the SHH ligand from attaching to its receptor, PTCH1. Roboticininin, a synthetically generated molecule, is recognized to provide an inhibition activity on the secondary signalling pathway through extracellular binding with SHH, in addition towards the HH antibody 5E1 [46,47]. It may also prevent the SHH ligand from binding with the PTCH1 receptor [48].
Inhibition of the SMO protein [49] is another approach. Due to promising pre-clinical outcomes in clinical studies, SMO inhibitors such cyclopamine and vismodegib are increasingly being utilised as monotherapies to prevent medulloblastoma and basal cell carcinoma [13,50-51]. Inhibition of Gli activity is the final option. This can be done in two ways: indirectly by inhibiting SMO transport (HPI-4: Ciliobrevin A) and directly by interfering with GLI binding to the promoter (GANT 58, GANT 61) [17,46,49,50,51,52,53,54].
The present finding suggests that ligand-dependent SHH communication pathway activation in recurrence spinal chordomas considering the high transcript of SHH and GLI1 in all investigated recurrent spinal chordoma samples. By inhibiting the ligand SHH's binding to PTCH1 with 5E1, robotnikinin, or RU-SKI 43, tumour growth could be slowed. Blotta et al. proposed that using GLI- and SMO-inhibitors together in multiple myeloma might be a more powerful combination therapy for suppressing the HH signalling cascade at several interfaces. [31]. According to our findings, GLI inhibitors such as GANT 58, GANT 61, and HPI-1-4, which act directly on the target protein, may be able to limit chordoma tumour growth. The indirectly inhibition of GLI by SMO inhibitors does have the potential to disrupt the dynamic HH signalling pathways, which needs to be further explored [43,18,41,54,55].

Conclusion

The Shh/Gli1 pathway has an aberrant activation pattern in recurrent spinal chordoma, which could have prognostic implications, according to this study. The Shh/Gli1 pathway may help recurrent spinal chordoma become more invasive. As a result, the Shh/Gli1 route could be a good target for treatment recurrent spinal chordoma.

Figure 1: Demonstrate competence of Gli1 expression in a spinal chordoma. A score of 3 correlates to the intensity of the tumour cells' cytoplasmic granular staining (magnification 100).

Figure 2:

A recurring spinal chordoma from a patient following a positive biopsy was compared. Immunohistochemistry staining (Shh (+) and in situ hybridization (Ptch1 (+), Gli1 (+)):

(**A**): In Situ Hybridization PTCH1 at 200× magnification.

(**B**): In Situ Hybridization GLI11 at 200× magnification.

(**C**): Immunohistochemistry of SHH, score 3, at 200× magnification.

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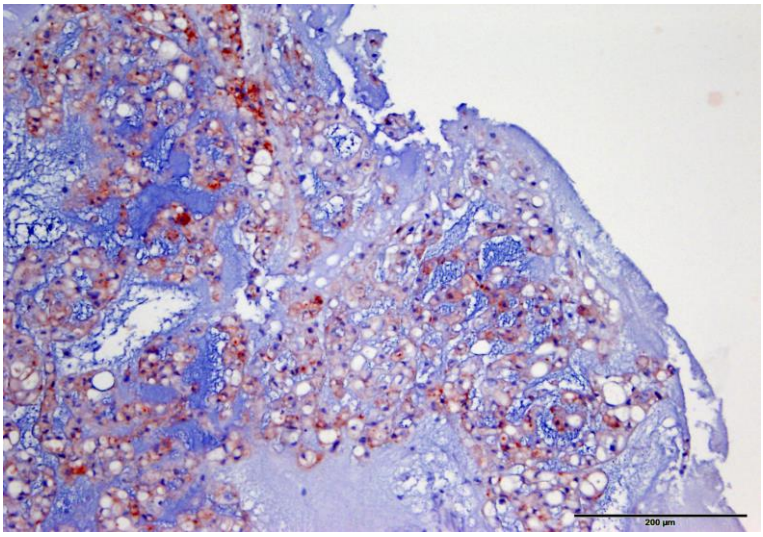

Figure 1

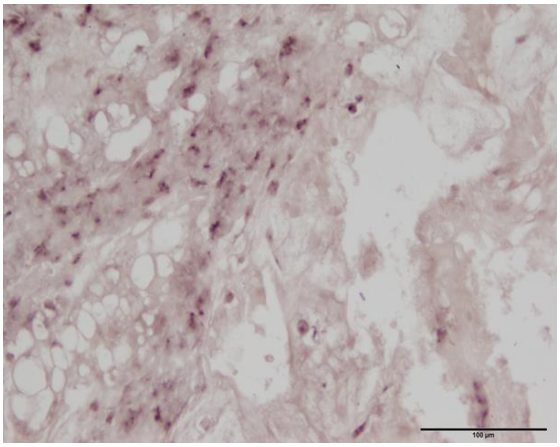

Figure 2A

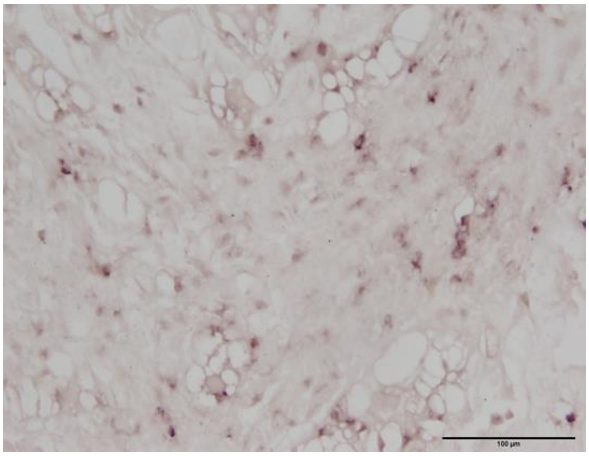

Figure 2B

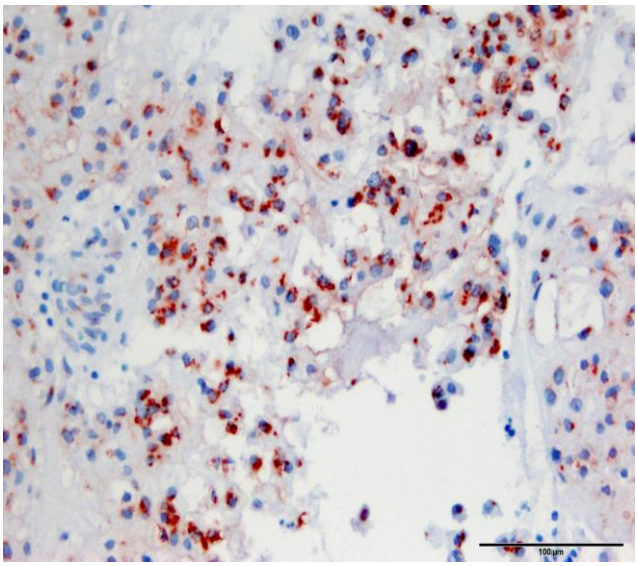

Figure 2C

Table 1

Recurrent Spinal Chordoma	SHH (+)	GLI1 (+)	PTCH1 (+)
Score 0	0 (0%)	0 (0%)	0(0%)
Score 1	1 (4%)	0 (0%)	2 (9%)
Score 2	3 (13%)	2 (9%)	3(13%)
Score 3	19 (82%)	21 (91%)	18 (78%)

In situ hybridization and immunohistochemistry: Positive expression for PTCH1, GLI1 and SHH was found in 18, 21and 19 patients.