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Clinicopathological significance of p16, cyclin D1, Rb and MIB-1 levels in skull base chordoma and chondrosarcoma

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Abstract *Objective:* To investigate the expression of p16, cyclin D1, retinoblastoma tumor suppressor protein (Rb) and MIB-1 in skull base chordoma and chondrosarcoma tissues, and to determine the clinicopathological significance of the above indexes in these diseases.

Methods: A total of 100 skull base chordoma, 30 chondrosarcoma, and 20 normal cartilage tissue samples were analyzed by immunohistochemistry. The expression levels of p16, cyclinD1, Rb and MIB-1 proteins were assessed for potential correlation with the clinicopathological features.

Results: As compared to normal cartilage specimen (control), there was decreased expression of p16, and increased expression of cyclin D1, Rb and MIB-1 proteins, in both skull base chordoma and chondrosarcoma specimens. MIB-1 LI levels were significantly increased in skull base chordoma specimens with negative expression of p16, and positive expression of cyclin D1 and Rb ($P < 0.05$). Significantly elevated MIB-1 LI was also detected in skull base chondrosarcoma tissues, while there was negative expression of p16, cyclin D1 and Rb ($P < 0.05$). In skull base chordoma, p16 negatively correlated with cyclin D1 and Rb, while cyclin D1 positively correlated with Rb. Additionally, p16, cyclin D1, Rb, or MIB-1 expression showed no correlation with age, gender, or pathological classification of patients with skull base chordoma ($P > 0.05$). However, p16 and MIB-1 levels correlated with the intradural invasion, and expression of p16, Rb and MIB-1 correlated with the number of tumor foci ($P < 0.05$). Further, the expression of p16 and MIB-1 appeared to correlate with the prognosis of patients with skull base chordoma.

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Conclusions: The abnormal expression of p16, cyclin D1 and Rb proteins might be associated with the tumorigenesis of skull base chordoma and chondrosarcoma.

Introduction

Skull base chordoma and chondrosarcoma are unusual central skull base tumors which differ in origin, histopathology, and therapeutic outcomes.[1,2] Chordoma, a low-grade malignancy, is a midline primary tumor arising from the ectopic remnants of embryonal notochord,[3,4] while chondrosarcoma is of mesodermal origin.[5,6] Chordoma and chondrosarcoma of the skull base are both invasive tumors, with similar clinical symptoms, anatomic location, as well as radiological findings.[1] The prognosis of these diseases largely depends on the treatment strategy such as, extent of surgical resection and adjuvant radiotherapy.[7]

Some studies have examined the molecular basis of carcinogenesis by investigating the types and status of proteins that are linked with prognosis in patients with chordoma.[3] For instance, MIB-1 (or Ki-67) labeling index (LI), which is related to tumor cell proliferation, was shown to correlate with the doubling time of skull base chordoma lesions.[8] Low MIB-1 value is thought to predict the low-grade propensity of most chordomas.[9] MIB-1, cyclin D1 and p53 were identified as important biomarkers for predicting recurrence in chordomas.[10] In addition, biomarkers that can distinguish skull base chordoma from chondrosarcoma have also been identified. CD24 and brachyury are known to be solely expressed in chordoma,[11] while podoplanin is known to be selectively expressed in chondrosarcoma.[12] However, the accuracy and sensitivity of these biomarkers still needs to be determined.

The retinoblastoma (Rb) pathway, consisting of p16-cyclin D1—dependent kinases 4/6 (CDK4/6)-Rb proteins, plays a pivotal role in regulating cell proliferation by modulating the transition of G1/S phase of the cell cycle.[13]

Mutations of this pathway have been detected in different types of malignancies in humans.[14] Nevertheless, evidence supporting the involvement of Rb pathway in tumorigenesis of skull base chordoma or chondrosarcoma, is rather limited.

In order to understand the clinicopathological significance of the critical proteins in Rb pathway, we assessed the expression of p16, cyclin D1, Rb proteins in tissue specimens of chordoma, chondrosarcoma and normal cartilage tissues. Profiling the respective molecular differences may provide valuable insights into the molecular mechanisms underlying carcinogenesis, and may facilitate the differential diagnosis and patient management.[15] Our results highlight the crucial role of Rb signal pathway in the development and progression of skull base tumors.

Materials and methods

Sample collection

We collected samples from patients undergoing surgical resection of skull base chordoma ($n = 100$) and chondrosarcoma ($n = 30$), at Xuanwu Hospital Capital Medical University, Beijing, China between July 2005 and December 2012. The average age of these patients was 40 years. Among the 100 cases of skull base chordoma, 55 were male and 45 were female. The demographic and clinicopathological characteristics of patients are listed in Table 1. Skull base chordoma was identified by pathological examination. Among 100 cases of skull base chordoma, 79 were identified as standard chordoma, and 18 cases were identified as cartilaginous chordoma according to pathological morphology and the immunohistochemical staining of tumor cells. Similarly, 30 samples of chondrosarcoma, included 21 cases of chondrosarcoma NOS ("Not Otherwise Specified"), 5 cases of myxoid chondrosarcoma, and 4 cases of mesenchymal chondrosarcoma. Twenty normal cartilage tissue specimens obtained from Department of Pathology served as controls for the purpose of this study. Ethical approval for this study was granted by Xuanwu Hospital Capital Medical University, Beijing, China.

Reagents

Primary antibodies, including mouse anti-human anti-p16 antibody, anti-cyclin D1 antibody, anti-Rb antibody, anti-MIB-1 antibody, and 3, 3′-diaminobenzidine tetrahydrochloride (DAB) kit were purchased from Maxin Biotech Inc., Fuzhou, China. Other chemicals and reagents were obtained from Beijing Chemical Reagent Company, China, unless stated otherwise.

Table 1 Average MIB-1 LI levels in skull base chordoma (CD), and chondrosarcoma (CHS) tissues positively or negatively express p16/cyclin D1/Rb.

	CD		CHS	
	MIB-1 LI	*P* value	MIB-1 LI	*P* value
p16				
Negative	10.6	0.015	7.4	0.008
Positive	4.2		2.2	
Cyclin D1				
Negative	3.9	0.015	3.1	0.025
Positive	9.4		2.2	
Rb				
Negative	5.4	0.025	4.3	0.018
Positive	8.6		2.7	

*MIB-1 LI is the positive rate of immunohistochemistry staining with anti-MIB-1.

Histological and immunohistochemical examination

Paraffin-embedded tissue samples were sectioned using an automatic tissue processor (S325, Shandon, UK). Four micrometer-thick sections were deparaffinized in xylene and rehydrated in an ethanol series (100% for 5 min, 95% for 5 min, 95% for 5 min, and distilled water). Following this, samples were washed with phosphate buffered saline (PBS) and incubated with 3% hydrogen peroxide for 15 min at RT. Sections were heated in citrate buffer (0.1 mmol/L, pH 6.0) for 15 min at 92°C—98 °C. After blocking with normal goat serum, samples were incubated with primary antibody at appropriate dilution (anti-p16, 1:80; anti-cyclin D1, 1:40; anti-Rb, 1:100; anti-MIB-1, 1:80) for 60 min at 37 °C followed by overnight incubation at 4 °C. After washing with PBS, sections were probed with secondary antibody using MaxVision™ HRP-Polymer anti-Mouse/Rabbit IHC kit according to manufacturer's instructions (Maxin Biotech Inc., Fuzhou, China). Immunostaining was visualized by DAB. The nuclei were counterstained with hematoxylin solution. Samples were then dehydrated through ethanol series, cleared in xylene, and mounted. Negative control samples were incubated with PBS instead of primary antibody. Some sections were stained with hematoxylin and eosin (HE) for histological examination.

Imaging analysis

Images were captured using optical microscope (BX51, Olympus, Japan) at 400× magnification. A total of 100 cells were randomly selected from each visual field and data were quantified from five fields. Cells with yellow/brown stain in cytosol (e.g. p16, Rb) or in the nucleus (e.g. cyclin D1, Rb, MIB-1) were considered as positive cells.

To semi-quantify the MIB-1 staining, cells with yellow brown or dark brown staining in nuclei were identified as MIB-1-positive cells. The percentage of MIB-1-positive cells in each visual field (magnification 400×) was calculated. The average number of cells was calculated from five non-overlapping fields. MIB-1 labeling index (LI) was calculated using the equation: LI = (Number of MIB-1-positive cells/Total number of cells) × 100.

Follow up study of patients with skull base chordoma

Among 100 cases with skull base chordoma, 72 patients received total removal of tumor mass in our hospital,62.50% of which (45 cases)were initially diagnosed as chordoma, and 28 patients received subtotal resection, 17.85% of which (5 cases)were initially diagnosed as chordoma. They were followed for 3 months to 10 years. The average time for follow up study was 23 months.

Statistical analysis

Data were analyzed using SPSS 17.0 software (IBM SPSS). Categorical variables were compared with Wilcoxon rank sum test. The correlation between the protein expression levels was analyzed using chi square test, and the correlation coefficient r was determined. $P < 0.05$ was considered statistically significant.

Results

Expression of p16, cyclin D1, Rb and MIB-1 proteins in skull base chordoma, chondrosarcoma and normal cartilage tissues

The cancer samples of chordoma, chondrosarcoma and normal cartilage tissues were subjected to IHC staining using specific antibody against p16, cyclin D1, Rb and MIB-1 proteins. As shown in Fig. 1, p16 was mainly located in cytosol; cyclin D1 and MIB-1 were mainly expressed in nuclei; while Rb was detected in both cytosol and nuclei. Compared to the normal cartilage control, the positive expression of p16 decreased in skull base chordoma and chondrosarcoma tissues. In addition, significant differences were found between the two types of sarcomas with respect to expression of p16, cyclin D1, Rb and MIB-1 ($P < 0.05$ for all indexes) in comparison with the control tissues. Besides, among 100 cases of skull base chordoma, 79 cases were identified as standard chordoma, and 18 cases were identified as cartilaginous chordoma. There was no significant difference in expression of these 4 proteins between standard and cartilaginous chordoma. ($P > 0.05$ for all indexes) (Table 2).

Correlation between MIB-1 and p16/cyclin D1/Rb in skull base chordoma and chondrosarcoma

We further investigated the correlation between expression of MIB-1 and p16/cyclin D1/Rb proteins in chordoma and chondrosarcoma samples. For this purpose, the average MIB-1 LI value was determined in both types of sarcomas, and was further correlated with positive or negative expression status of p16, cyclin D1, and Rb. The average MIB-1 LI level was higher in skull base chordoma tissues than in chondrosarcoma (Table 1). MIB-1 LI value was significantly increased in skull base chordoma tissues and correlated with negative expression of p16, and with positive expression of cyclin D1 and Rb ($P < 0.05$). Additionally, a significantly elevated MIB-1 LI expression was also noted in chondrosarcoma tissues with negative expression of p16, cyclin D1 and Rb ($P < 0.05$). Spearman correlation analysis revealed that in the skull base chordoma samples, p16 negatively correlated with cyclin D1 and Rb (p16 vs. cyclin D1, $P < 0.01$, correlation coefficient $r = -0.514$; p16 vs. Rb, $P < 0.01$, $r = -0.403$), while cyclin D1 positively correlated with Rb ($P < 0.01$, $r = 0.677$) (Table 3). Among 30 cases of chondrosarcoma, the MIB-1 LI value for 70% of chondrosarcoma NOS 16.7% of myxoid chondrosarcoma and 13.3% of mesenchymal chondrosarcoma was 2.1, 3.1, and 7.4, respectively ($P < 0.05$).

Association between p16/cyclin D1/Rb/MIB-1 and the demographic and clinical characteristics of patients with skull base chordoma

We investigated potential association of expression levels of p16/cyclin D1/Rb/MIB-1 with the demographic and
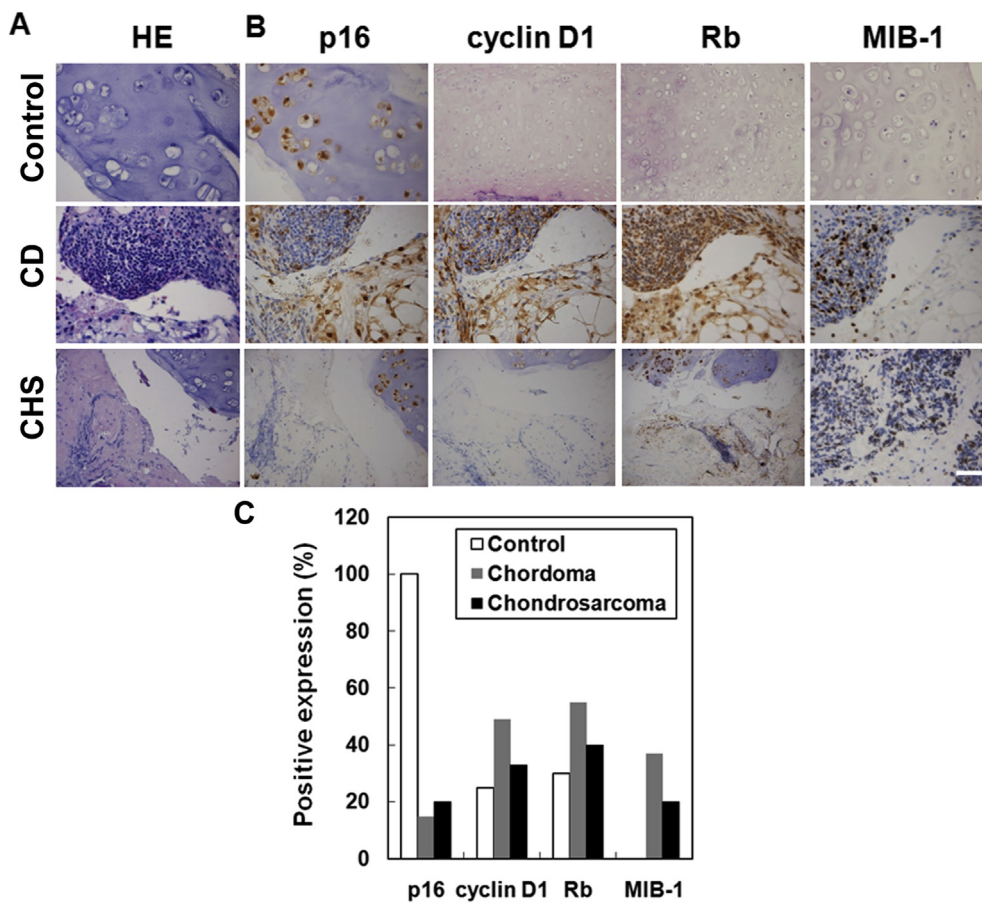
Fig. 1 Histopathological and immunohistochemical study of skull base chordoma and chondrosarcoma specimens. A) HE stained tissue sections; B) Tissue sections immunostained with specific antibodies against p16, cyclin D1, Rb and MIB-1 proteins. Nuclei are counterstained with hematoxylin. Scale bar, 50 μm; (C) Positive expression rate of p16, cyclin D1, Rb and MIB-1 proteins by study group.

clinical characteristics of enrolled patients. As shown in Table 4, the expression of p16, cyclin D1, Rb, or MIB-1 was not associated with age, gender, or pathological features of the patients with skull base chordoma ($P > 0.05$ for all indexes). Although expression of cyclin D1 and Rb did not correlate, the expression of p16 and MIB-1 appeared to correlate with intradural invasion ($P < 0.05$), while no such association was found for cyclin D1 and Rb proteins ($P > 0.05$). Moreover, the expression of p16, Rb and MIB-1, but not cyclin D1, was related to the number of tumor foci ($P < 0.05$).

Table 2 Expression levels of p16/cyclin D1/Rb/MIB-1 between standard and cartilaginous chordoma. Data are presented in terms of frequencies.

	Standard CH	Cartilaginous CH	P value
	Positive rate	Positive rate	
p16	9/79	5/18	>0.05
cyclin D1	40/79	9/18	>0.05
Rb	44/79	10/18	>0.05
MIB-1	10/79	3/18	>0.05

*Positive Rate is the percentage of positive sections with immunohistochemistry staining.

Follow up of patients with skull base chordoma

Among 24 cases with skull base chordoma, 15 cases (Group A) had disease recurrence 2 years after surgical treatment, with an average recurrence time of 7 months, while 9 cases (Group B) were disease free. There was negative expression of p16 in 8 cases (53.3%) of Group A), while it was absent only in 2 cases (22.2%) of Group B. Moreover, expression of MIB-1 was detected in 13 cases of Group A (86.7%), while it was found in only 3 cases in Group B (33.3%). These findings suggest that the expression of p16 and MIB-1 might be related to prognosis in patients with skull base chordoma.

Table 3 Correlation of p16, cyclin D1 and Rb protein levels in skull base chordoma samples. Data are presented in terms of frequencies.

	p16		*P* value *r* value	Rb		*P* value *r* value
	Negative	Positive		Negative	Positive	
Cyclin D1						
Negative	38	13	$P < 0.01$ $r = -0.514$	39	12	$P < 0.01$ $r = 0.677$
Positive	47	2		6	43	
Rb						
Negative	32	13	$P < 0.01$			
Positive	53	2	$r = -0.403$			

Table 4 Association between expression levels of p16/cyclin D1/Rb/MIB-1 and the demographic and clinical characteristics of patients with skull base chordoma. Data are presented in terms of frequencies.

	n	p16			Cyclin D1			Rb			MIB-1		
		Negative	Positive	*P* value	Negative	Positive	*P* value	Negative	Positive	*P* value	Negative	Positive	*P* value
Age (yr)													
<40	47	41	6	>0.05	32	15	>0.05	29	17	>0.05	32	15	>0.05
≥40	53	44	9		36	17		32	21		34	19	
Gender													
Male	55	46	9	>0.05	38	17	>0.05	35	20	>0.05	30	25	>0.05
Female	45	39	6		30	15		26	19		36	9	
Intradural invasion													
Yes	28	24	4	<0.05	14	14	>0.05	17	11	>0.05	4	24	<0.05
No	55	21	34		30	25		29	26		40	15	
Number of tumor foci													
Single	37	20	17	<0.05	17	20	>0.05	13	15	<0.05	12	25	<0.05
≥2	50	37	13		22	28		39	11		31	19	
Pathological type													
Standard chordoma	79	70	9	>0.05	20	59	>0.05	15	64	>0.05	6	73	>0.05
Cartilaginous chordoma	18	12	2		4	14		3	15		2	16	

Discussion

In the present study, we investigated the expression of the key mediators in the Rb signaling pathway in skull base chordoma and chondrosarcoma specimens using immunohistochemical analysis. We observed an association of the expression of p16, cyclin D1, Rb and MIB-1 proteins with the clinicopathological parameters in skull base tumors.

Cell cycle is important for the duplication of cells and growth of an organism, hence it is tightly regulated. It is accepted that tumor cells may evolve partially by overriding the cell cycle regulation.[16] Cyclin D1 is one of the essential mediators in the regulation of cell cycle. Cyclin D1 binds to and induces the activation of CDK4 and CDK6, which in turn results in phosphorylation of Rb, leading to progression of cell cycle from G1 phase into S phase.[17] Inhibition of CDK, for example, by the tumor suppressor p16, can prevent the G1 to S phase transition.[18,19] Here, we found that the positive expression of p16 decreased,

whereas there was increased expression of cyclin D1, and Rb in skull base chordoma and chondrosarcoma tissues, when compared to that in normal cartilage controls. The pathological findings implicate the derangement of multiple components of the Rb signal pathway in the causation and progression of skull base tumors.

p16 is a widely accepted tumor suppressor protein that regulates cell cycle.[20] The expression of p16 is reportedly downregulated in several tumors, such as glioblastomas, lymphomas, leukemia, liver, lung, bladder, and breast cancers.[20] Consistent with these findings, we too detected reduced p16 expression in skull base chordoma and chondrosarcoma. Besides, we observed a close association between the expression of p16 and MIB-1 LI, since the MIB-1 LI level was significantly increased in skull base chordoma and chondrosarcoma samples that showed a concomitant loss of p16 expression. The negative expression of p16 was associated with the development of intradural invasion and an increased number of tumor foci in patients with skull base

chordoma, which suggests a close correlation of p16 protein loss with disease progression. However, the level of p16 showed no correlation with variables such as age, gender or pathological classification of skull base chordoma. Since increased MIB-1 LI value correlated with increasing histological grade of most chordoma samples,[9] it appears to be a potential risk factor for aggressive disease progression and reduced survival time in these cancers.[21] Some reports have indicated a correlation between loss of p16 expression and high-grade central chondrosarcoma.[22] Further, p16 and MIB-1 LI levels might be of value in predicting the histological grade and progression of skull base tumors. Further, MIB-1 expression and loss of p16 expression was found to be associated with tumor recurrence. Our findings indicate the prognostic significance of p16 and MIB-1 in patients with skull base tumors.

Cyclin D1 was found to be highly expressed in 49% of skull base chordoma and 33% of chondrosarcoma samples, compared to its low expression in 25% of normal cartilage samples. Consistent with our findings, another study claimed that 62% (17 out of 27) cases of high-grade chondrosarcoma express cyclin D1.[23] The cyclin D1 expression correlates with the high MIB-1 LI value and the recurrence of chordomas.[10] We did not detect any association between cyclin D1 expression and the demographic or clinicopathological variables including age, gender, intradural invasion, number of tumor foci, and tumor classification.

Positive expression of Rb was found in 55%, 40%, and 30% of the chordoma, chondrosarcoma and control samples, respectively. In addition, the level of Rb was found to be closely associated with the number of tumor foci, which suggested that the increased Rb expression might correlate with enhanced tumor proliferation and invasion. These results are in accordance with previous report of the alternation of Rb pathway in high-grade chondrosarcoma.[23] We speculate that the reduced p16 expression, and thereby enhanced cyclin D1 and Rb activity, may contribute to tumorigenesis of skull base tumors.

In summary, our current study demonstrates the clinicopathological significance of the p16-cyclin D1-Rb pathway and the MIB-1 level in skull base chordoma and chondrosarcoma. We believe that therapies targeting the cell cycle regulation, by exogenous over-expression of p16,[20,24] or introduction of the anti-sense oligonucleotides of cyclin D1,[25] or interference of Rb activity, may prove valuable in the treatment of cancer.[26] In addition, the molecular mechanisms involved in the Rb pathway (CDK4/6 pathway) and tumorigenesis of skull base tumors are being researched.[27] Future studies should employ both *in vitro* studies of cultured tumor cells, as well as animal disease models to unravel the underlying mechanisms. The small sample size is one of the limitations of our study. Future studies involving larger sample sizes could provide more definitive evidence and help substantiate the leads emanating from this study. The association between the Rb signal pathway regulators and disease prognosis is of particular clinical import.

Conflicts of interest

We declare that we have no conflicts of interest.

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