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Correlation between the methylation of LIVIN geneand the pathogenesis of bone tumor

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Abstract. – OBJECTIVE: We investigated the correlation between the methylation of LIVIN gene and the pathogenesis of bone tumor at the molecular level, in order to improve the treatment method and enhance the cure rate of bone tumor.

PATIENTS AND METHODS: The expression level of Livin protein was detected using Western blot analysis, and its expressions in control group and patients were detected by immunohistochemistry. The methylation frequency of LIVIN gene was calculated by direct sequencing. Finally, the prognosis of treatment was investigated by follow-up.

RESULTS: The experiment found that Livin protein was not expressed in normal cells, while its expression rate was about 71.4% in 112 patients. The methylation frequency of LIVIN gene was gradually decreased with the increase of clinical stage, and had no significant relationship with age and sex. The prognosis experiment indicated that the lower the methylation frequency of LIVIN gene was, the shorter the survival time would be.

CONCLUSIONS: The methylation of LIVIN gene was closely related to the pathogenesis of bone tumor, which may be one of the important factors to induce the formation of a bone tumor. In addition, the methylation frequency of LIVIN gene could be used as a biomarker for the prognosis of bone tumor treatment.

Key Words
LIVIN gene, Gene methylation, Bone tumor, Prognosis.

Introduction

Bone tumor is a kind of malignant tumor, which often occurs in youngsters with rapid skeleton growth[1,2]. The disease affects four in one million people. Although the incidence is not high, the survival rate of patients is very low. Some findings have showed that the survival rate of patients treated with surgery or chemotherapy within 5 years was only 60%[3,4]. Generally, the treatment method is determined by recognition of the pathogenesis of the disease, and a clear understanding of its formation mechanism, so as to enhance the cure rate of bone tumor patients. At present, many studies on bone tumor are still limited to the location of disease and cell morphology, so this study is designed to investigate the occurrence and development mechanism of bone tumor at the molecular level to further understand the pathogenesis of the disease. It is well known that the formation of tumor cells is closely related to the expression of genes affected by DNA methylation, so DNA methylation has an inextricable relationship with tumor cells. At present, the research trend worldwide is to find the key factors, which can regulate tumor cells. Kasof et al[5] have found the LIVIN gene, which encodes a kind of inhibitor of apoptosis protein, and its expression, can directly intervene in the proliferation and metastasis of tumor cells. In recent years, a large number of researches[6-8] have indicated that LIVIN gene has an important relationship with the formation mechanism of many tumor cells. Researches on LIVIN gene reported that Livin protein could be detected in serum of patients with lung cancer, and there is negative correlation between Livin expression and caspase-3 gene[9], which may be caused by the anti-apoptosis of LIVIN gene. Livin protein is also expressed in patients with colorectal cancer[10], and the study has investigated and found the expression of Livin protein was associated with the

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clinical stages and prognosis of colorectal cancer. It was shown that there is a great relationship between LIVIN gene and tumor cells, while the correlation between LIVIN gene, lung tumor and colorectal cancer was also explored worldwide. However, there are relatively few studies on the correlation between methylation of LIVIN gene and bone tumor. Therefore, this study was designed to further understand the pathogenesis of bone tumor, in order to find new chemotherapy drugs and surgical methods according to the formation mechanism of tumor cells.

Patients and Methods

Patients

We selected 112 patients, who were admitted to our hospital and diagnosed with bone tumor from 2012 to 2013. There were 71 cases (male) ranging from age 29 to 59 years old (average age = 47.3 yrs) and 41 cases (female) ranging from age 31 to 61 years old (average age = 45.6). The 112 patients were divided into I-IV stage (21, 28, 31, 26 cases, respectively) according to the standard recommended by the International Union Against Cancer (UICC). There were 40 cases in healthy control group with age ranging from 30 to 60 years old (average age = 48.2 yrs). Statistical analysis showed that there was no significant difference in the age and gender ratio between bone tumor patients and healthy control group. This study was approved by the Ethics Committee of Jinan Maternity and Child Care Hospital. Signed written informed consents were obtained from all participants before the study.

Western Blotting Analysis

Specific methods were used as per Ida et al[11].

Immunohistochemistry Detection

Specific methods were used as per Kruglova et al[12], and then the results were analyzed by microscopy.

Direct Sequencing (BSP)

The method used was as per Chen et al[13]. Specific methods of PCR amplification were done as per Hassani et al[14].

Follow-up of Prognosis of Bone Tumor Patients

Specific survey methods were as per Sanz-Ramos et al[15] with follow-up for 36 months after treatment.

Kaplan-Meier Survival Analysis

Specific methods were as per van Walraven et al[16] with a little modification.

Statistical Analysis

Statistical Product and Service Solutions 14 software (SPSS Inc., Chicago, IL, USA) were applied for statistical analysis. X^2-test was used, with the test level as α=0.05.

Results

Expression of Livin Protein in Bone Tumor Cells

In order to investigate the correlation between the methylation level of LIVIN gene and the pathogenesis of bone tumor, the expression of Livin protein in bone tumor cells was analyzed primarily. First, the expressions of Livin protein in control group and bone tumor cells were compared by Western blotting analysis (Figure 1). Results showed that Livin protein was not expressed in healthy control group, but it was expressed in bone tumor cells. The quantitative results were shown in Figure 2.

To determine the quantitative expression of the Livin protein in the different tumor cells, the Livin protein was also detected by immunohistochemistry and its expression in different cells tissues was observed by microcopy. Bone tumor cells were graded into different clinical stages, according to Chen et al[17] (Figure 3).

As shown in Figure 3, panel A demonstrated normal cells and panels B, C and D revealed the malignant degrees of bone tumor cells that were divided into Grade I-III. The expressions of Livin protein were compared according to the different grades, the specific data of which were shown in Table I. The results showed that the Livin protein was not expressed in 40 cases of healthy control

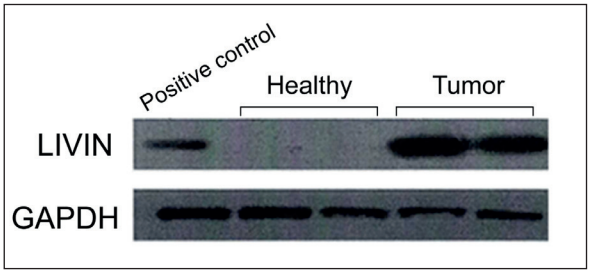

Figure 1. Expression of Livin protein in different cell tissues (1 and 2 were from healthy control group cells, 3 and 4 were from bone tumor cells).

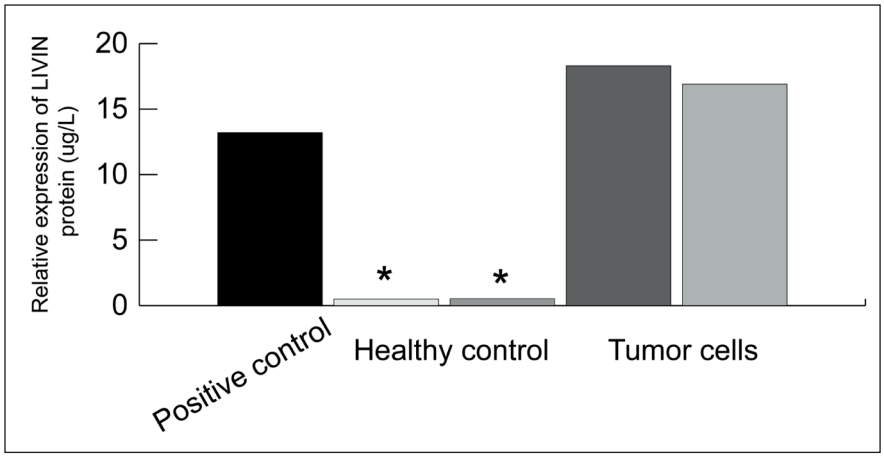

Figure 2. Expression of Livin protein in different cell tissues ("*" indicated a significant difference between groups).

group, while it was not completely expressed in some patients with bone tumor either. The positive expression rate was about 71.4% in 112 patients. In addition, the expression of Livin protein had no significant relationship with the age and gender of patients with bone tumor (p>0.05), but there was a distinct relationship between the expression of Livin protein and the clinical stage of bone tumor (p<0.05). Therefore, the study would focus on the correlation between LIVIN gene methylation and clinical stage.

Analysis of the Methylation Frequency of LIVIN Gene

A literature search showed that methylation occurs in the promoter sequence of the LIVIN gene. We used the DNA man software to design primers and pyrosequencing (BSP) to study methylation in the promoter. The differences of methylation frequency of LIVIN gene in control group and patient group with positive expression of Livin protein as well as in different clinical stages were compared. (Table II). It is showed that the methylation frequency of LIVIN gene had no significant relationship with the age and gender of patients with bone tumor (p>0.05), which was similar to the expression of Livin protein in bone tumor cells (Table II). However, its methylation frequency at clinical stages gradually decreased, with significant difference (p<0.05).

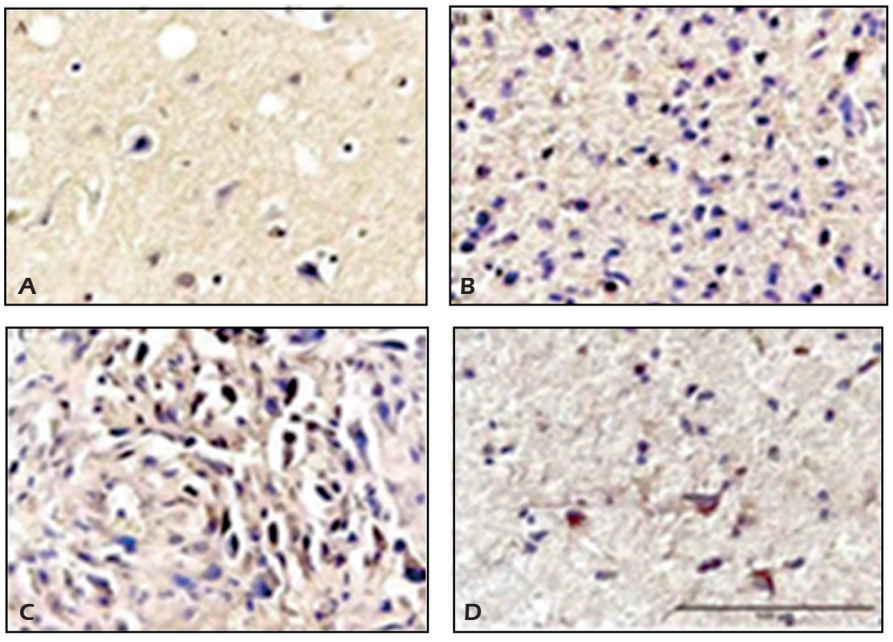

Figure 3. Expression of Livin protein in bone tumor cells (**A**, normal cells; **B**, bone tumor in Grade I; **C**, bone tumor in Grade II; **D**, bone tumor in Grade III).

Table I. Expression of Livin protein in cancer cells of 112 patients with bone tumor.

Clinical features	Cases (n)	Expression of Livin protein				Positive ratio (%)	*p*
		Negative	Grade I	Grade II	Grade III		
Control group	40	40	0	0	0	0	<0.05
Patient group	112	32	65	21	4	71.4	
Gender							
Male	71	20	33	14	4	71.8	>0.05
Female	41	12	22	7	0	70.7	
Age							>0.05
≥ 50	63	19	31	11	2	69.8	
< 50	49	15	22	10	2	69.3	
Clinical stage							<0.05
I	27	9	15	3	0	66.7	
II	28	8	13	6	1	71.4	
III	31	7	19	4	1	77.4	
IV	26	5	11	8	2	80.7	

Methylation of LIVIN Gene and Prognosis

The relationship between LIVIN gene methylation and prognosis was analyzed by follow-up, with the follow-up for 36 months after treatment (Table III). Figure 4 showed that the lower the methylation frequency of LIVIN gene was, the shorter the survival time after treatment and this observation had significant difference (p<0.05).

Discussion

In recent years, the research on the occurrence and development mechanism of tumor was not confined to observe the relationship between tumor position and anatomy as per tradition. With the development of molecular biology, more studies are focusing on tumor formation mechanism[17]. For malignant bone tumor, there are no significantly effective therapies. With a low cure rate and high mortality, bone tumor has been a threat to the safety and patients' life for a long time. Therefore, it is of great significance to understand the pathogenesis of bone tumor, so as to improve the treatment method[18]. Takenchi et al[19] showed that tumor cells were radically caused by aberrant methylation of gene, while LIVIN was a gene which was closely related to the occurrence and development of the majority of tumor cells[20].

Table II. Analysis on the methylation frequency of LIVIN gene in patients with bone tumor (%±SD).

Clinical features	Cases	Methylation frequency	*p*
Gender			>0.05
Male	71	45.23±14.15	
Female	41	44.17±11.28	
Age			>0.05
≥ 50	63	45.55±17.32	
< 50	49	43.21±9.18	
Clinical stage			<0.05
I	27	64.17±17.68	
II	28	50.23±15.32	
III	31	37.24±9.38	
IV	26	26.81±7.58	

The methylation frequency of LIVIN gene had no significant relationship with the age and gender of patients with bone tumor (p>0.05), which was similar to the expression of Livin protein in bone tumor cells. However, its methylation frequency in clinical tumor stages gradually decreased, with significant difference (p<0.05).

Table III. Relationship between methylation frequency of LIVIN gene and prognosis.

Clinical groups	Cases (n)	Positive rate of Livin protein (%)	Methylation frequency (%)	Survival time after treatment (month)
I	27	68.4	64.17±17.68	33.8±2.3[a]
II	28	74.6	50.23±15.32	30.1±1.7[ab]
III	31	78.6	37.24±9.38	25.5±1.9[abc]
IV	26	83.6	26.81±7.58	15.6±2.6[abcd]

Notes: [a-d] indicated there were significant differences ($p<0.05$).

Therefore, the present study was designed to investigate the correlation between the methylation of LIVIN gene and the pathogenesis of bone tumor.

First, the expression levels of Livin protein in normal human cells and bone tumor cells were analyzed: 40 cases (healthy volunteers) and 112 patients with bone tumor were respectively and randomly selected. Expression of Livin protein in tissue cells was analyzed by Western blotting methodology. We showed that Livin protein was only expressed in bone tumor cells and not expressed in tissue cells of two healthy volunteers. In order to verify the result, tumor tissues of 112 patients with bone tumor were also analyzed for the expression of Livin protein using immunohistochemistry. Patients were categorized in groups Grade I-III according to the severity. The experiment found that the expression of Livin protein was negative in healthy control group (n=40), while it was not completely expressed in some of the 112 patients with bone tumor either. The positive expression rate was about 71.4%. The result indicated that the expression of Livin protein was associated with the formation of bone tumor. As shown in Table I, it could be seen that there was a distinct relationship between the expression of Livin protein and the clinical stage of bone tumor ($p<0.05$), suggested that its expression was related to the severity of bone tumor.

Next, the relationship between LIVIN gene methylation and bone tumor was analyzed. The methylation frequency of LIVIN gene was calculated by bisulfite pyrosequencing (BSP) method. The methylation frequency of LIVIN gene had no significant relationship with the age and gender of patients with bone tumor, which was gradually decreased in clinical stages. The data confirmed the results, which indicated that the low methylation frequency of LIVIN gene affected the occurrence and development of bone tumor.

When the prognosis effect of bone tumor treatment was investigated, all the experimental data were summarized, and the correlation between them was analyzed. The experiment showed that the positive expression rate of Livin protein was gradually increased with the increase of clinical stage, but average methylation frequency was gradually reduced. Although positive expression rate increased, the number of key methylation gene resulting in bone tumor formation did not increase, thus it may explain the reduction of methylation

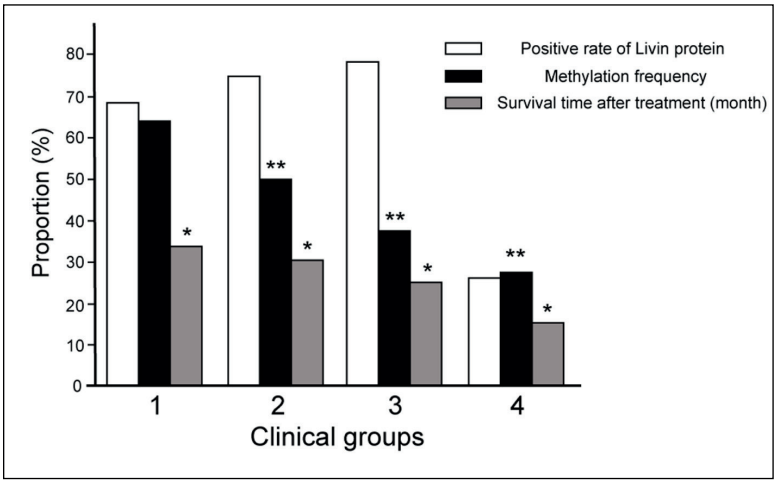

Figure 4. The relationship between methylation frequency of Livin gene and prognosis ("*" indicated significant differences among the groups).

frequency. However, the lower the methylation frequency was, the shorter the survival time after treatment, which indicated the low methylation frequency of LIVIN gene was associated with the prognosis of treatment. Based on this observation in patients of clinical Stage IV, the positive expression rate of Livin protein was the highest, which was most likely due to low methylation, thus the high positive expression rate could result from the decrease of overall LIVIN gene methylation frequency. We speculated that the methylation of LIVIN gene was responsible in inducing the formation of bone tumor.

Conclusions

We found that the methylation frequency of LIVIN gene was closely related to the clinical stages and prognosis of bone tumor, indicating that it may be one of the important factors affecting the formation of bone tumor. Further investigations would be performed on the basis of this work.

Conflict of Interests

The authors declared no conflict of interest.

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