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The relationship between the expression of TAM, survivin and the degree of necrosis of the tumor after cisplatin treatment in osteosarcoma

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Abstract. – OBJECTIVE: To explore the relationship between the expression of TAM, survivin and the degree of necrosis of the tumor after cisplatin treatment in osteosarcoma.

MATERIALS AND METHODS: The mice model of osteosarcoma S180 were injected with 6 mg/kg/day of cisplatin (observation group) or the same amount of normal saline (control group) for 4 weeks. Mice were sacrificed at days 1, 4, 9, 14, 18, 22 and 28, respectively, 24 h before administration of the drug or saline, and tumor tissues were collected. The size of the tumor samples was measured and the correlation of TAM, survivin expression in osteosarcoma and necrosis degree of tumor tissue after cisplatin treatment was studied using various methods including fluorescence quantitative PCR, enzyme linked immunosorbent assay (ELISA), Western blotting and immunohistochemistry.

RESULTS: Fluorescence quantitative PCR showed that the expression of TAM, survivin mRNA in the control group was significantly higher than that in the observation group. Also, the ELISA monitoring showed that the expression of mice TAM, survivin protein *in vivo* was significantly lower than TAM, survivin protein expression of mice *in vivo* in the observation group (2.3 μg/l, 1.6 μg/l) relatively to the control group (9.7 μg/l, 10.3 μg/l). Consistent with the Western blot data, ELISA results showed that the expression of survivin and TAM protein decreased gradually with the prolongation of drug treatment along the time in the observation group. The volume and weight of the tumor in the observation group were significantly less than that of the control group. Additionally, the tumor necrosis of mice in the observation group was more significant, suggesting that the meant of the size of tumor tissue decreased significantly with the extension of the time of drug treatment. Immunohistochemical results showed that the rate of the positive cell of TAM and survivin in the observation group (82.3%) was significantly higher ($p<0.05$) than that in the control group (19.5%). However, the rate of the positive cell of survivin and TAM gradually declined at the level of the trend with the extension of the time of drug treatment in the observation group.

CONCLUSIONS: Cisplatin treatment can inhibit the expression of TAM and survivin in osteosarcoma tissue sand then, promote the necrosis of tumor tissue.

Key words:
Osteosarcoma tissue, TAM, Survivin, Cisplatin treatment, Tumor necrosis.

Introduction

Research results show that[1] in recent years, the incidence of osteosarcoma was increasing year by year in our country. In 2015, data showed about 10 million people suffering from a different degree of osteosarcoma[2], a proportion that is still increasing with no clear pathogenesis. Accordingly, it is still difficult to diagnose and evaluate therapeutically effects of drugs in the treatment of osteosarcoma disease. However, data from a study[3] showed that the related drug resistance of tumor cells mainly comes from two aspects including the higher rate of mutation of genome and the probability of significant increases of a certain class of drug resistance[4], and the DNA mismatch repair mechanism of tumor cells and the significant ability of improvement and repair of the damaged DNA by external drug[5]. Relevant research[6] showed that cisplatin, as an effective drug for the treatment of osteosarcoma, plays an important role in the treatment of osteosarcoma, but its mechanism is not clear although clinical statistics had shown that cisplatin and other drugs have good therapeutic effect for the treatment of

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osteosarcoma. Indeed, data from the clinical research showed that cisplatin had a certain inhibitory effect on the proliferation of osteosarcoma cells, with significant effect when used earlier and decreased effect on the late treatment in combination with various drugs. A research[7] showed that inhibitory protein of apoptosis can significantly inhibit the development of the process of apoptosis. In particular, the expression of the tumor cells, significantly elevated are inhibited by survivin, a new type of apoptosis inhibitory protein found in lung cancer and pancreatic cancer, even if its role is still unknown[8].

However, TAM gene, also acting as a class of genes related to apoptosis, is involved in the process of cell apoptosis[9]. There are few reports about the correlation between the expression of TAM and survivin, and the degree of necrosis of the tumor after cisplatin treatment. Therefore, to clarify the mechanism of interaction we study the relationship between the expression of TAM and survivin, and the degree of necrosis in the tumor tissue after cisplatin treatment.

Materials and Methods

Samples and Materials

In the current study, we used S18 type mice with osteosarcoma disease. The observation group (12 rats) was treated with cisplatin (Hengrui Pharma, Nanjing, China) (6 mg/kg/d). The average age was 3.8 ± 0.3 weeks with average body weight of 14.3±3.6 g. The control group (12 rats) was injected with the same amount of normal saline. Their average of age was 4.2 ± 0.3 weeks and their body weight was 15.2±2.8 g. The different treatments lasted 4 weeks and some mice were respectively sacrificed on day 1, 4, 9, 14, 18, 22, 28, 24 h before injections.

Methods for mice sacrifice and tumor obtaining: the mice were killed by cervical dislocation method and tumor was cut for sample preparation.

Main reagent: DMEM medium Gibco (Rockville, MD, USA), fetal bovine serum (FBS) (China, Suzhou Alpha Company), cisplatin (Guangzhou National Medicine, Guangzhou, China), polyclonal murine TAM first anti (Axygen Waltham, MA, USA), polyclonal murine TAM first anti (Axygen Waltham, MA, USA), HRP labeled Goat anti-mouse second antibody (Axygen Waltham, MA, USA).

Main instrument: low temperature high speed centrifuge (Thermo Fisher Scientific, Waltham, MA, USA) and analytical balance (Olympus, Tokyo, Japan), Thermo Cell (Tiangen, Beijing, China), ultra-low temperature freezer (Haier, Shanghai, China), fluorescence quantitative PCR instrument (Thermo Fisher Scientific, Waltham, MA, USA), gel imager Bio-Rad (Hercules, CA, USA).

Methods

Fluorescence Quantitative PCR

RNA Extraction

We used RNA extracted from different osteosarcoma cells kept at -80°C in refrigerator and determined the quality of extraction.

Fluorescence Quantitative PCR

To study differences in mRNA expression between TAM and survivin in treatments, cDNA was obtained from RNA as a template, and the fluorescence quantitative PCR test was carried out. The primer sequences were shown in Table I.

Enzyme-Linked Immune Response (ELISA)

The experimental method was carried out according to the specification of ELISA Kit (TAKARA Company, Tokyo, Japan) that was improved according to Yin et al[12]. ELISA standard protein sample was diluted with Assay Buffer (Biosharp, Hefei, Anhui, China) in accordance with the ratio of 1:50, and the specification of the operation the standard curve was produced. The extracted total protein was taken as the object of study. They were diluted with phosphate buffered saline (PBS) (pH 7.2) (Biosharp, Hefei, Anhui, China) according to the scale of 1:100, then 80 μl samples were discarded to be tested by adding 35 μl protein detection solution, and incubating them at room temperature 37°C for 2 h. Thereafter, TMB was added for chromogenic substrate and the light value of absorption was measured at 495 nm. The content and concentration of TAM and survivin in various samples were calculated according to the standard curve measured.

Western-Blotting Experiment

Western blotting of TAM and survivin protein in different samples were measured through animal cell total protein extraction kit that extracted total protein in accordance with "the operation guide to molecular cloning, the third edition". Briefly, after the first antibody diluted in accor-

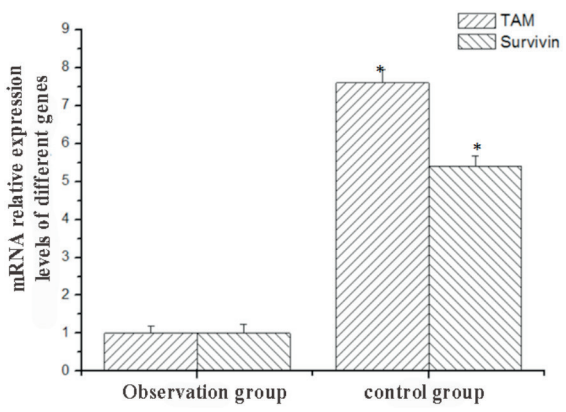

Figure 1. TAM and survivin mRNA expression in the observation group as well as the control group. "*" shows significant difference (p<0.05).

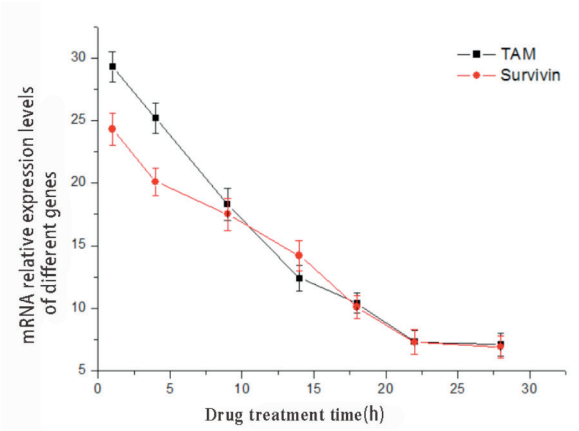

Figure 2. Treatment time "*" shows significant difference (p<0.05).

ding to the ratio 1:800, the second antibody was used after a dilution of 1:500 ratio according to Qin et al[10].

Immunohistochemistry

TAM and survivin protein of different samples were determined by immunohistochemistry SP method. With the consent of the patients, family members and the hospital Ethics Committee, Kidney tissues and normal renal normal tissue of mice were taken for immunohistochemical experiments. The samples were immersed in xylene for about 10 min and then washed by anhydrous ethanol for 1 min and this was repeated for 2 times. Then, samples were dyed with hematoxylin and blocked with 1% hydrochloric acid for 20 s. Then, they were stained with eosin solution. The neutral resin was used for mounting. Detailed protocols for immunohistochemical staning were according to a previous report[11].

Tumor Tissue Acquisition and Weighing

We took different treatment of mice tumor tissue respectively on day 1, 4, 9, 14, 18, 22, 28, and treatment method was referred to literature. According to the formula $V=1/2ab^2$ (a means tumor tissue length, b means short radius of tumor tissue), we calculate tumor size in mice with different treatments.

Statistical Analysis

Software SPSS 20.0 (SPSS Inc., Chicago, IL, USA) was used for the data analysis. Inspection standards α=0.05, p<0.05 as a significant difference, α=0.01, p<0.01 as an extremely significant difference.

Results

mRNA Expression of Survivin and TAM in Both Observation and Control Groups.

Compared with mice tumor tissue samples of the control group, the mRNA contents of TAM and survivin gene in the tumor tissue samples of the observation group were relatively higher (Figure 1). Through the comparison of TAM and survivin mRNA expression in mice tissue samples of observation group, it was found that TAM gene mRNA expression was 7.6 times of the expression in mice tumor tissue samples compared with sample from the control group (p<0.05). At the same time, the expression of survivin gene in the observation group was 5.4 times of that in the control group (p<0.05).

Time Course of TAM and Survivin mRNA Expressions in the Observation Group According to the Length of Treatment.

TAM and survivin gene mRNA expression in different time points were measured by fluorescence quantitative PCR method and compared (see Figure 2). Data showed that with the length of treatment of the cisplatin treatment of mice, TAM and survivin mRNA showed a declining trend, but the decline rate was showing a gradual decrease trend, suggesting that cisplatin treatment can inhibit TAM and survivin mRNA expression in osteosarcoma cell.

Expression of Protein TAM and Survivin in Both Observation and Control Groups

The expression of TAM and survivin protein in tumor tissue samples from both observation

Table I. Fluorescence quantitative PCR primer.

Primer name	Sequence
TAM-F	AGTCGTAGCTAGCTAGCTACG
TAM-R	GTCGTGATCGATCGAGCTCGC
Survivin-F	ATGCTGCGCTAGCTCGATCGC
Survivin-R	ATCGCTGCTAGCACAGACGTCAG
GAPDH-F	TGCTAGGCTAGGACGCTAGCTAC
GAPDH-R	CTGGGCTAGATCGACGAGAGCTC

and control groups were determined by ELISA method (see Figure 3). TAM protein expression of the tumor tissue samples in the control group (9.7 ± 1.2) μg/l was significantly higher (p<0.05) than the expression of mice in tumor tissue in the observation group after cisplatin treatment (2.3 ± 0.73) μg/l. At the same time, survivin protein expression in the tumor tissue of control mice (10.3±1.04) μg/l was significantly higher (p<0.05) than that in the observation group (1.6±0.15) μg/l.

Time Course of TAM and Survivin Protein Expression in Observation Group with Treatment

We selected mice tumor tissue samples at different time points of the observation group, by enzyme-linked immunosorbent assay method determined TAM and survivin mRNA expression at different time points and results were as shown in Figure 4. As observed, the extension of the time of cisplatin treatment, lead to downstream of the expression of TAM and survivin proteins. The rate of the decline of protein content was gradual until a trend of decrease. These suggested that cisplatin treatment could inhibit time-dependently TAM and survivin protein expression in osteosarcoma cell.

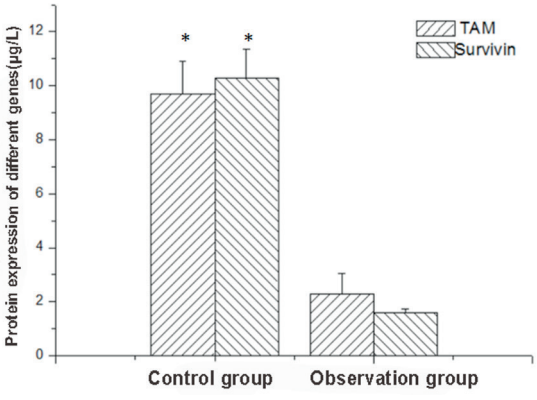

Figure 3. Protein expression of TAM and survivin in the observation group and the control group."*" shows significant difference (p<0.05).

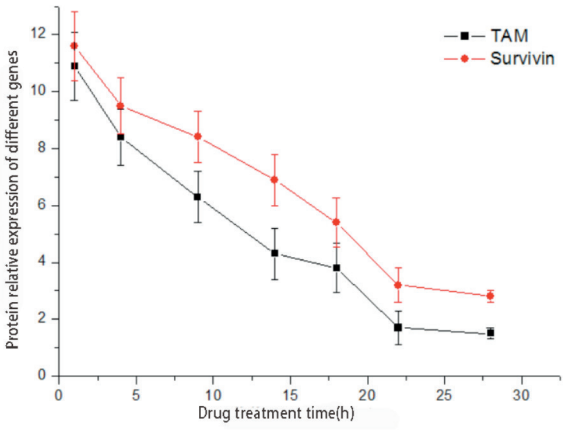

Figure 4. Protein expression of TAM and survivin samples of the observation group with different treatment time."*" shows significant difference (p<0.05).

The Expression of TAM and Survivin in the Observation Group and the Control group Detected with Western-Blotting.

The expressions of proteins TAM and survivin in the tumor tissues of both observation and control groups were measured by Western-blotting method (see Figure 5). In the control group, TAM and survivin protein content in the tumor tissue samples were significantly higher (p<0.05) than those of observation group after cisplatin treatment. This data was consistent with the results of ELISA (Figure 5B).

Immunohistochemical Results of the Observation Group and the Control Group

Through immunohistochemical detection of tumor tissue samples in both observation and control groups, we found that the TAM positive cells were more in tumor tissues from control group while TMA positive cells in the tumor tissues of the observation group were significantly decreased (Figure 6) after cisplatin treatment, and there was a significant difference between the two groups. Through the number of the positive cells in tumor tissue in the control group and the observation group (Table II), it was found that the rate of TAM positive cell (92.5%) in tumor tissue of the control group was significantly higher (p<0.05) than that in the observation group (16.5%).

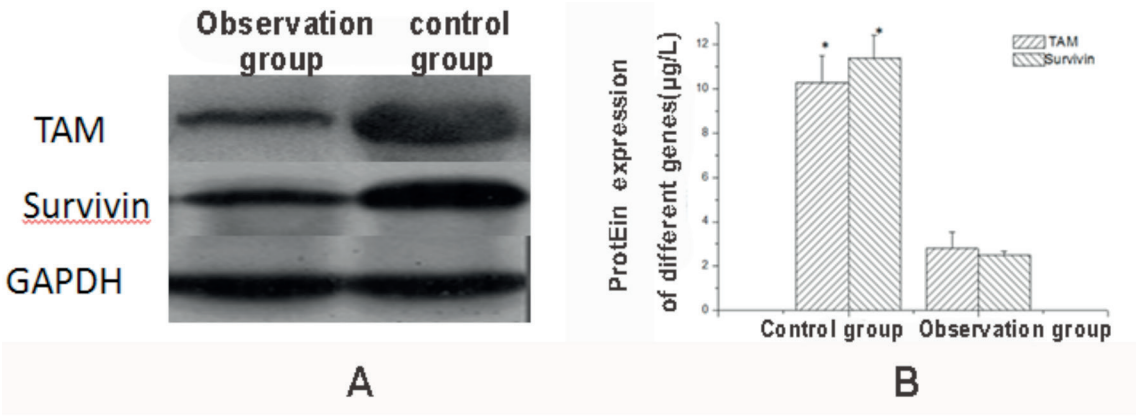

Figure 5. The expression of TAM and survivin in the observation group and the control group was detected with Western-blotting. ***A,*** Western-blotting detection result. ***B,*** Western-blotting semi quantitative results. "*" shows significant difference ($p<0.05$).

Table II. Immunohistochemical positive cell number in the observation group and control group.

Group	Total cell count	Number of positive cells	Positive cell rate (%)	Number of negative cells	Negative cell rate (%)
Control group	400	365	91.25	35	15.5
Observation group	400	62*	8.85*	338*	84.5*

Note: *p shows significant difference, **p shows a marked significant difference.

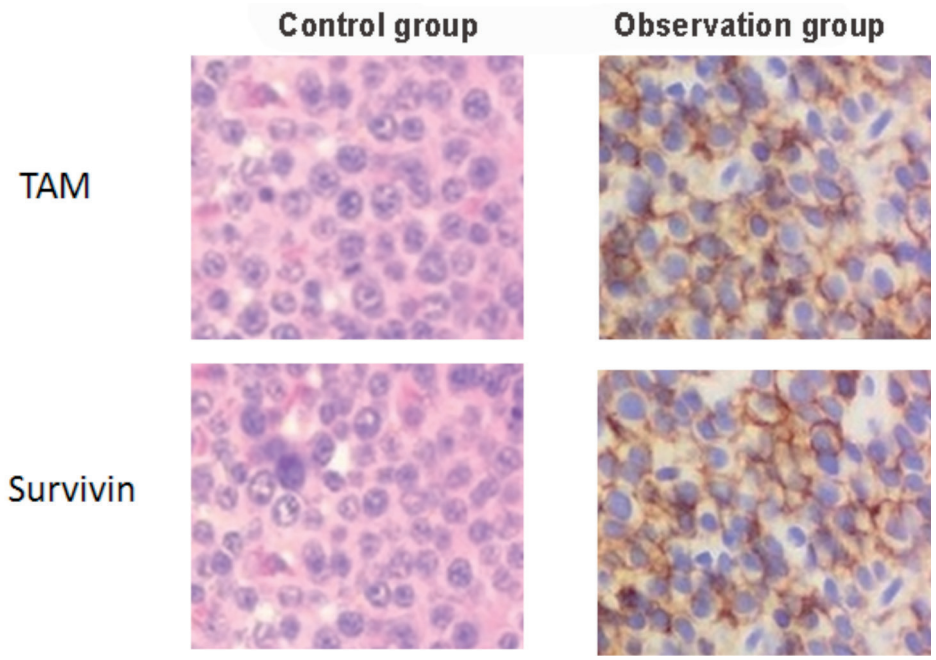

Figure 6. Immuno-histochemistry in the observation group and control group. Pink indicates positive cells, purple represents negative cells.

Tumor Size Measurement in the Observation Group of Different Treatment Time

In the observation group, the measurement of the size of the tumor tissue of mice, was analyzed regarding the different time of cisplatin treatment (Table III). With the prolongation of the time of drug treatment, the tumor tissue volume showed a gradual trend of decrease. There was

Table III. Tumor size in the observation group of different treatment time.

Treatment time	0	1	4	9	14	18	22	28
Tumor tissue size (square centimeter)	9.35	7.25	5.32	3.28	1.54	1.01	0.67	0.63*

Note: *p shows significant difference, **p shows an extreme significant difference.

a significant difference in the tumor size from 1 h to 14 h of treatment ($p<0.05$) while, after 22 h of treatment, the tumor size change was not significant.

Discussion

Studies have indicated that osteosarcoma is mainly due to the intermediate tissue lesions of the mesenchymal tissue, but it is not clear about the pathogenesis of osteosarcoma[12]. In recent years, with the development of molecular biology, some progresses have been made in the study of osteosarcoma[13]. Data from research have shown that[14] the most of the tumor pathogenesis, osteosarcoma tumor cells in the human body are a result of some mutations, apoptosis and disorder of cell proliferation. In this process, the role of apoptosis-related signaling pathway decreases, resulting in the proliferation of osteosarcoma cells in the human body. This abnormal proliferation of cells largely destroys the balance of the body inside the cell[15]. Due to the cell apoptosis and the decreased related to regulation and control function, the gene mutation frequency was increased in the process of proliferation[16]. Additionally, activation of some proto-oncogenes or methylation of anti-oncogene promoter often result in loss of function of tumor suppressor gene and increase of proto-oncogene. This can lead to the tumor cells generation and abnormal proliferation in cell. As an important regulator of apoptosis, the inhibitor of apoptosis protein, which is found in recent years, plays an important role in the process of apoptosis. The results of the study indicate that the apoptosis-inhibiting protein can inhibit many kinds of genes such as bcl-1 family protein in the human body[17]. It can inhibit the transcription and translation of oncogenes by the co-action with transcription factors and regulate the process of apoptosis of some cancer cells. As an important member of apoptosis protein, TAM and survivin have been proved to play important roles in the pathogenesis of breast cancer, ovarian cancer and colon cancer[18,19]. For example, studies have shown that survivin gene in breast cancer cells can, through NK-KB signaling pathway, be involved in the inhibition of cancer cell group protein translation process. Also, due to the histone proteins in DNA replication and cell proliferation in the process has an important role in regulation. Therefore, survivin gene can inhibit cancer cell proliferation process through the regulation of cell histone synthesis. Also, for example, the results show that[20] survivin can improve the permeability of mitochondria and other organelle membrane, promote the release of mitochondria and promote the apoptosis of protein - cytochrome C. It has been shown that there is a strong correlation between tumor formation and blood vessels. For example, results[21] showed that VEGF can promote the expression of survivin gene in vascular endothelial cells, and its expression is significantly higher than that of normal vascular endothelial cells, although it has been demonstrated that TAM and survivin are associated with colon cancer, lung cancer, and other cancer-related[22]. However, the mechanism of TAM and survivin in the treatment of osteosarcoma is not clear. In our study, we found that cisplatin treatment could significantly inhibit the expression of TAM and survivin gene in tumor tissues, and it has been proved that TAM and survivin genes are closely related to apoptosis. In our study, we first found that cisplatin treatment could inhibit TAM and survivin expression and significantly reduce the size of tumor lesions. For example, compared with the control group, the size of the tumor tissue in the mice treated with cisplatin was significantly decreased. This indicated that cisplatin treatment could promote the necrosis of osteosarcoma tumor tissue. In our study, we observed osteosarcoma lesions size with medication in mice for different days, and the lesions gradually had a significant difference; that was due to the extension of time for drug. Tumor lesion tissue decreased gradually, consistent with earlier results, but in the later stage of the treatment, the correlation between tumor decrease or necrosis and the time of drug taking was gradually reduced. This showed that

cisplatin for the treatment of osteosarcoma had a certain time effect, and this was the reason because multiple drugs were used in the current clinical treatment of osteosarcoma by cisplatin. Then, we found that the effect of cisplatin on the treatment of osteosarcoma was significantly correlated with the expression of TAM and survivin in the cells. Further, the results showed that with the increase of TAM and survivin expression. The necrosis of osteosarcoma tissue increased, indicating that the treatment of cisplatin for osteosarcoma was mainly to improve the expression of TAM and survivin in the cell. However, due to the experimental conditions and time constraints, this study does not have a deep study on the specific mechanism of cisplatin affecting the change of the expression of TAM and survivin, and this was the main direction of the follow-up study.

Conclusions

Based on the results of our study, it can be found that cisplatin treatment can inhibit the TAM and survivin gene expression in tumor cells to promote tumor necrosis and thus, help patients with cancer rehabilitation.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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