Expression of B7-H3 in cancer tissue during osteosarcoma progression in nude mice

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ABSTRACT. Immune cells might participate in the ontogenesis of osteosarcoma. B7-H3 is a new discovered T cell co-stimulatory molecule that was found to be overexpressed in malignant tumors. We aimed to investigate the dynamic expression level of B7-H3 in nude mice with osteosarcoma. A nude mouse osteosarcoma model was successfully established. B7-H3 expression and distribution changes in the early, middle, and late phases of osteosarcoma formation after tumor implantation were observed. Reverse transcription-polymerase chain reaction and western blot analyses were applied to measure the B7-H3 mRNA and protein dynamic changes. Confocal microscopy and immunohistochemistry were used to determine B7-H3 localization and CD3+ T cell expression, respectively, in osteosarcoma tissue. B7-H3 mRNA and protein levels fluctuated during the process of osteosarcoma formation in the nude mouse model. Expression levels were lower in the early and middle phases, while B7-H3 mRNA and protein were overexpressed in the late stage. Accordingly, CD3+ T cell numbers in the early, middle, and late phases in osteosarcoma tissue were 93 ± 13, 92 ± 12, and 46 ± 15, respectively; they can be seen to have decreased significantly in the late stage ($P < 0.05$). Overall, our results indicated that the B7-H3 expression level is correlated with tumor

volume and severity; therefore, it might serve as a tumor biomarker for osteosarcoma.

Key words: Osteosarcoma; Nude mice; B7-H3; T cell

INTRODUCTION

Osteosarcoma is the most common primary bone tumor. Though high-dose cytotoxic chemotherapy and surgical resection can improve prognosis, it still has high possibility of metastasis and recurrence. The exact pathogenic mechanism of osteosarcoma is still poorly understood. Recent studies have shown that immune cells might participate in osteosarcoma occurrence, development, and progression (Botter et al., 2014; Fleuren et al., 2014; Luetke et al., 2014). For example, programmed death 1 (PD-1), a receptor expressed on the surface of T cells, has been found to be overexpressed in the peripheral blood of patients with osteosarcoma; this is closely related to the progression of the disease (Zheng et al., 2014). Further animal experiments have confirmed that injecting specific T cells into osteosarcoma animal models can prevent disease recurrence and metastasis (Merchant et al., 2007). A series of *in vitro* and *in vivo* experiments have found that cytokines produced by T cells are also involved in disease progression, and can intervene the animal model under certain conditions (Loeb, 2009; Moore et al., 2010; Li et al., 2011; Segaliny et al., 2014).

Traditionally, lymphocyte function is regulated by major histocompatibility antigens and costimulatory molecules; the latter are mainly composed of members of the B7/CD28 family including B7-H1, PD-1, B7-DC, ICOS, ICOSL, B7-H3, and B7-H4 (Chapoval et al., 2001). B7-H3 is a newly discovered molecule with 28% homology to the other B7/CD28 molecules. To date, its role in cell biology is still controversial. It has been reported that B7-H3 can present positive synergetic stimulation in the immune system and promote T cell proliferation to induce TH1 cell generation and increase the activity of cytotoxic T cells (Sun et al., 2002). Other studies have found that it exhibits negative synergetic stimulation to inhibit the proliferation of activated T cells, thus inhibiting cytokine synthesis (Steinberger et al., 2004; Crispen et al., 2008; Wang et al., 2014).

Several studies have shown that B7-H3 is overexpressed in multiple malignant tumors including lung cancer and that its expression might relate to prognosis (Zheng et al., 2014). Recently, a series of studies have suggested that B7-H3 also has certain non-immune functions under specific conditions. For example, B7-H3 is associated with tumor size in a mouse hepatic cancer model and can be used for combined therapy (Sun et al., 2006; Roth et al., 2007; Zang et al., 2007; Zhang et al., 2008; Yamato et al., 2009; Zhang et al., 2009; Sun et al., 2010).

Until now, there have not been any studies regarding the role of B7-H3 in osteosarcoma. Here, we investigated the dynamic expression levels of B7-H3 mRNA and protein during different stages in a nude mouse osteosarcoma model by reverse transcription-polymerase chain reaction and western blot analyses. We also detected the distribution of B7-H3 and the CD3+ T cell number in tumor tissue to further clarify the role of B7-H3 role in osteosarcoma.

MATERIAL AND METHODS

Animals

In total, 40 BALB/c-nu/nu nude mice aged 4 to 6 weeks and weighing approximately 20 g were provided by the Chinese Academy of Sciences (Shandong). The mice were bred in an

aseptic laboratory at constant temperature and received sterile operations. Mice were used for all experiments, and all procedures were approved by the Animal Ethics Committee of our hospital.

Nude mice and tumor implantation

Human osteosarcoma cells from nude mice provided by Chinese Academy of Sciences were revived from liquid nitrogen. After digestion by trypsin and being washed with Hank's buffered saline solution, the cells were high speed centrifuged three times. The osteosarcoma cells were resuspended to 2.2 x 10^7/mL, and 0.2 mL aliquots were implanted into the necks of nude mice.

Observation

The general status of the mice was observed daily. Tumor volume was calculated as follows: V = length x width x 0.5. Osteosarcomas formed at least 1 week after inoculation in all mice, with volumes of about 40 cm^3 to obtain osteosarcoma tissue. Mice were sacrified by neck snap.

Western blot

Osteosarcoma tissue was washed using phosphate buffered saline and digested with 450 µL cell lysis solution (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1 mM PMSF, 1 mM EDTA, 5 µg/ml Aprotinin, 5 µg/ml Leupeptin, 1% Triton x-100, 1% Sodium deoxycholate, 0.1% SDS, 7M urea, 2M thiourea and proteinase K) (Zhongzhi biotechnology, China). Total proteins were separated on 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis gels (Bio-Rad Laboratories, Berkeley, CA, USA) and transferred onto nitrocellulose membranes (Millipore Corp., Bedford, MA, USA). Membranes were probed with B3-H7 (1:250, Millipore Corp.) or β-actin (1:1000, Santa Cruz Biotechnology, Dallas, TX, USA) antibodies, followed by horseradish peroxidase-tagged secondary antibody. Specimens and first antibody was incubated overnight at 4°C, and secondary antibody was incubated at room temperature for 2 h.

RT-PCR

Total RNA was extracted from the 2 g osteosarcoma tissues and reverse transcribed to cDNA for PCR amplification in according with kit instruction (Life Technologies, USA). The PCR primers were as follows: B7-H3 sense, 5'-AGC ACT GTG GTT TGG TAT CTG TCA G-3'; anti-sense, 5'-CAC CAG CTG TTT GGT ATC TGT CAG-3'; β-actin sense, 5'-GGT GTG ATG GTG GGT ATG GGT-3'; anti-sense, 5'-CTG GGT CAT CTT TTC ACG GT-3'. The cycling conditions in according with kit instruction (Life Technologies, USA) consisted of an initial, single cycle of 5 min at 94°C, followed by 30 cycles of 60 s at 94°C, 60 s at 60°C, and 3 min at 72°C. PCR products were tested by 1% agarose gel electrophoresis. Weused Primer 5.0 software to design the primers and analyzed data by an optimized comparative Ct (ΔΔCt) value method.

Confocal microscopy

Confocal microscopy (Nikon, Japan) was used for osteosarcoma specimen scanning. The secondary antibody labeled with PE emits green fluorescence, whereas DAPI emits blue fluorescence which indicates the cell nucleus. After fusing the B7-H3 and DAPI images, red

fluorescence could be observed at the location of the blue light, indicating that B7-H3 was expressed primarily in the nucleus.

Immunochemistry

To prepare specimens for immunohistochemistry mice were euthanized and the tumors were fixed in formaldehyde and embedded in paraffin. The tissue sections (5-7u) were baked for approximately 1 h and dewaxed. After being blocked by a 3% H_2O_2 solution for 30 min, 10% citric acid was used for antigen repair. The slices were then blocked with normal rabbit serum for 30 min, to which CD3+ antibody (1:100) was added and the sections were incubated overnight at 4°C. After being rewarmed for 30 min, the slices were washed with phosphate buffered saline, and rabbit anti-goat IgG secondary antibody was added and the slides were incubated at room temperature for 1 h. Mould avidin horseradish enzyme marker chain working liquid was then added to the slice and incubated at 37°C for 30 min. After DAB colorization, hematoxylin redyeing, and neutral balsam mounting were performed. The slices were observed by following method to determine numbers of CD3 + T cells. We used a grid system, the lattice is 1 cm2, which was mounted on a 10X eyepiece and divided into 4 small squares. Per sections randomly counted CD3+ T cells numbers in 10 fixed boxes in the view at high magnification. Numbers were adjusted to cm2.

Statistical analysis

All statistical analyses were performed using SPSS17.0 software (SPSS, Chicago, IL, USA). Numerical data were presented as means and standard deviation (±SD). Differences between means were analyzed using one-way ANOVA or paired *t*-tests when necessary. $P < 0.05$ was considered to indicate a statistically significant result.

RESULTS

B3-H7 RT-PCR

Real time PCR showed that B3-H7 was expressed both in the early and middle phases of osteosarcoma formation. However, expression reached a maximum during the late phase. Notably, B3-H7 mRNA expression was closely related to tumor volume. In other words, the higher the expression level, the larger the associated tumor volume (Figure 1).

B3-H7 protein results

Our analyses indicated that B3-H7 protein was expressed both at the early and middle phases of osteosarcoma formation. However, expression reached a maximum during the late phase (Figure 2). Our results suggested that B3-H7 protein expression was closely related to tumor volume. As for B3-H7 mRNA, the higher the expression level, the larger the associated tumor volume.

B3-H7 location in osteosarcoma tissue

Confocal microscopy was applied for osteosarcoma slice scanning, and B7-H3 was found to be primarily distributed in the nucleus (Figure 3).

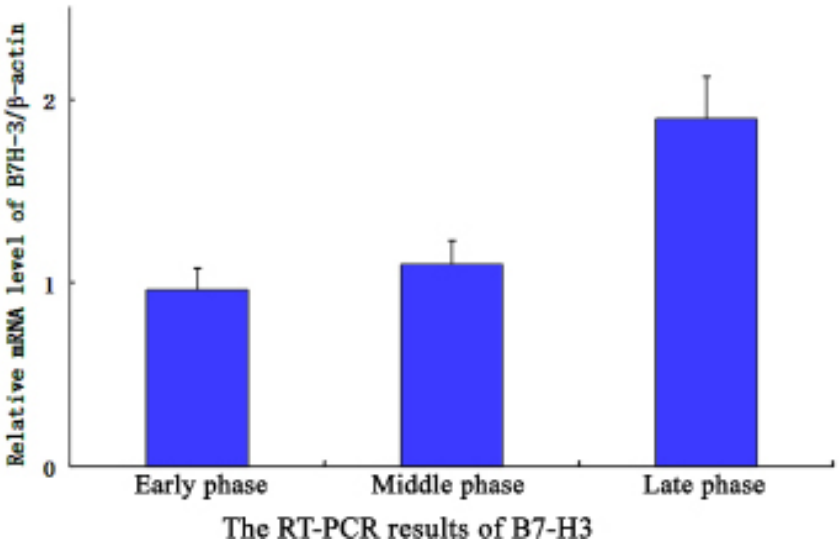

Figure 1. Relative *B3-H7* mRNA expression levels. Tumor volumes were 10, 15, and 40 cm^3 in the early, middle, and late phases, respectively.

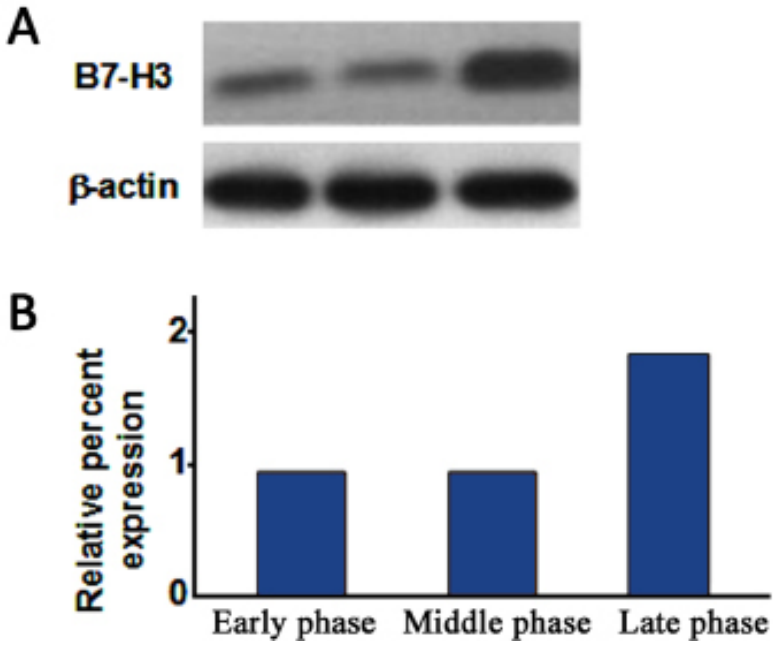

Figure 2. B3-H7 protein expression levels. Tumor volumes were 10, 15, and 40 cm^3 in the early, middle, and late phases, respectively. **A.** B7-H3 protein Western blot result. **B.** The semi-quantitative results of B7-H3 protein.

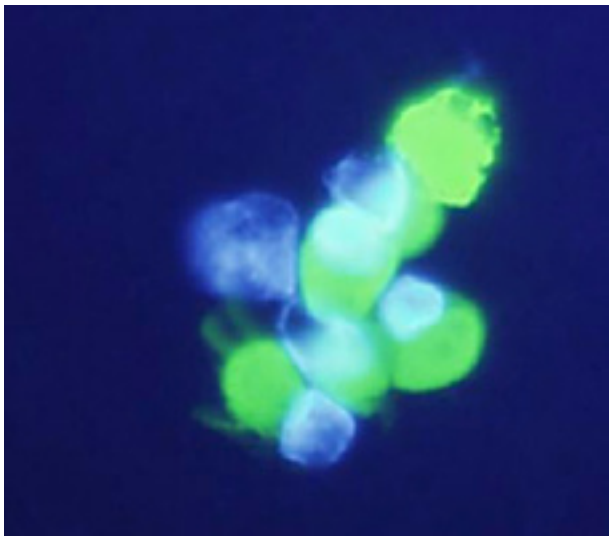

Figure 3. B3-H7 localization in osteosarcoma tissue. B7-H3's position in osteosarcoma. Green fluorescence indicated osteosarcoma cells, blue fluorescent indicated B7-H3.

CD3+ T cell expression in osteosarcoma tissue

CD3+ T cell numbers in osteosarcoma tissue in the early, middle, and late phases were 93 ± 13, 92 ± 12, and 46 ± 15, respectively. The cell number decreased significantly during the late stage ($P < 0.05$) (Figure 4).

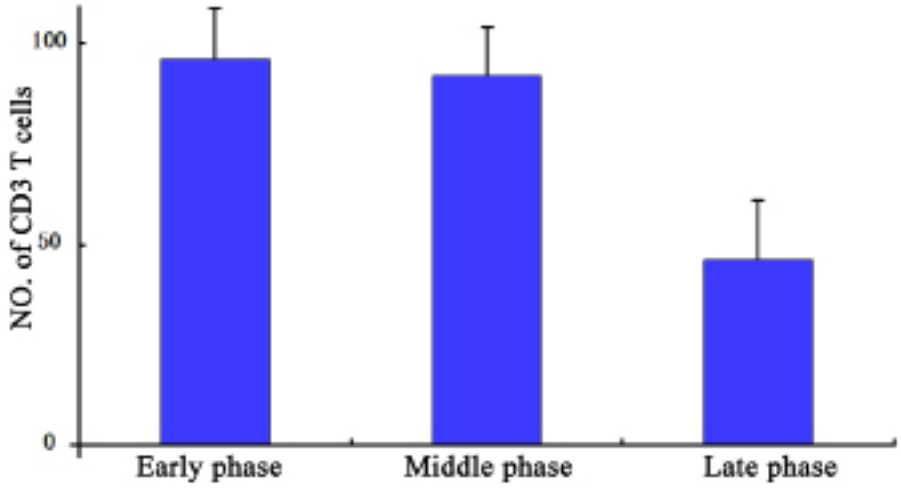

Figure 4. CD3+ T cell quantification results.Expression of CD3+ t cells in bone sarcomas. Columns indicated the number of CD3+T cells.

DISCUSSION

Lymphocytes play a central role in the process of tumor immunity, which is regulated by major histocompatibility antigens and costimulatory molecules; the latter is mainly composed of members of the B7/CD28 family including the newly discovered B7-H3 protein (Chapoval et al., 2001; Yi and Chen, 2009; Loos et al., 2010). Previous studies have shown that B7-H3 is expressed in numerous organs at the level of transcription, whereas it exhibits limited expressed in normal osteoblasts, fibroblasts, a fraction of epithelial cells, and in activated lymphocytes. Its expression, however, has been shown to be increased in tumor tissues. Our study provides the first demonstration that both B7-H3 mRNA and protein are overexpressed in an osteosarcoma mouse model. In addition, our results also revealed that the expression levels of B7-H3 fluctuate during disease procession. B7-H3 mRNA and protein are expressed at lower levels during the early and middle phases, whereas they are overexpressed during the late stage. In recent years, B7-H3 expression has been found to be correlated with survival time and high recurrence rates in different cancers, such as renal clear cell carcinoma, prostate cancer, and cervical cancer. Its expression level has also been associated with the severity of clinical pathology (Sun et al., 2006; Roth et al., 2007; Zang et al., 2007; Zhang et al., 2008; Yamato et al., 2009; Zhang et al., 2009; Sun et al., 2010). Our results also suggested that B7-H3 expression levels are associated with tumor volumes, and might be related to prognosis in the clinic; however, this suggestion will require confirmation through large-scale clinical research.

T cell activation and proliferation depend on a dual signal; that is, the major histocompatibility complex antigen peptide complex on antigen presenting cells specifically combined with T cell receptors (TCR) to transmit the initial signal. The second signal is transmitted by members of the B7/CD28 family to determine whether T cell will be in a state to enhance, inhibit, weaken, or have no response. Costimulatory molecules of the B7/CD28 family, including B7-H3, play a very important role in determining the T cell immune response. In recent years, a large number of *in vitro* and *in vivo* experiments have confirmed that B7-H3 might be an inhibitory molecule that can be characterized by negative synergetic stimulation to inhibit activated T cell proliferation and cytokine synthesis (Chapoval et al., 2001; Yi and Chen, 2009; Loos et al., 2010). In addition, overexpressed B7-H3 has been negatively correlated with multiple malignant tumor prognoses (Zang et al., 2007; Zhang et al., 2008; Yamato et al., 2009; Sun et al., 2010). For example, a recent clinical study investigated the relationship of B7-H3 expression with the prognosis of patients with lung cancer and the degree of infiltration by CD3+ T lymphocytes. In that study, B7-H3 expression and CD3+ T lymphocyte infiltration degree were detected by immunohistochemistry, which found that B7-H3 was overexpressed and that B7-H3 expression was negatively correlated with patient survival and T cell invasion. Thus, B7-H3 exhibited a potential value in clinical application for lung cancer diagnosis and prognosis evaluation (Zhang et al., 2009).

In addition, it has also been suggested that increased B7-H3 content might inhibit T cell activity to facilitate tumor growth (Chapoval et al., 2001; Sun et al., 2002; Yi and Chen, 2009; Loos et al., 2010). Whereas our results did not include immunologic analyses, they suggested that B7-H3 was overexpressed in osteosarcoma tissue, which in turn promoted tumor cell proliferation. Thus, osteosarcoma tissue growth might be associated with B7-H3 expression level. Tumor cell proliferation, as a basic feature, is known to be influenced by many factors. We believe that overexpressed B7-H3 is likely to facilitate its binding with corresponding ligands, and thus activate cell proliferation related genes directly, or stimulate secretion of certain cytokines to

influence cell proliferation indirectly. Future research could be focused on the signaling pathways activated by B7-H3.

In addition, as a transmembrane glycoprotein, B7-H3 contains an extracellular domain, a transmembrane region, and intracellular domains. Our study found that B7-H3 primarily exists in the cell nuclei within osteosarcoma tissue, indicating it might play a role in protein transcription, and might also activate downstream signaling pathways in the cell nucleus. Other studies have found that microRNA29 participated in the B7-H3 posttranscriptional regulatory mechanism. In normal tissue, the expression level of microRNA29 is high, which was shown to reduce the half-life of B7-H3 and also to inhibit the B7-H3 translational process. MicroRNA29 expression was shown to be markedly decreased in colon cancer tissue, resulting in insufficient levels of miRNA29 to negatively regulate the B7-H3 posttranscriptional mechanism (Chapoval et al., 2001; Sun et al., 2002; Steinberger et al., 2004; Yi and Chen, 2009; Loos et al., 2010).

Recently, tumor biological therapy, which mainly includes gene therapy and immune therapy, has increasingly become a hot clinical topic. The latter is closely related to the T cell-mediated immune response (Chapoval et al., 2001; Luetke et al., 2014), in which costimulatory molecules play an important role. Our study confirmed that upregulated B7-H3 inhibited the activity and quantity of infiltrated CD3+ T cells, which alters the tumor microenvironment and promotes tumor proliferation. Furthermore, inhibition of B7-H3 on T cells also can down-regulate a variety of cytokines secreted by T cells such as IFN-α, IL-2, IL-17, and IL-10. Thus, B7-H3 might play an important role in tumor immunity. Our study also suggested that osteosarcoma cells might escape immune surveillance through B7-H3; for example, overexpressed B7-H3 might down-regulate T cell mediated antitumor immunity (Chapoval et al., 2001; Sun et al., 2002; Steinberger et al., 2004; Yi and Chen, 2009; Loos et al., 2010; Wang, Kang et al., 2014). Therefore, if we could reduce the increased B7-H3 level through molecular biology methods such as siRNA or gene knockout to maintain the antitumor immune function of T cells, it would provide a new strategy for osteosarcoma treatment.

Our study indicated that B7-H3 plays the role of a negative synergetic stimulating molecule in the process of tumor occurrence and development. It has a central role in the tumor cell immune escape mechanism by inhibiting the activation and proliferation of T cells to negatively regulate the immune response. Furthermore, B7-H3 expression is also closely related to tumor proliferation, invasion, and metastasis. Numerous tumors escape immune surveillance by down-regulating positive synergetic stimulators and up-regulating negative synergetic stimulating signals. Blocking the negative synergetic stimulating pathway can enhance anti-tumor immunity, and can be effected with traditional cancer treatments in clinical or preclinical studies (Chapoval et al., 2001; Sun et al., 2002; Steinberger et al., 2004; Yi and Chen, 2009; Loos et al., 2010; Wang et al., 2014).

Our experiments had several limitations, as our observations were limited to B7-H3 expression in a mouse model but not in clinical patients. In addition, the question of whether the overexpressed B7-H3 in osteosarcoma tissue is or functions the same as the normal B7-H3 still needs further verification.

In summary, this study demonstrated the presence of B7-H3 mRNA and protein expression in an osteosarcoma mouse model for the first time. Our results also showed the fluctuation of B7-H3 expression during the disease process. B7-H3 was expressed at lower levels in the early and middle phases, while it was overexpressed in the late stage. Accordingly, T cell numbers were decreased in the osteosarcoma tissue. Our research also revealed that the B7-H3 expression

level is associated with tumor volume. Thus, the level of B7-H3 expression might serve as an osteosarcoma marker, a proposition that needs further in-depth research.

Conflicts of interest

The authors declare no conflict of interest.

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