

B7-H3 protein expression in a murine model of osteosarcoma

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Abstract. Osteosarcoma is an aggressive type of bone tumor that commonly occurs in pediatric age groups. The complete molecular mechanisms behind osteosarcoma formation and progression require elucidation. B7-H3 is a protein of the B7 family that acts as a co-stimulatory molecule with a significant role in adaptive immune responses. The link between B7-H3 expression and its role in different types of cancer remains unclear. B7-H3 protein exhibits different functional roles in *in vivo* and *in vitro* conditions that remain controversial. In the present study, a murine model of osteosarcoma was successfully established using a modified protocol so as to easily obtain a low grade and metastatic form of osteosarcoma tissue without complication. Histological data showed that a less organized and highly proliferative mass of cells was observed in the osteosarcoma tissue. A higher expression level of B7-H3 protein was also observed at each advanced stage of osteosarcoma, which indicated the contributory role of the protein in the development of the primary and metastatic forms of osteosarcoma. Immunohistochemistry was performed, which showed that the overexpression of B7-H3 protein in the metastatic form of osteosarcoma may be associated with its migration and invasion.

Introduction

The structural framework of the human body is provided by the internal skeletal system. At birth, humans have a total of 270 bones. However, during the various developmental processes, certain bones fuse with one another and their number is reduced to 206 in adults (1). Osteosarcoma is a malignant tumor of the bones that occurs due to the abnormal

proliferation of osteoblast cells. Although it occurs in all age groups (2,3), it is more common in young adults and growing children. The survival rate of osteosarcoma patients is <20%, as they are often diagnosed at advanced stages due to a lack of definitive diagnostic techniques (4,5). Currently, the metastatic form of the disease is treated with chemotherapy, however patient responses to chemotherapy are not positive enough to ensure long overall survival times (6).

The molecular mechanisms behind osteosarcoma growth and metastasis are not fully understood (7). Previous studies have reported that the frequent alterations of tumor suppressor genes, such as tumor protein 53 and retinoblastoma 1, play a role in osteosarcoma (8-10). In recent years, the role of Wnt signaling has been revealed in osteosarcoma development (11). The expression of the B7-H3 protein is reported in numerous studies of malignant tissues of the human lungs, stomach, prostate and other organs (12-15). B7-H3 protein is a recently identified B7 family member whose roles remain controversial (16). A recent study revealed that B7-H3 protein expression is elevated in osteosarcoma and assists in tumor progression (16). Further studies on B7-H3 protein are necessary to identify its role in the primary and metastatic forms of osteosarcoma in order to assist in understanding the invasive nature of the tumor. The aim of the present study was to successfully develop a murine osteosarcoma model by injecting mice with K7M2 cells, which have the potential to cause osteosarcoma. The B7-H3 protein expression profile was then analyzed in the primary and metastatic stages of osteosarcoma.

Subjects and methods

Experimental animals. Female, 9-month-old, BALB/c mice (mean weight, 30 g) (Jackson Laboratory, Ben Harbor, ME, USA) were maintained under pathogen-free conditions in the Experimental Therapy Unit, Department of Orthopedics, The First Affiliated Hospital of Soochow University (Suzhou, China). All the animals (n=18) were kept under regular supervision as per the institutional guidelines. The protocol of the present study was approved by the Institutional Animal Care and Ethical Committee (The First Affiliated Hospital of Soochow University), which was formed for the specific purpose of this project. The animals were regularly provided with food and water, and handled according to the local ethical regulations.

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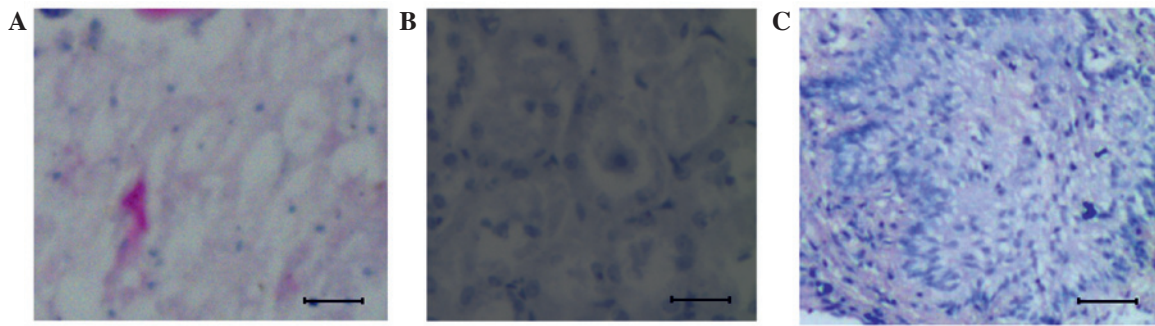


Figure 1. Histological appearance of normal and osteosarcoma tissues (hematoxylin and eosin stain). (A) Histological section of control bone tissue, which shows differentiated cells that are well organized. (B) Histological section of low-grade osteosarcoma (primary form) with enlarged nucleus. (C) Histological section of metastatic osteosarcoma showing a diffuse cellular pattern and the heavy replication of individual cells. Scale bar, 10 μ m.

Murine osteosarcoma model. The mouse model of osteosarcoma was developed by injecting K7M2 cells (dilution ratio, 10^4 cells/20 μ l) into the BALB/c strain. The K7M2 cells were obtained from American Type Culture Collection (Manassas, VA, USA), and stored at -130°C and 25% relative humidity. Cells were grown in Dulbecco's modified Eagle's medium (Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (Sigma-Aldrich, St. Louis, MO, USA) at 37°C and 95% relative humidity with 5–6% CO_2 . The exact site of injection was within the bone marrow space of the femoral bone. The BALB/c strain of mice was chosen, as these animals have a good ability to develop osteosarcoma (17). The BALB/c strain of mice developed spontaneous tumors and the metastatic form of osteosarcoma following the injection. The mice took 12 days to form the tumors, as in humans. Mice were sacrificed on the 12th and 20th days following injection with K7M2 cells, coinciding with the development of primary and metastatic forms of osteosarcoma. Of the six mice induced to form the primary tumor, all of the mice developed a primary tumor on 12th day and three mice developed tumors at two sites; therefore, a total of nine primary tumor samples were obtained. Of the six mice induced to form the metastatic tumor, five developed a metastatic tumor on 20th day and two developed metastatic tumors at two sites; therefore, a total of seven metastatic tumor samples were obtained. Control tissue samples were also obtained from the healthy thigh bones of 6 mice.

Western blot analysis. Samples from normal tissues, and from the primary and the metastatic forms of osteosarcoma tissue were dissected and cell lysate was prepared. The protein samples from the cell lysate were prepared (80 μ g/well) and resolved in a 12% gel for sodium dodecyl sulfate polyacrylamide gel electrophoresis, following the previously described protocol (18). The proteins were then transferred to PVDF membranes (Abcam, Cambridge, MA, USA), and the membrane was blocked with 5% milk in Tris-buffered saline with Tween 20. The primary antibody (monoclonal rat anti-mouse anti-B7-H3 antibody; catalog no. 135605; BioLegend, San Diego, CA, USA) was used at a 1:500 dilution and further developed with a secondary antibody [horseradish peroxidase-conjugated goat anti-rat immunoglobulin (Ig) G; catalog no., sc-2032; Santa Cruz Biotechnology, Dallas, TX, USA] at a 1:3,000 dilution. Diaminobenzidine (Abcam) was applied as the chromogen to visualize

the proteins. Monoclonal mouse anti-rabbit glyceraldehyde 3-phosphate dehydrogenase antibody (catalog no., ab8245; 1:500; Abcam) was used as the loading control. Protein signals were visualized using a microscope (Ti-S; Nikon Corporation, Tokyo, Japan) and signal intensity was analyzed using ImageJ software (National Institutes of Health, Bethesda, MD, USA).

Immunohistochemistry. For immunohistochemistry, the tissues were initially fixed with 10% formalin and paraffin-embedded. Next, slides with 5- μ m consecutive sections of tissue were deparaffinized with xylene and then hydrated. Endogenous peroxidase activity was inhibited by immersing the slides in freshly prepared 10% H_2O_2 and 10% methanol in 1X phosphate-buffered saline (PBS) for 30 min. In order to assess the target protein, the tissue sections were incubated with 0.1% trypsin in 0.1% CaCl_2 at 37°C for 10 min. The processed sections were incubated with mouse monoclonal rabbit anti-human anti-B7-H3 antibody (1:500; catalog no., SP206; Sigma-Aldrich) overnight in a humid chamber at 4°C . Subsequently, the sections were washed thoroughly with 1X PBS and successively incubated with a suitable secondary antibody (polyclonal horseradish peroxidase-conjugated goat anti-rabbit IgG; 1:4,000; catalog no., 6721; Abcam) for 1 h. After being washed, the sections were stained with diaminobenzidine, which was used as a chromogen. The sections were counterstained with Ehrlich hematoxylin (Sigma-Aldrich) to assist in locating the individual cells.

Results

Development of primary and metastatic osteosarcoma model. For formation of a murine osteosarcoma model, the standard protocol is for 4-month-old mice to be injected with aggressive K7M2 cells (10^6 cells/20 μ l). The injected mice then develop a spontaneous metastatic form of cancer on the 8th day (16). Using this protocol, it is difficult to differentiate and isolate the primary and metastatic forms of the disease due to the aggressive nature of injected K7M2 cells at a dosage of 10^6 cells/20 μ l. In the present study, the protocol was slightly modified by decreasing the dosage of K7M2 cells (10^4 cell/20 μ l) and by increasing the age of the mice (9 month old) so that the aggressive development of osteosarcoma was limited. Using this modified protocol, optimized and well controlled isolation of primary and metastatic forms of osteosarcoma was managed. The mice developed

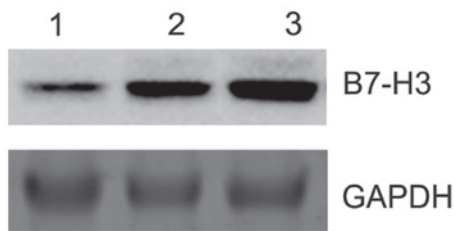


Figure 2. B7-H3 protein expression in normal tissue, and in the primary and metastatic forms of osteosarcoma. B7-H3 expression was validated by western blot analysis: Lane 1, B7-H3 protein expression in normal tissue; lane 2, B7-H3 protein expression in the primary form of osteosarcoma; and lane 3, B7-H3 protein expression in the metastatic form of osteosarcoma. For control purposes, glyceraldehyde 3-phosphate dehydrogenase was used as a loading control.

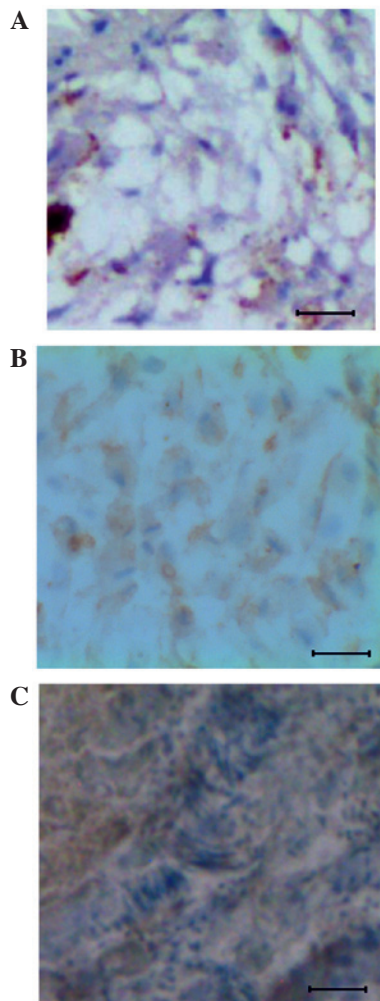


Figure 3. Validation of B7-H3 expression by immunohistochemistry. (A) Control tissue showing the weak expression of B7-H3 protein. (B) Primary tissues showing the moderate expression of B7-H3 protein. (C) Metastatic osteosarcoma tissue showing the overexpression of B7-H3 protein. Scale bar, 20 μ m.

the primary and metastatic forms of osteosarcoma on the 12th and 20th days, respectively.

Histological observation. In order to understand the cellular level changes that were taking place at the time of osteosarcoma formation in the mouse model following injection with

K7M2 cells (10^4 cell/ 20μ l), tissue samples from the 12th and 20th days post-injection were subjected to histological analysis. Results were compared against data obtained from the control mouse tissues. The data clearly showed that in the control, the bone tissues are well organized, as shown in the Fig. 1A. By contrast, in the mice injected with K7M2 cells, a primary form of osteosarcoma was present on 12th day (Fig. 1B) and a metastatic form of osteosarcoma on the 20th day (Fig. 1C). The primary form of osteosarcoma showed a less organized tissue structure, whereas in the metastatic form of osteosarcoma, the individual cells were heavily replicated.

B7-H3 protein expression in control tissues, and primary and metastatic forms of osteosarcoma. To examine B7-H3 expression, the protein samples were prepared from the control tissue, and the osteosarcoma tissues of primary and metastatic form, and then subjected to western blotting analysis using the antibody against B7-H3 protein. The expression pattern of B7-H3 protein in the control, primary and metastatic tissues is shown in Fig. 2A-C. The results were assessed based on the intensity of the signals obtained. When compared with the control tissue, the primary form of osteosarcoma exhibited 3-fold increased expression of B7-H3 protein. The metastatic form of osteosarcoma showed a 5-fold higher level of B7-H3 protein expression compared with the control and primary osteosarcoma tissues.

Overexpression of B7-H3 protein in the proliferative cells of osteosarcoma tissues. The contribution of B7-H3 protein in the formation of the primary and metastatic forms of osteosarcoma was studied by immunohistochemistry using the anti-B7-H3 antibody. For this study, control tissues and mouse osteosarcoma samples from the 12th and 20th day post-injection were subjected to immunohistochemistry. The results showed that the B7-H3 expression was restricted in the control tissue (Fig. 3A), whereas the expression was uniformly distributed among the cells at the primary osteosarcoma stage. It was also noted that the protein was highly expressed in the plasma membrane and cytoplasm of these cells (Fig. 3B). In the metastatic form of osteosarcoma, a higher expression level of B7-H3 protein was observed (Fig. 3C).

Discussion

The functional role of B7-H3 protein is controversial; two different theories exist, as it has been identified as with immunoinhibitory and immunoenhancing roles (19). Recent studies have linked the overexpression of the protein with a range of cancers, including lung (20), prostate (21), breast (22) and gastric (23) cancer, and osteosarcoma (16), while the knockdown of the protein is linked with other cancer types, for example, human oral squamous cell cancer (24). Therefore, research is required in order to provide further elucidation of the functions of the B7-H3 protein in different cancerous tissues, as well as to study its role in different model systems.

Osteosarcoma is a malignant tumor of the bone that mostly affects younger individuals. A good model system that mimics the disease in humans is necessary for performing research that assists in refining the knowledge in this field. Controversy exists with regard to the B7-H3 protein, with one debatable

point being the different responses in the *in vitro* and *in vivo* study of various cancer types (25). Mice have a natural ability to form osteosarcoma spontaneously (26) and exhibit useful responses after triggering the disease condition artificially. The present study was performed using the BALB/c strain of mice, which has the potential to form the primary and metastatic forms of osteosarcoma in the 12th and 20th days post-injection, respectively. This method of osteosarcoma formation assists in evaluating the failure of the immune system that supports osteosarcoma formation and progression.

In the present study, the histological data suggested that the well-organized structure of the tissues becomes disturbed with osteosarcoma progression (Fig. 1A-C). This particularly occurs in the metastatic form of osteosarcoma, where clumps of proliferative cancerous cells are observed on the 20th day (Fig. 1C), thus showing the aggressive nature of osteosarcoma development. The gradual increase in the expression of B7-H3 protein as the osteosarcoma progressed indicated that the disease progression is associated with the level of B7-H3 protein expression (Figs. 2 and 3). Also, this may suggest that it is directly or indirectly involved in the development of osteosarcoma. Wang *et al* (16) also proposed a possible association between osteosarcoma and B7-H3, stating that B7-H3 inhibits CD8⁺ T cell infiltration into the tumor tissue and thereby suppresses the development of tumor immunogenicity. The highest disease severity is achieved in the metastatic form of osteosarcoma and at that stage, significant overexpression of B7-H3 protein may support tissue dissemination. A previous study has suggested that the overexpression of B7-H3 protein may reduce the survival time of an affected patient to 5 years, and that this may be due to the worsening of immune system function as the disease progresses (16). Further research is required in this area to identify the functional aspect of this misbehavior.

In summary, the present study indicates that the primary and metastatic forms of osteosarcoma show varied histological characteristics. The data also supports that the costimulatory molecule B7-H3 elicits higher levels of expression as the disease progresses. Overall, this data may aid in developing a therapeutic intervention to access this type of cancer.

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