Prevalent expression of MHC class I chain-related molecule A in human osteosarcoma

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Received September 13, 2007

MHC class I chain-related molecule A (MICA) is one of the major ligands for activating immune-receptor NKG2D which is expressed on NK cells and cytotoxic T lymphocytes. The release and sustained expression of MICA protein can impair NKG2D-mediated cytotoxic activity by reducing NKG2D receptor on immune effector cells. The aim of the study was to investigate the expression and release of MICA in human osteosarcoma. RT-PCR, immunohistochemistry, western blotting and flow cytometry were used in analyzing the expression of MICA. Serum level of soluble MICA was quantitated by ELISA. Our data showed that MICA is prevalently expressed in osteosarcoma both in mRNA and protein level. Upregulation of MICA was found in osteosarcoma compared with benign tumors and normal bone tissues. Higher level of soluble MICA in serum can be detected in osteosarcoma patients. In conclusion, prevalent expression of MICA and higher serum level of soluble MICA may suggest a deficiency of MICA-NKG2D mediated immunosurveillance in osteosarcoma patients. Restoring the expression of NKG2D receptor on immune effector cells may contribute to a therapeutic strategy for human osteosarcoma.

Keywords: osteosarcoma; MICA; NKG2D; sMICA

Osteosarcoma is a common primary bone malignancy. Although efforts have been made on the exploration of chemotherapy and surgery, the 5-year survival rate is 20-30% for patients with metastasis or recurrences and has not be improved significantly over the last 20 years [1, 2]. In a murine model of osteosarcoma metastases, T cell immune responses played a critical role in determining whether metastases developed [3]. Although infiltration of cytotoxic CD8+ T-lymphocytes was observed in 68% osteosarcoma (24/35) by immunohistochemical studies [4], they failed to eradicate the tumor cells. Therefore, a more comprehensive understanding of tumor immune evasion is required for the treatment of osteosarcoma.

NKG2D receptor is expressed on NK cells, $CD8^+\alpha\beta$T cells, $\gamma\delta$T cells and a subset of $CD4^+\alpha\beta$T cells. Human NKG2D ligands (NKG2DLs) include the MHC class I chain-related molecule A (MICA) and B (MICB), and the retinoic acid early transcript-1 (RAET1) gene family, which used to be called the UL16-binding protein (ULBP) family [5]. The interaction between NKG2D and NKG2DL directly triggers NK cell cytotoxic activity and costimulates $CD8^+$ T cell cytotoxic activity [5, 6]. NKG2DLs are absent or show low expression on normal tissues. Up-regualtion of NKG2DL expression can be found in cells after stress suffering, bacteria/viral infection and neoplastic transformation. NKG2D-mediated cytotoxic response can eradicate transformed cells through the recognition to NKG2DLs. However, tumor cells have developed some mechanisms to evade such tumor immunosurveillance. It is reported that soluble MICA (sMICA) shedding from membrane binding MICA on tumor cells impaired NKG2D-mediated cytotoxitivity by reducing NKG2D receptor through internalization and degradation [7]. Sustained expression of NKG2DL in vivo also contributes to NKG2D downregulation and a defect in NK cells and $CD8^+$ T cells mediated cytotoxicity [8, 9]. Therefore, up-regulation of MICA protein and higher serum level of sMICA may suggest a defect in NKG2D-mediated immunosurveillace in patients with tumor.

To the best of our knowledge, no information about MICA expression and serum level of sMICA in osteosarcoma pa-

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Abbreviations: MICA, MHC class I chain-related molecule A; NKG2DLs, NKG2D ligands; sMICA, soluble MICA

tients is available. In the current study, we estimated MICA expression and serum level of sMICA in human osteosarcoma.

Materials and Methods

Patient clinicopathologic characteristics and tissues and cell lines. Sixty-six formalin-fixed, paraffin-embedded, and decalcified osteosarcoma specimens of bone, diagnosed by the department of pathology, Sun Yat-Sen University, Guangdong, China during 2003 to 2006 were studied retrospectively. Among them, 57 were needle biopsy specimens prior to neoadjuvant chemotherapy and 9 were surgical resection specimens. There were 40 males and 26 females overall. The age of the patients ranged from 8 to 52 years, with a mean age of 19.02±8.69 (Mean±SD) years. Eleven osteoblastoma, eight ossifying fibroma and six normal bone tissues were studied too. The histopathological diagnoses of all the specimens were reviewed by two pathologists, according to WHO 2002 on osteosarcoma classification standard. Patients' clinical characteristics were summarized in Table 1. Sera of 16 patients were obtained at the time of diagnosis prior to neoadjuvant chemotherapy. Informed consent was obtained from all patients. Human osteosarcoma cell lines Saos-2, U2OS, HOS and MG63 were purchased from American Type Culture Collection (Manassas, Virginia, USA) and the cell line OS732 was purchased from Jishuitan Hospital (Peking, China). All cell lines were cultured in Dulbecco's Minimum Essential Medium (Gibco) supplemented with 10%FBS in a humidified atmosphere of 5% CO_2 at 37°C.

Reverse transcription-PCR. Total RNA of fresh tumor tissues and cultured cells was extracted with TRIzol (Invitrogen) and transcribed to cDNA using commercial cDNA Synthesis kit (Fermentas). The following PCR primers designed to span more than 1 intron were used as follow [10]:

MICA sense 5'-GTGCCCCAGTCCTCCAGAGCTCAG-3',
antisense 5'-GTGGCATCCCTGTGGTCACTCGTC-3' (635 bp);

GAPDH sense 5'-CACTGACACGTTGGCAGTGG-3',
antisense 5'-CATGGAGAAGGCTGGGGCTC-3' (410 bp)

The resultant cDNA and primers were added to PCR premixture (Takara, Japan). For negative control, cDNA was replaced by distilled water. The conditions for PCR were: 5 min at 95°C, 30 cycles of 40 sec at 95°C, 1 min at 65°C, 1 min at 72°C, and 10 min at 72°C. PCR products were separated by electrophoresis in 1.5% agarose gel with ethidium bromide. HeLa cells, a human cervical carcinoma cell line, served as a positive control.

Immunohistochemistry. Immunohistochemical staining were performed on 5μm thick formalin-fixed deparaffinized tissue sections with goat polyclonal anti-MICA antibody (R&D Systems, Abingdon, UK, dilution 1:100). This antibody had been well characterized in other studies using similar conditions. All stains were based on the labeled streptavidin-biotin peroxidase method with microwave antigen retrieval for 10 minutes in 10mM citrate buffer (pH6.0). Endogenous peroxidase activity was blocked with 3% H_2O_2 in distilled water for 15 minutes. Sections were treated with normal non-immunized serum for 10 minutes, and then incubated with optimal concentration of primary antibody at 4°C overnight. In negative control sections, primary antibody was replaced by normal goat IgG or PBS. After rinsing, sections were incubated with appropriate biotinlated antibody and the HRP-conjugated Streptavidin, 15 minutes each at room temperature. Immune complexes were detected with diaminobenzidine-H_2O. Tissue sections were counterstained with Mayer's hematoxylin. Sections known to be positive MICA expression were used as positive control.

Immunohistochemical staining was evaluated independently by two pathologists using a scoring method based on intensity and percentage of the stained cells. The staining intensity was scored as: 0 (no staining), 1 (mild staining), 2 (strong staining). The percentage of the positive cells was scored as: 0 (<5%), 1 (5%~25%), 2 (26%~50%), 3 (>50%). Immunostaining score for each specimen was produced by adding the scores of staining intensity and percentage. The scores were graded as: 0, negative expression (-); 2, weak expression (+); 3 and 4, moderate expression (++); 5, strong expression (+++).

Western blotting analysis. Total cellular proteins were prepared using lysis buffer containing PBS, 1% NP40, 0.1% SDS,

Table 1 Clinicopathologic characteristics of osteosarcoma patients

Characteristics	N (%)
Age	
≤ 16 years	34
> 16years	32
Gender	
Male	40
Female	26
Histological subtypes	
Osteoblastic type	40
Chondroblastic type	10
Fibroblastic type	6
Mixed type	4
Telangiectatic type	5
Periosteal type	1
Histological grade	
Well differentiated	10
Moderate/Poor differentiated	56
Tumor sites	
Femur	30
Tibia	27
Radius	2
Humerus	4
Fibula	1
Ilium	1
Scapula	1
Metastasis	
Present	13
Absent	53
Recurrence	
Present	12
Absent	54

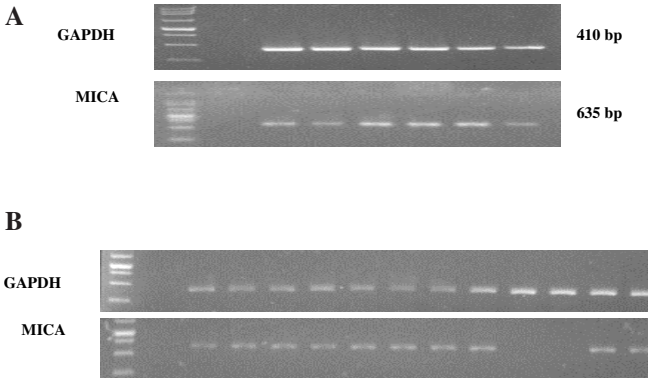

Figure 1. Expression of MICA mRNA in human osteosarcoma cell lines and human osteosarcoma tissues as examined by RT-PCR. Housekeeping gene GAPDH was tested as control. (A) From left to right: molecular weight markers; negative control, cDNA was replaced by water; positive control, Hela cells; Soas-2 cells; U2OS cells; MG63 cells; HOS cells and OS732 cells. (B) From left to right: molecular weight markers; negative control, cDNA was replaced by water; positive control, Hela cells; eleven osteosarcoma tissues.

5mM EDTA, 0.5% Sodium Deoxycholate, 1mM Sodium Orthovanadate and 1/100 PMSF. Protein concentration of lysates was measured with the Micro BCA kit (Pierce, Rockford, IL).The same protein amount from different samples were separated by 10% SDS-PAGE and were transferred to PVDF membrane (Amersham Pharmacia Biotech).The membrane was blocked in 5% nonfat dry milk in PBS containing 0.1%TWEEN 20 for 3 hr and inoculated with goat polyclonal anti-MICA antibody (R&D Systems, Abingdon, UK, dilution 1:750) at 4°C overnight. Normal goat IgG served as a negative control. After washing, the membrane was inoculated with peroxidase-conjugated anti-goat IgG for 1 hr at room temperature. Blot was detected with the enhanced chemiluminescence reagent (Cell Signal). HeLa cells were used as a positive control.

Flow Cytometry. To detect the surface MICA expression on osteosarcoma cells, they were harvested and washed for two times, then incubated with goat polyclonal anti-MICA antibody or goat IgG followed by FITC-conjugated rabbit anti-goat IgG and analyzed by a Beckman Coulter (Miami, FL) flow cytometer.

ELISA. The serum levels of sMICA were detected with commercial ELISA Kits((R&D Systems, Abingdon, UK), based on quantitative "sandwich" technique. Plates were coated with capture monoclonal anti-MICA antibody at room temperature overnight, and then blocked by BSA for 60 min at 37°C. After washing, the standard or diluted serum samples were added triplicatly and incubated for 90 min at 37°C. Then the plates were coated with detection antibody for 90 min at 37°C, followed by addition of streptavidin-HRP for 20 min at room temperature. Afterwards, plates were added substrate solution and incubated for 20 min at room temperature. Then, stop ELISA samples were read at a wavelength of 450nm with a wavelength correction of 540nm. A standard curve of the logarithmic relationship between concentration and absorbance was used to calculate the sMICA concentration in samples.

Statistical analysis

Statistical analyses were performed using SPSS 11.0 software (SPSS, Chicago, IL). Fisher's exact test was used to compare MICA expression among different categories. The statistically significant difference of serum level of sMICA between osteosarcoma patients and the healthy was assessed with Mann-Whitney text. The values $P<0.05$ were regarded statistically significant.

Results

MICA expression in osteosarcoma specimens and cell lines. MICA mRNA was examined in 5 osteosarcoma cell lines (Figure 1A) and 11 fresh-frozen surgical resection specimens of osteosarcoma (Figure 1B). All of the cell lines and nine osteosarcoma specimens consistently expressed MICA mRNA. To explore MICA protein expression on human primary osteosarcoma, immunohistochemical analysis was performed on 66 primary osteosarcoma specimens. MICA protein expression was heterogenous in several histological components of osteosarcoma. Immunoreactivity was detected in the cytoplasm of osteosarcoma cells with varied intensity (Figure 2A). The immunostaining pattern showed 12 (18.2%) for strong staining, 22 (33.4%) for moderate staining, 16 (24.2%) for weak staining and 16 (24.2%) for no staining (Table 3). Most osteoblastomas (Figure 2B) and total ossifying fibromas (Figure 2C) showed none or very weak immunostaining. No MICA expression was detected in 6 normal bone tissues (Figure 2D). Significantly higher MICA expression was found in osteosarcoma, compared with osteoblastoma (Table 2, Fisher's exact test, $p=0.01$), ossifying fibroma (Table 2, Fisher's exact test, $p=0.006$) and normal tissues (Table 2, Fisher's exact test, $p=0.026$). But, MICA expression neither correlated with recurrence, nor with metastasis(data not show). Surface MICA protein was investigated in five osteosarcoma cell lines by flow cytometry (Figure 3). The cell lines Saos-2, MG63 and HOS showed positive surface MICA expression, while the U2OS and OS732 showed very limited expression. To confirm the results of immunohistochemistry, we also performed Western blotting analysis on 9 fresh-frozen osteosarcoma specimens and Hela cells were used as a positive control (Figure 4). All of them including positive control showed a band of 55 kDa and five of them showed another band of 65 kDa too. These forms of MICA protein had been reported [11, 12]. The result of Western blotting analysis was consistent with immunohistochemistry with more sensitivity. Normal goat IgG served as a negative control and no immunoreactivity was detected.

Higher serum level of sMICA in OS patients. Sera from 16 osteosarcoma patients and 10 healthy controls were examined

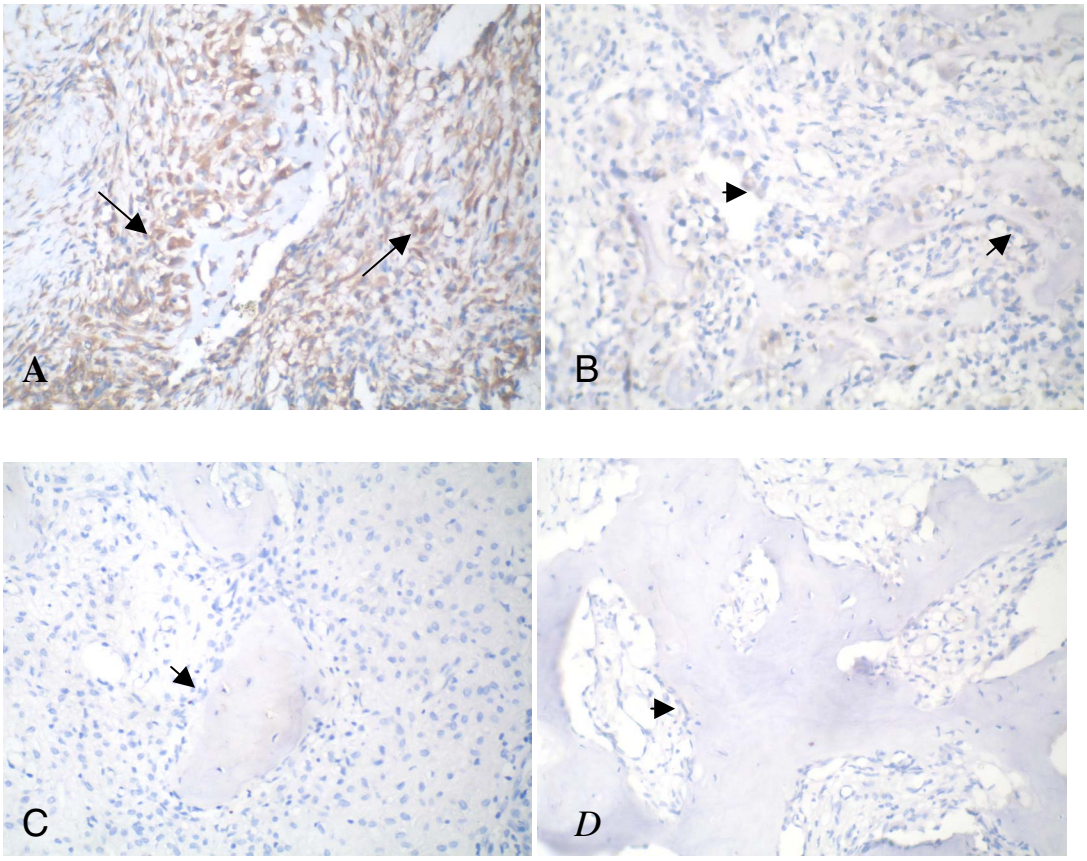

Figure 2. Immunohistochemical expression of MICA in normal bone tissues, ossifying fibroma, osteoblastoma and osteosarcoma. (A) Osteosarcoma showed strong cytoplasmic immunostaining of MICA (DAB ×200). (B) Osteoblastoma showed focal weak cytoplasmic immunostaining of MICA (DAB ×200). (C) Ossifying fibroma showed negative immunostaining of MICA (DAB ×200).(D)Normal bone tissues showed negative immunostaining of MICA (DAB ×200). Arrows indicated osteosarcoma cells and arrowheads indicated osteoblast cells.

Table 2 MICA expression and serum levels of sMICA in some osteosarcoma patients

Patients	Age	Recurrence	Metastasis	MICA mRNA	MICA Protein		sMICA (pg/ml)
					Western blot	Immunostaining	
p1	14	-	-	NT	NT	+	26
p2	21	-	+	NT	NT	+	92
p3	21	-	-	+	+	+++	0
p4	18	-	+	+	+	++	35
p5	24	-	+	+	+	+	46
p6	38	+	-	NT	NT	++	0
p7	19	-	-	+	+	+	0
p8	18	-	-	NT	NT	++	0
p9	23	-	-	+	+	++	0
p10	13	-	-	NT	NT	++	0
p11	33	+	+	+	+	+	0
p12	16	-	-	NT	NT	-	75
p13	9	-	-	NT	NT	+++	0
p14	12	-	-	+	+	+	0
p15	16	-	-	-	NT	-	0
p16	16	-	+	-	NT	-	0
p17	21	-	-	+	+	+++	NT
p18	10	-	-	+	+	-	NT

Serum level of sMICA was examined for sixteen patients (patients p1-16). NT, not tested.

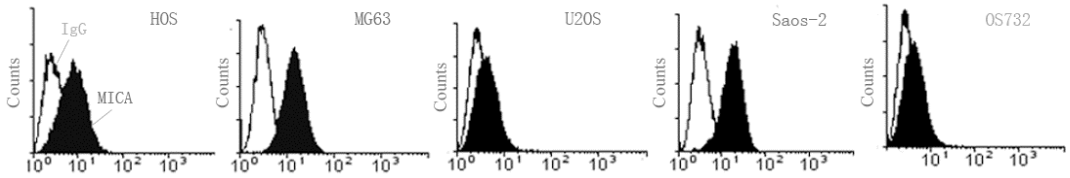

Figure 3. Flow cytometry analysis of surface MICA expression on osteosarcoma cell lines HOS, MG63, U2OS, Saos-2 and OS732. The cell lines HOS, MG63 and Saos-2 showed surface MICA expression, while the cell lines U2OS and OS732 showed very limited expression..

Figure 4. Western blotting analysis of MICA expression in six representative osteosarcoma tisssues out of nine analyzed. From left to right: positive control, Hela cells; Osteosarcoma tissues from patient 3-5, 7,9 and 17 showed positive MICA expression. The experiment was repeated for three times.

the sMICA by sandwich ELISA. sMICA was detectable in sera from 5 (31%) patients, ranged between 26 and 92pg/ml, with a mean of 54.80±27.80pg/ml (Mean±SD,). In these five patients, one had high differentiated osteosarcoma, the others had moderate/poor differentiated osteosarcoma. None of them had recurrence of thedisease and 3 of 5 patients had metastatic disease at the time their sera were tested. No detectable sMICA was found in health controls (lower than detection limit of 10pg/ml). The difference of serum sMICA level between patients and the healthy was statistically significant (Figure 5, Mann-Whitney Test, P=0.039). There was a trend that sMICA$^+$ osteosarcoma had low MICA expression and intended to develop metastasis, for 4 of 5 sMICA$^+$ osteosarcomas showed weak immunohistochemical staining for MICA and 3 of 5 sMICA$^+$ osteosarcomas had metastasis.

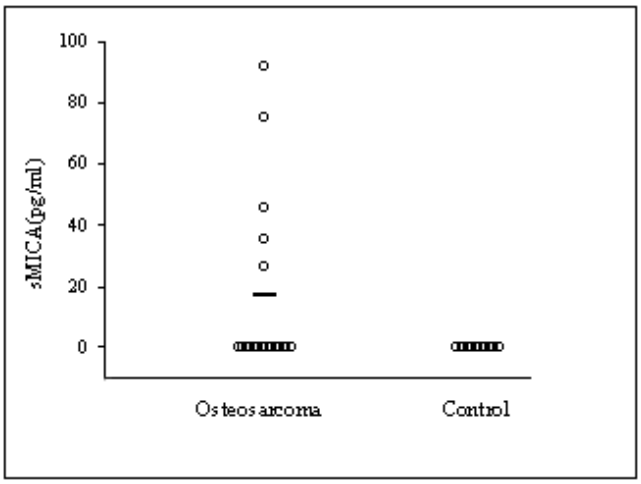

Figure 5. Serum level of sMICA in osteosarcoma patients (n=16) and the healthy (n=10). Data shown represents mean values of three independent experiments. Serum level of sMICA in osteosarcoma patients was significantly higher than that in the healthy (P=0.039).

Discussion

MICA gene locates in the human HLA locus, encoding type I transmembrane protein. MICA protein can be recognized by NKG2D receptor on immune effector cells. It had been well demonstrated that MICA constitutively expressed on many epithelial cancers, including colon cancer [13], prostate cancer [14], hepatocellular carcinoma [15], lung cancer [16], thyroid cancer [17], hematopoietic malignancy [18]. Although upregulation of NKG2DLs had been observed in experimental sarcomas in mouse [19, 20], no information about MICA expression on human sarcomas was available.

In the current study, we investigated MICA expression and serum level of sMICA in osteosarcoma. To our knowledge, this is the first time to show MICA expression in human osteosarcoma both at mRNA and protein level. Significantly higher MICA expression in osteosarcoma compared with benign bone tumors and normal bone tissues suggested that

Table 3 MICA immunohistochemical expression in osteosarcoma, benign bone tumors and normal bone tissues

Tissues	*n*	MICA expression				*p*
		-	+	++	+++	
Osteosarcoma	66	16	16	22	12	
Osteoblastoma	11	4	6	1	0	0.01*
Ossifying fibroma	8	6	2	0	0	0.006*
Normal bones	6	6	0	0	0	0.026*

*To compare MICA expression in different tissues, MICA expression was grouped as -/+ expression group and ++/+++ expression group, and Fisher's exact test was used.

MICA protein might associate with malignant transformation. The mechanism for MICA expression during cell transformation is poorly understood. It was reported that MICA expression could be triggered by some oncogenes, such as BCR/ABL oncogene [18] and adenovirus E1A oncogene [19]. Some DNA damage reagents can also induce MICA expression via DNA damage pathway [21]. Thus, anticancer activity of chemotherapy drugs may partly depend on the upregulation of MICA and it is interesting to investigate the relation between MICA expression and chemotherapy response in osteosarcoma patients.

Experimental evidences indicated that ectopic expression of MICA protein rendered tumor cells more susceptible to NKG2D-mediated cytotoxic activity and induced tumor rejection [10, 16]. However, MICA expression on tumor cells is a two-edged sword. Membrane surface MICA protein sheds to form sMICA which impairs anti-tumor immunosurveillance by inducing NKG2D receptor internalization and degradation [7]. This view is supported by accumulating clinical evidences that higher serum level of sMICA and lower level of NKG2D expression on NK cells and $CD8^+$ T cell with function deficiency are found in cancer patients [7, 8, 14–16].

In this study, sMICA in sera was detectable in some osteosarcoma patients, suggesting an impairment of NKG2D-mediated immunosurveillance in these patients. Moreover, MICA expression in $sMICA^+$ osteosarcoma was low, indicating that MICA shedding not only down-regulated NKG2D receptor on immune effector cells, but also diminished immuno-genicity of tumor cells by reducing MICA surface expression on osteosarcoma cells. Recently, it is reported that sustained NKG2DL expression in vivo also contributes to NKG2D down-regulation and a defect in NK cells and $CD8^+$ T cells mediated cytotoxicity [8, 9]. Kriegeskorte et al. [22] demonstrate that NKG2D ligand H60 and MICA can also mediate strong suppression on T cell proliferation. These findings might explain the observation that most tumor cells prefer to retain NKG2DLs. In our study, half cases of osteosarcoma tissues showed moderate to strong MICA expression. The constitutive expression of MICA and higher serum level of sMICA might suggest a deficiency in MICA-NKG2D mediated immunosurveillance in osteosarcoma patients. Further investigations of NKG2D expression in circulating peripheral blood mononuclear cells and tumor-infiltrating lymphocytes and their cytotoxic activity in $MICA^+$ osteosarcoma are urgently needed. The down-regulation of NKG2D can be restored after treatment with IL-2 or IL-15 [14]. These cytokines might be of clinical significance for eradicating $MICA^+$ tumor cells through enhancing MICA-NKG2D mediated cytotoxic activity.

In summary, this study described the prevalent expression of MICA and higher serum level of sMICA in osteosarcoma patients. The expression and release of MICA might be a new potential mechanism for osteosarcoma cells to evade immunosurveillance. Our data showed that $sMICA^+$ osteosarcomas (3/5) intended to develop metastasis. Owing to the limited number of cases with detectable sMICA in this study, further investigations are needed to know whether MICA or sMICA can be regarded as a marker of metastasis and survival in human osteosarcoma.

This study was supported by Chinese National Natural Science Foundation 30300354.

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