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Effect of Thermal Stress on MICA/B Induction in a Human Liposarcoma Cell Line

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ABSTRACT

Background: A possible mechanism by which hyperthermia enhances tumor immunogenicity is the induction of NKG2D ligands on tumor cells. Although the expression of MHC class I chain-related protein A and B (MICA/B) has previously been reported in different carcinomas, there is no information about MICA/B expression in liposarcomas. **Objective:** To investigate MICA/B induction in a human liposarcoma cell line (SW-872) after thermotherapy. **Methods:** SW-872 and HeLa cell lines were subjected to thermal stress for 1 h at 42, 44 and 46°C, and after 2, 4 and 6 h of incubation at 37°C, MICA/B expression was assessed at the mRNA and protein levels. **Results:** Despite high levels of MICA/B transcripts in SW-872 cells at baseline, the expression of these genes decreased significantly at both the mRNA and protein levels after almost all thermal treatments. **Conclusion:** Our data conclude that thermotherapy under 42-46°C had no effect on MICA/B induction on SW-872 liposarcoma cell line but the effects of fever-range temperatures remain to be tested on this cell line.

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Keywords: Hyperthermia, Liposarcoma, MICA/B

INTRODUCTION

Natural killer (NK) cells and cytotoxic T lymphocytes (CTL) are the main immune effectors of tumor cell clearance. Because defects in MHC class I expression are key factors in the immune evasion of tumors from CTL functioning, NK cell cytotoxicity can be helpful in the control of tumor growth (1,2). NKG2D is an activating receptor expressed on NK cells and some T cells (3). NKG2D ligands are stress molecules; for example, MHC class I chain-related proteins A and B (MICA and MICB) and UL16-binding proteins are mostly detected on malignant and virally-infected cells (4). Although T cells receive costimulatory signals through NKG2D molecules, the engagement of these

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molecules with their corresponding ligands is sufficient for NK cell activation (5,6). MICA and MICB expression has been detected in many tumors of epithelial origin such as ovarian, breast, lung, colon and kidney cancers (7). MIC molecules are induced by thermal stress in some tumor cell lines (8). Despite data based on the thermal tolerance of mammalian cells to moderate temperatures and cell toxicity at higher temperatures (9), hyperthermia is still a promising approach for controlling tumor cells (10).

In hyperthermia, both the temperature and the duration of heating are important (11). Due to its lower toxicity for adjacent normal cells, fever-range hyperthermia is considered more applicable in clinical trials, although some studies have used higher temperatures in tumor therapy (12). Thermosensitivity might be sustained for a variable period after thermotherapy depending on tumor type, pathologic grade, tumor size and location (13). Therefore, in vitro analyses of different tumors can predict tumor behavior in clinical trials. Although the expression of MICA/B has previously been reported in different carcinomas (7), there is no information about MICA/B expression in liposarcomas. The aim of the present study was to investigate MICA/B induction in a human liposarcoma cell line (SW-872) after thermotherapy.

MATERIALS AND METHODS

Cell lines. In the present study we used SW-872 (a human liposarcoma cell line) and HeLa (a human carcinoma cell line). HeLa cells were used as a positive control because the upregulation of MIC molecules were previously reported in these cells after thermal stress (8,14). Cells were cultured in RPMI 1640 (Biosera, Ringmer, UK) supplemented with 10% fetal bovine serum (Gibco BRL, Gran Island, NY, USA), penicillin (100 U/ml) and streptomycin (100 μg/ml).

Heat Treatment. Cell lines were grown at 37°C to more than 85% confluency, then half of the supernatant was replaced by fresh complete media and the cells were subjected to heat treatment at 42, 44, 46°C for 1 h in a CO_2 incubator (Binder, Tuttlingen, Germany). Immediately after heat treatment, the cells were transferred to 37°C and incubated for 2, 4, or 6 h. Then the cells were harvested by mild trypsinization and MICA/B expression was determined at the mRNA and protein levels.

Real-Time PCR Analysis. Total RNA was extracted from 1×10^6 cells with RNX-Plus (CinaGene, Tehran, Iran). RNA samples were treated with RNase-free DNase I (Fermentas, Vilnius, Lithuania) and then transcribed to cDNA with the RevetAid H Minus kit (Fermentas). Real-time PCR was performed in triplicate with SYBR Green (ABI, Foster City, CA, USA) to determine MICA/B mRNA levels, which were calibrated to GAPDH with the following forward and reverse primers respectively:

5′GGACCAGAAAGRAGGCTTGCATTCCC3′, 5′AGAGGAAGAGCTCCCCATCGT AGT3′ for MICA/B and 5′GGCTGGGGCTCATTTGCAGG3′, 5′AGTTGGTG GTGCAGGAGGCA3′ for GAPDH.

Real-time PCR conditions were: 10 min at 95°C, 40 cycles of 15 s at 95°C, 1 min at 60 C. After amplification, the melting curve was obtained by collecting fluorescence data while increasing the temperature from 60°C to 95°C over 1 min.

Flow Cytometric Analysis. To evaluate cell surface MICA/B expression, viability of the harvested cells was determined with trypan blue, and 0.5×10^6 cells were stained with PE-conjugated anti-human MICA/B monoclonal antibody (BD Biosciences, Santa Cruz, CA, USA) according to the manufacturers' recommended procedure. The same

process was also used for PE-conjugated mouse IgG2aκ as an isotype control (BD Biosciences). Samples were analyzed on a FACSCalibur flow cytometer (BD Biosciences) and the results were analyzed with Cell Quest Pro software (BD Biosciences, Franklin Lakes, NJ, USA). The average percentages of cells with MICA/B cell surface expression were determined in two separate experiments and reported as the mean ± SE.

Statistical Analysis. One-way ANOVA and unpaired Student's *t-tests* were used to compare each thermal state with the corresponding cell line under control conditions (at 37°C). All statistical analyses were done with SPSS 13.0 software and $p<0.05$ was considered significant.

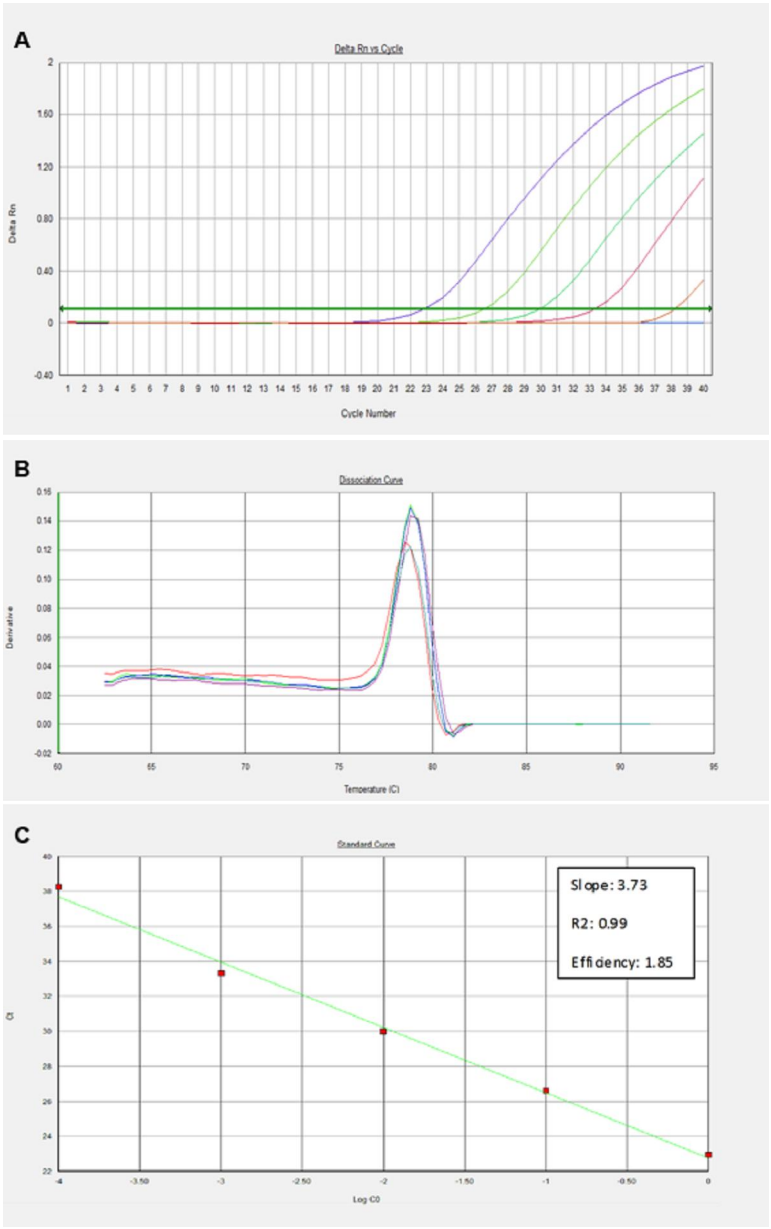


Figure 1. Validation of real-time PCR assay for MICA/B transcripts using serial dilution of cDNA (undiluted, 1:10, 1:100, 1:1000 and 1:10000) from the untreated HeLa cell line: amplification plot (A), melting curve (B) and standard curve (C).

Induction of MICA/B Transcripts by Heat Shock in Human Cell Lines. To investigate the effect of heat shock on MICA/B induction, tumor cells were treated with heat shock and total RNA was extracted and analyzed by real-time PCR.

Baseline mRNA levels of MICA/B at 37°C were significantly higher by 23-fold in the SW-872 cell line than in HeLa cells. Validation of the real-time PCR assay for MICA/B transcripts from the untreated HeLa cell line is shown in Figure 1.

Although real-time PCR analysis revealed significantly enhanced MICA/B mRNA expression approximately 4-6 h after incubation at 44°C in HeLa cells, the transcription of MICA/B seemed to be downregulated in SW-872 cells at all temperatures (Figure 2).

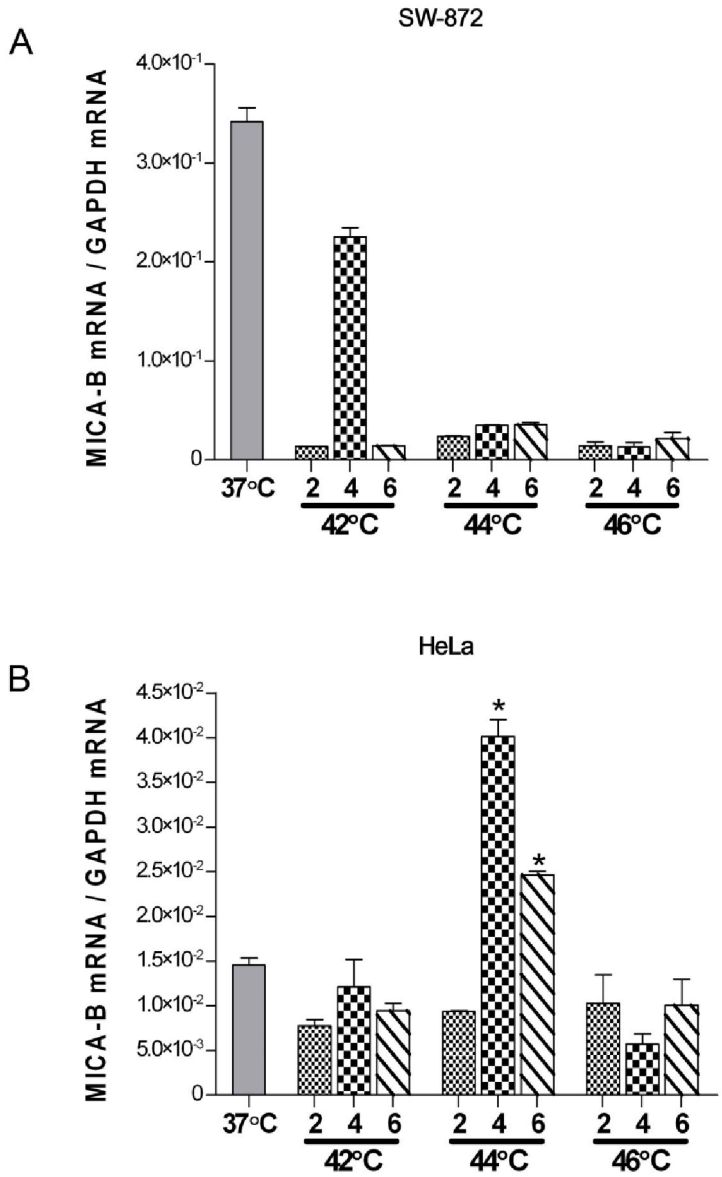
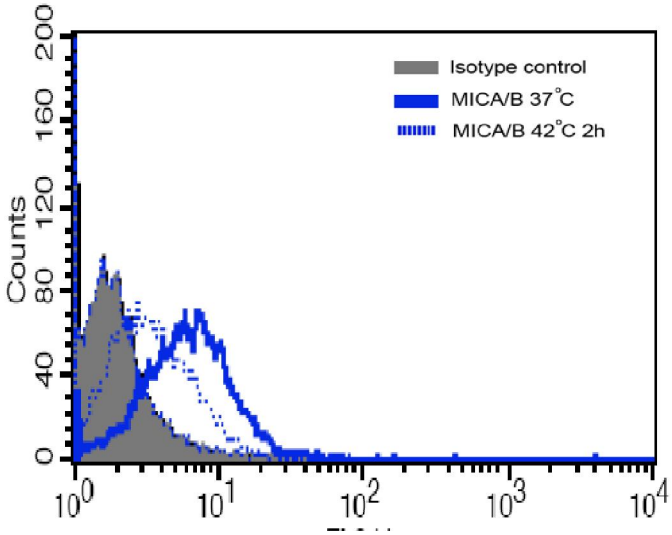
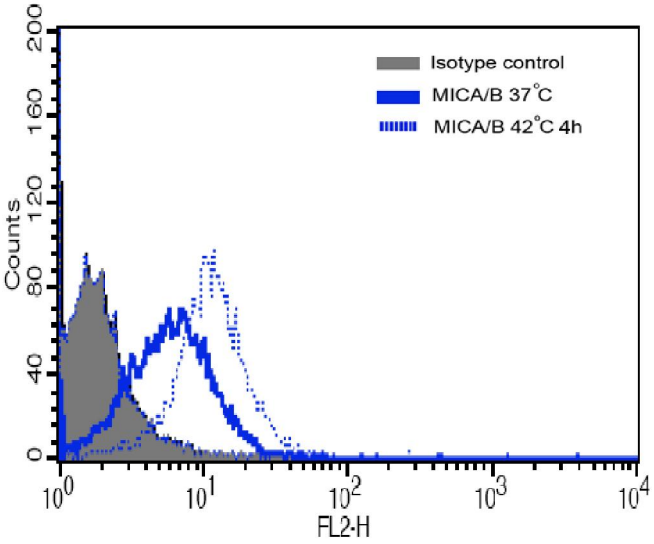


Figure 2. Induction of MICA/B mRNA in human cancer cell lines by thermal stress. SW-872 (A) and HeLa (B) cells were treated at 42, 44 and 46°C for 1 h and were recovered at 37°C for 2, 4 and 6 h. MICA/B mRNA levels were determined by real-time PCR using SYBR green and calibrated to GAPDH. Significant enhancements are marked with an asterisk.





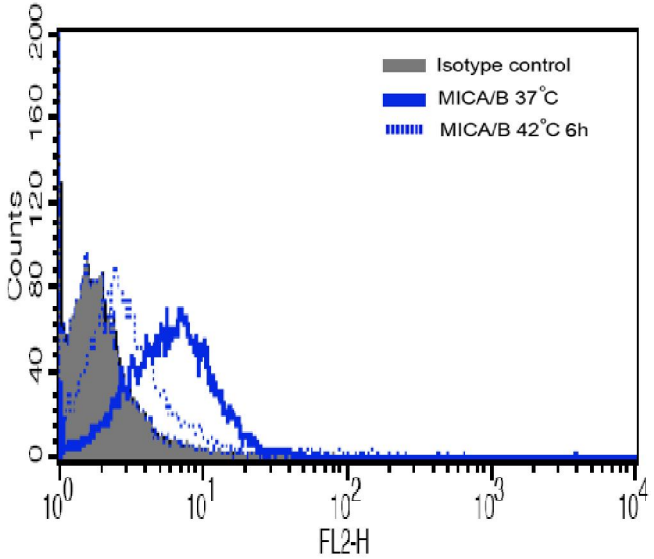


Figure 3. Comparison of MICA/B cell surface expression on heat-treated SW-872 cells and untreated SW-872 cells.

Cell Surface Expression of MICA/B after Heat Shock in Human Cancer Cell Lines. The levels of MICA/B cell surface expression were examined with flow cytometric analysis. Baseline cell surface expression of MICA/B did not differ between the two cell lines. The flow cytometric results showed high levels of basal MICA/B expression on the surface of both cell lines at 37°C; however, we found no significant enhancement in the cell surface expression of these molecules after thermal treatment. MICA/B surface expression increased in SW-872 cells 4 h after thermal stress at 42°C but this increase was not statistically significant in comparison to 37°C (Figures 3 and 4).

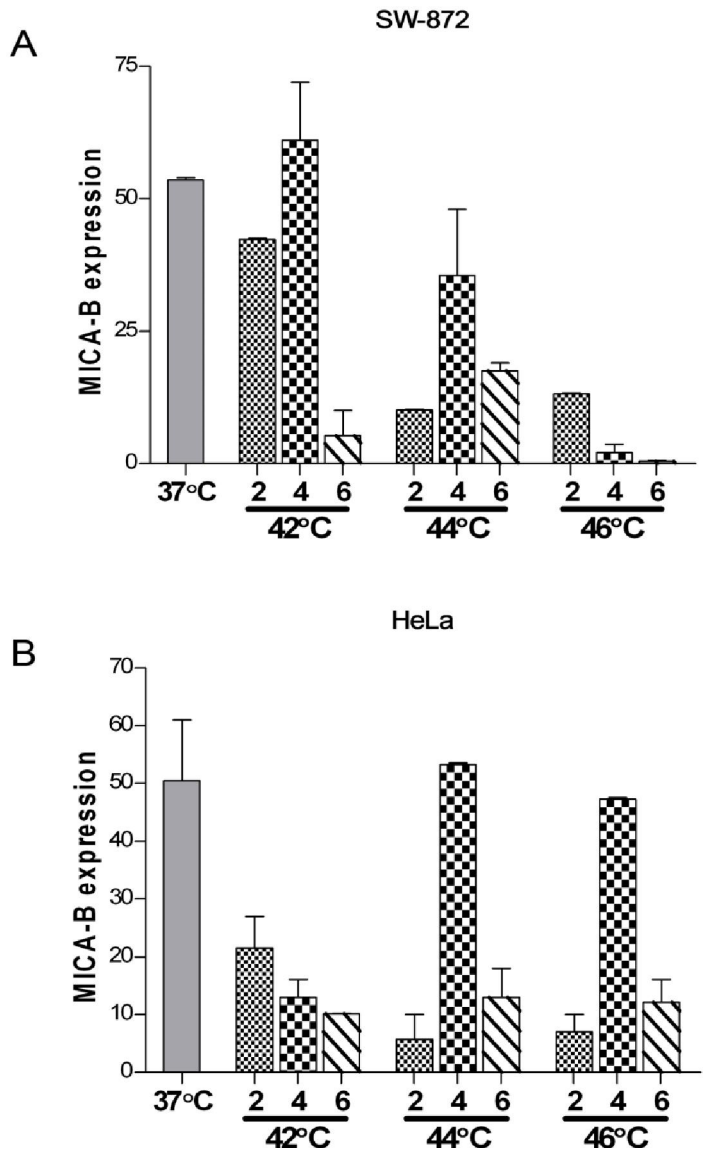


Figure 4. The percentage of MICA/B cell surface expression in human cancer cell lines after thermal stress. SW-872 (A) and HeLa (B) cells were treated at 42, 44 and 46°C for 1 h and were recovered at 37°C for 2, 4 and 6 h. MICA/B cell surface expression levels were determined by flow cytometry using PE-conjugated monoclonal antibody calibrated to the isotype control.

DISCUSSION

Identification of the NKG2D receptor has provided a molecular basis for NK cells as the first line of immune response against malignant diseases. Natural killer cell activation through NKG2D engagement can occur even in the presence of normal levels of MHC class I molecules on target cells (15). Furthermore, stress-induced upregulation of NKG2D ligands in tumor cells may trigger an additional signal that augments NK cell-mediated cytolysis (16). Nevertheless, tumor cells can escape from immune surveillance if the expression of NKG2D ligands and HLA class I molecules is defective. The role of HLA class I antigen abnormalities in tumor immune evasion has been well documented (17-19); however, the role of NKG2D ligand defects in tumor escape is less well understood.

As one of the oldest methods of tumor therapy, hyperthermia has been tested in clinical trials alone or in combination with other treatment modalities (20,21). Previous reports have shown that heat shock treatment leads to the upregulation of MICA/B, apparently because of the presence of putative heat shock elements in the 5'-flanking region of these genes (8,22,23).

In addition to the dose and duration of heat, the origin of tumor cells is an important factor in their heat responsiveness because the effects of hyperthermia vary in different types of tumors (24). To elucidate the response of human liposarcomas to hyperthermia, we investigated MICA/B inducibility in the SW-872 cell line at different temperatures and various recovery times after heat treatment. Our results showed that MICA/B transcription in SW-872 cells was much higher than in HeLa cells before thermal stress. Differences in the transcription levels of these genes may be due to the chromatin structure in regulatory regions (25).

The cell surface expression of MICA/B did not differ significantly between the two cell lines. The inconsistency between mRNA levels and the cell surface expression of MICA/B in these two cell lines suggests that additional mechanisms are involved in the regulation of gene expression after transcription (26,27). Increased levels of MICA/B mRNA were observed in HeLa cells 4 to 6 h after thermal stress at 44°C, although cell surface expression of these molecules showed no significant changes. In contrast to our results, Groh and Kim observed a correlation between MIC transcripts and cell surface protein expression when they subjected HeLa cells to thermal stress (8,28). This discrepancy may be due to differences in the thermal treatment protocols, or the use of a CO_2 incubator in this study versus water baths in others. Furthermore, we used real-time PCR to evaluate mRNA levels, whereas reverse transcriptase-PCR was used by other groups (8,28). The monoclonal antibody clone used to detect MIC molecules by flow cytometry in these studies was also different. Discrepancies between MICA/B mRNA levels and cell surface protein expression have also been reported by others (29). These findings probably reflect the regulation of gene expression at the post-transcriptional level (23).

Because MIC molecules are highly glycosylated (4), the accumulation of translated proteins in the Golgi apparatus and delayed cell surface expression can also be considered a probable reason for this difference. Moreover, MICA/B shedding may reduce the concentration of these molecules on the cell surface (30-32). Therefore, in this situation the simultaneous evaluation of cytoplasmic and membrane MICA/B molecules by flow cytometry can provide useful information. On the other hand, because soluble MIC molecules induce NKG2D internalization and degradation in effector cells (32), the determi-

nation of soluble MIC molecules in the culture supernatant after heat treatment may be useful to explain the effect of hyperthermia on NK cell activity against tumor cells.
Despite extensive research on MIC expression in human carcinomas (4), information about MIC expression in sarcomas is limited. The only report published to date involved patients with osteosarcoma in which, Lu et al. found an inverse relationship between tumor cell MICA molecules and serum concentrations of the soluble form of MICA (33).
Although we observed high levels of MICA/B mRNA in SW-872 cells at 37°C, the expression of these genes decreased significantly at both the mRNA and protein levels after almost all thermal treatments. Because the temperatures we tested here did not induce MICA/B cell surface expression, future studies in this cell line should test fever-range temperatures. Heat treatment for shorter durations may also be informative.

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