Sesamin induces cell cycle arrest and upregulation of NKG2D ligands in MG-63 cells and increases susceptibility to NK cell cytotoxicity

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Abstract. Osteosarcoma is a common solid malignancy in children and adolescents. Despite a success of standard therapeutic approaches, an effective therapeutic strategy is in a great need for improved outcomes. The aim of the present study was to investigate the anticancer effect of sesamin and its influence on NKG2D ligand expression in human osteosarcoma MG-63 cells and to compare the differences in NK cell elimination efficiency before and after treatment. Cell viability of MG-63 cells treated with serial concentrations of sesamin (0-100 μM) was measured using MTT. Induction of cell cycle arrest was determined with flow cytometry. Flow cytometry and reverse transcription-quantitative PCR were employed to detect the changes of protein and mRNA level of NKG2D ligands before and after treatment with sesamin. NK cell elimination assay was performed to determine the changes in NK cell-mediated cytotoxicity against MG-63 cells treated with sesamin. Sesamin induced G_2/M cell cycle arrest in MG-63 cells with increased p21 expression. Expression of MICA, MICB and ULBP1 at the protein and mRNA level were significantly increased (P<0.05). MG-63 cells treated with sesamin were more susceptible to NK cell-mediated elimination (P<0.05). Enhanced NK cell-mediated cytotoxicity was correlated with expression of NKG2D ligands (P<0.05). In conclusion, sesamin can induce cell cycle arrest and upregulate the expression of NKG2D ligands in MG-63 cells, thereby enhancing NK cell-mediated cytotoxicity against osteosarcoma cells.

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Introduction

Osteosarcoma is one of the most common malignant neoplasms of bone with rapid progression and poor prognosis. It is prevalent in children and adolescents. Common symptoms include pain at affected extremities, joint pain and a palpable mass upon examination. The current standard of care for osteosarcoma commonly involves surgery to remove the tumor and chemotherapy for metastatic disease. Combination of surgery with neoadjuvant and postoperative chemotherapy has revealed a 5 year survival rate of 68% in patients with primary osteosarcoma (1). Despite the success of combined treatments in primary osteosarcoma, the patients with pulmonary metastasis have poor prognosis with a 5 year survival of 33% (2,3). The development of treatments for osteosarcoma has been limited due to failures in optimizing existing treatments and unsuccessful discovery of new effective agents for last decades. Novel therapeutic strategies are required to fulfill the urgent clinical needs. Treatments harnessing immune system have gained attention for osteosarcoma owing to cellular signature and immune-features of osteosarcoma tumor (4). It is of interest to explore a therapeutic modality which combines anticancer agent and cellular immunotherapy.

Natural killer (NK) cells are a subgroup of large granular lymphocytes, which account for 5-15% of peripheral lymphocytes (5). NK cells play essential roles in immune responses against malignant transformation and viral clearance. The cells

recognize target cells through a complex set of activating and inhibitory receptors expressed on NK cell surface (6). Outcomes of NK cell cytolytic response to target cells depend on the balance between activation and inhibition signaling (7). NK group 2, member D (NKG2D) protein is one of NK activating receptors, which is expressed on NK cells, NK T cells and $CD8^+$ T. NKG2D recognize NKG2D ligands (NKG2DL) expressed on cancer cells, which include the MHC class I polypeptide-related proteins A (MICA), MICB and unique long 16 binding proteins 1 to 6 (ULBP1-6). Expression of NKG2DL is upregulated by stress situations such as viral infection or cancerous transformation (8,9). NKG2DL is expressed on a variety of cancer cells, suggesting NKG2DL to be putative targets for treatment (10). It is of interest to explore the use of potent tumoricidal agents which can increase the expression of NKG2DLs in a combination with NK cell infusion against osteosarcoma.

Sesamin is a predominant lignan in sesame seeds, which has been consumed in Asian countries for hundreds of years. It has been revealed to exert several pharmaceutical activities including anti-inflammation and anticancer activities (11). Sesamin has been demonstrated to inhibit cell proliferation through cell cycle arresting and apoptosis induction in various cancer cell types (12-15). Moreover, sesamin enhances anticancer effects of several chemotherapeutic agents on several malignancies (16,17). However, there is limited information about effects of sesamin on osteosarcoma and its use in combination with other therapeutic modalities.

It was hypothesized that NKG2DLs on osteosarcoma cells are inducible, leading to improved efficacy of NK cell-based immunotherapy for osteosarcoma. Modulations of NKG2DL expression mediated by sesamin in osteosarcoma were evaluated. The cytotoxicity of NK cells mediated by upregulated NKG2DL expression was examined.

Materials and methods

Cell culture. Human osteosarcoma cell line MG63 (CRL-1427) and human NK cell line NK-92 (CRL-2407) were purchased from the American Type Culture Collection. MG63 cells were cultured and maintained in Dulbecco's modified Eagle's medium (DMEM; cat. no. D6429; MilliporeSigma) supplemented with 10% fetal bovine serum (FBS; cat. no. F8192; MilliporeSigma) and 100 μg/ml penicillin/streptomycin (cat. no. P4333; MilliporeSigma) within a humidified atmosphere containing 5% CO_2 at 37˚C. Sesamin (cat. no. SMB00705; MilliporeSigma) was prepared in dimethyl sulfoxide at a concentration of 50 mM, stored as aliquots at -20˚C, and diluted with a cell culture medium for use. For treatments with sesamin, cells were treated with sesamin with serial concentrations in serum-free DMEM for 24 or 48 h. After treatments, resulting cells were washed with phosphate-buffered saline (PBS; pH 7.2) for following analyses. NK-92 cells were cultured and maintained in complete RPMI-1640 media (cat. no. R8758; MilliporeSigma) (10% FBS, 1% HEPES, 1% L-glutamine, 1% penicillin-streptomycin) and 300 U/ml recombinant human IL-2 (Proleukin; IOVANCE Biotherapeutics). NK-92 cells were activated by culturing in complete medium containing 500 IU/ml IL-2 and 50 ng/ml IL-15 (R&D Systems, Inc.) for 24 h. Activated NK-92 cells were harvested for following experiments.

Cell viability assay. Cell viability was evaluated using MTT assay. Cells ($x10^5$) in culture media treated with sesamin were incubated with (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide) (0.5 mg/ml) at 37˚C for 4 h. MTT solution was aspirated and the formazan crystals were dissolved in 200 μl DMSO. Number of viable cells was directly proportional to the production of formazan determined by measuring the absorbance at 570 nm.

Cell cycle analyses. After treatment with sesamin, $5x10^5$ of MG-63 cells were digested by trypsin, washed with PBS, and fixed with 70% alcohol on ice for 30 min. Cells were subsequently washed with cold PBS, suspended in 200 μl staining solution (PBS with 1% BSA, 10 mg/ml propidium iodide, 0.25 mg/ml RNase A) and incubated at 37˚C in the dark for 30 min. Resulting cells were analyzed using population of each phase of cell cycle, determined using BD FACS Canto II with the FlowJo v.10 software package (BD Biosciences).

Western blotting. After treatments, cells were lysed using lysis buffer (50 mM Tris-HCl, pH 7.5, 1% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride and 1 mM NaF). Cell lysates were centrifuged at 4˚C, 15,000 x g for 20 min. Supernatants were collected and assessed for protein concentration using the Bradford assay (cat. no. B6916; MilliporeSigma). A total of 20 μg of total protein was subjected to a 12.5% SDS-polyacrylamide gel electrophoresis followed by transferring onto a nitrocellulose membrane (MilliporeSigma). The resulting membranes were blocked with 5% w/v skimmed milk in PBS for 30 min at room temperature and then incubated for 2 h at room temperature with primary antibodies at a ratio of 1:1,000, which were against p21 (cat. no. MA5-31479), cyclin B1 (cat. no. MA1-155), cyclin-dependent kinase 1 (CdK1; cat. no. MA5-15631) and β-actin (cat. no. MA5-15739; all from Invitrogen; Thermo Fisher Scientific, Inc.), respectively. Blots were subsequently incubated with 1:2,000 dilution of horseradish peroxidase-conjugated secondary antibodies (cat. no. 31430; Invitrogen; Thermo Fisher Scientific, Inc.) for 1 h at room temperature and antigen-antibody complexes were displayed using ECL chemiluminescence (Millipore Sigma). The protein bands were quantified with the ImageJ software (version 1.54; National Institutes of Health).

RNA extraction and reverse transcription-quantitative PCR (RT-qPCR). Total RNA of cells after treatment was isolated using TRIzol® (MilliporeSigma) according to the manufacturer's protocol. Reverse transcription was performed using SuperScript IV VILO Master Mix (cat. no. 11756050; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. Quantification of mRNA levels of the genes of interest was performed using the ABI PRISM 7700 sequence detection system (Applied Biosystems; Thermo Fisher Scientific, Inc.) with designated primer pairs. FastStart Universal SYBR Green Master (Roche Applied Science) was used for qPCR. The thermocycling conditions for qPCR included initial denaturation at 95˚C for 5 min, 40 cycles of amplification (at 95˚C for 30 sec, 58˚C for 30 sec and 72˚C for 30 sec) and at 72˚C for 5 min. The threshold cycle numbers were calculated using the $2^{-\Delta\Delta Cq}$ relative value method (18) and normalized to GAPDH.

Table I. Primers used for reverse transcription-quantitative PCR analysis.

Gene name	Variant	Primer sequence (5'-3')
p21	NM_001374509.1	F: GAAGACCATGTGGACCTG
		R: GCGTTTGGAGTGGTAGA
Cyclin B1	NM_031966.4	F: CTTATACTAAGCACCAAATC
		R: GTTGGCTAAATCTTGAACT
CdK1	NM_001786.5	F: GACTAGAAAGTGAAGAGGAAG
		R: TACTGACCAGGAGGGATAGAA
MICA	NM_001177519.	F: GCCATGAACGTCAGGAATTT
		R: GACGCCAGCTCAGTGTGATA
MICB	NM_005931	F: TCTTCGTTACAACCTCATGGTG
		R: TCCCAGGTCTTAGCTCCCAG
ULBP1	NM_025218.4	F: CTTGACATTCAAGTGGAGAAT
		R: GTCCATTGAAGAGGAACTGCC
GAPDH	NM_002046.7	F: AATGGAAATCCCATCACCATCT
		R: CAGCATCGCCCCACTTG

CdK1, cyclin-dependent kinase 1; MICA, MHC class I polypeptide-related sequence A; MICB, MHC class I polypeptide-related sequence B; UPBL1, unique long 16 binding protein 1.

Pairs of primers used for genes of interest are listed in Table I. Each qPCR reaction was repeated at least three times.

NK cell elimination activities. A total of $5x10^3$ MG-63 cells were seeded into a well of the 96-well culture plates with complete DMEM media and incubated for 24 h, followed by treatments with or without sesamin for another 48 h. Resulting MG-63 cells were co-cultured with activated NK-92 cells at a range of effector: target (E:T) ratios (1:5, 1:3, 1:1, 3:1, 5:1 and 10:1) for 4 h at 37˚C with 5% CO_2. After 4 h of co-culture, NK-92 cells were removed and MTT assay was carried out to assess viable cells by measuring the absorbance at 570 nm.

ELISA. Concentrations of IFN-γ secreted by activated NK-92 cells in cultures with MG-63 cells were measured using ELISA according to manufacturer's protocol. The levels of IFN-γ in the culture medium was detected using IFN-γ ELISA Kit (R&D systems, Inc.).

Flow cytometry. A total of $1x10^6$ MG-63 cells were cultured with different concentrations of sesamin. The expression of the NKG2DLs was analyzed by immunofluorescence staining using anti-MICA (1:50; cat. no. MA5-36026; Invitrogen; Thermo Fisher Scientific, Inc.), anti-MICB (1:50; cat. no. MA5-29422; Invitrogen; Thermo Fisher Scientific, Inc.), anti-ULBP-1 (1:50; cat. no. MA5-38655; Invitrogen; Thermo Fisher Scientific, Inc.) and phycoerythrin. In all experiments, cells were stained at room temperature for 15 min with propidium iodide (1 μg/μl) to assess cell viability. Data acquisition and flow cytometric analysis were carried out on a BD FACSCanto II using the FlowJo v10 software package (FlowJo LLC).

Statistical analysis. Data are presented as the mean ± SD of the three independent experiments with conditions set up in three or six replicates. Comparisons were made by unpaired Student's t-test between two groups. One-way and two-way ANOVA were performed to assess the statistical significance of differences in measured variables between multiple groups with Tukey's post hoc test. Correlations between the protein expression levels with NK cell-mediated cytotoxicity were examined using Pearson's correlation analysis. All statistical analyses were conducted using SigmaPlot 10 (Systat Software, Inc.). $P<0.05$ was considered to indicate a statistically significant difference.

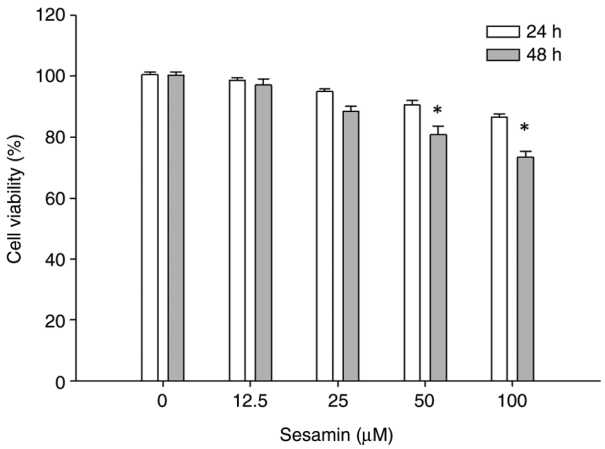

Figure 1. Effect of sesamin on the viability of MG-63 osteosarcoma cells. MG-63 cells were treated with sesamin at serial concentrations (0, 12.5, 25, 50 and 100 μM) for 24 and 48 h. Cell viability was measured using MTT assay. Experiments were performed in triplicate and the data were expressed as the mean ± standard deviation. *$P<0.05$ compared with 0 μM sesamin.

Results

Sesamin reduces cell viability of MG63 osteosarcoma cells. The effect of sesamin on growth of osteosarcoma cells was determined using MTT assay. MG-63 osteosarcoma cells were

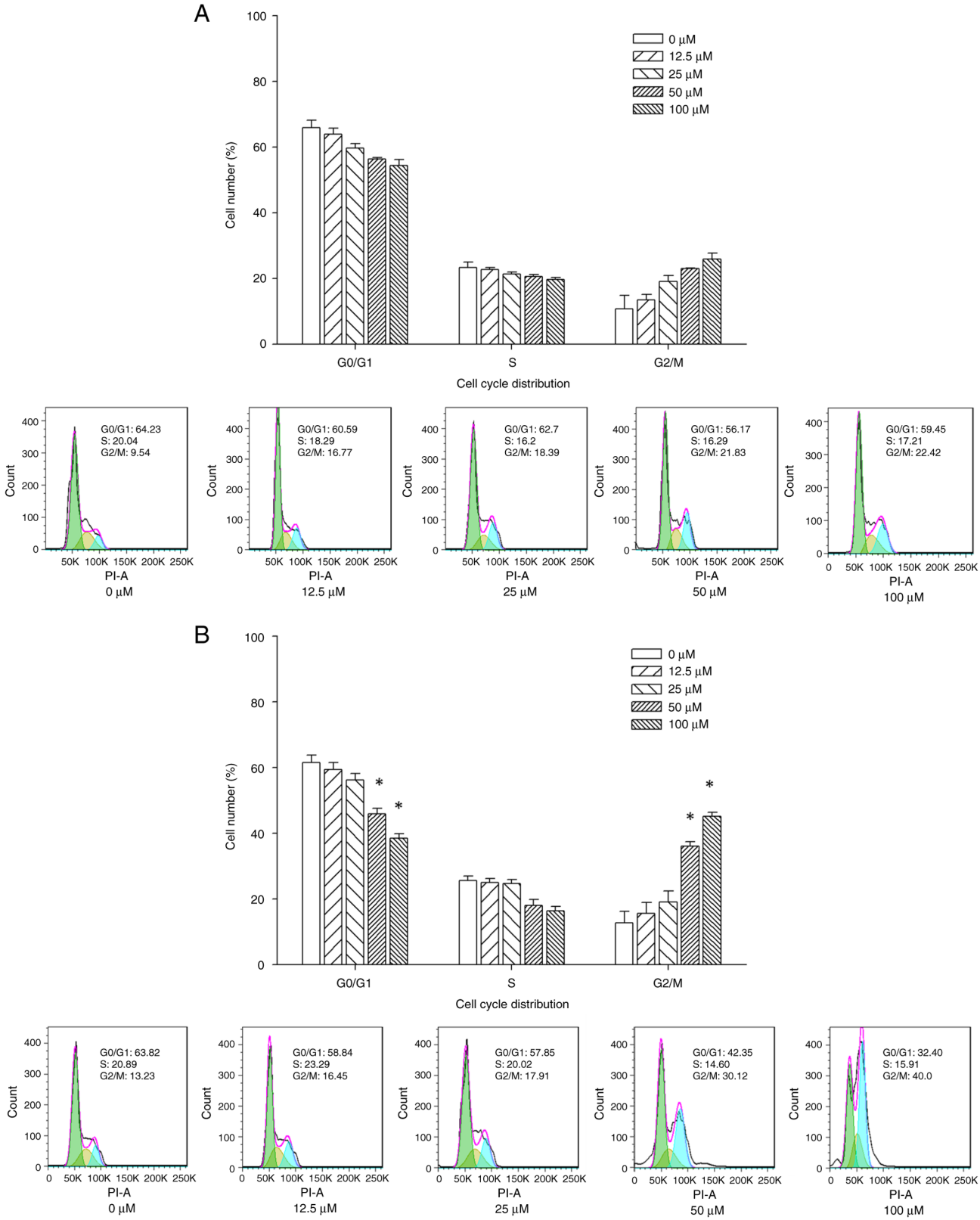

Figure 2. Induction of G_2/M cell cycle arrest in sesamin-treated MG-63 osteosarcoma cells. MG-63 cells were cultured with sesamin at indicated concentrations (0-100 μM) for 24 and 48 h. Distribution of MG-63 cells in the different cell cycle phase after treatments with sesamin for (A) 24 h and (B) 48 h was presented as the percentage of cells in each cell cycle phase. Results were expressed as the mean of three independent experiments ± SD. *P<0.05 vs. 0 μM sesamin.

treated at various concentrations of sesamin (0,12.5,25,50 and 100 μM) for 24 h or 48 h and measured for cell viability. No significant inhibitory effects were observed at 24 h. The cells responded time-dependently to sesamin treatment within the concentrations tested. The viability of MG-63 cells was decreased to 80.7±2.9 and 73.3±1.9% of controls in response

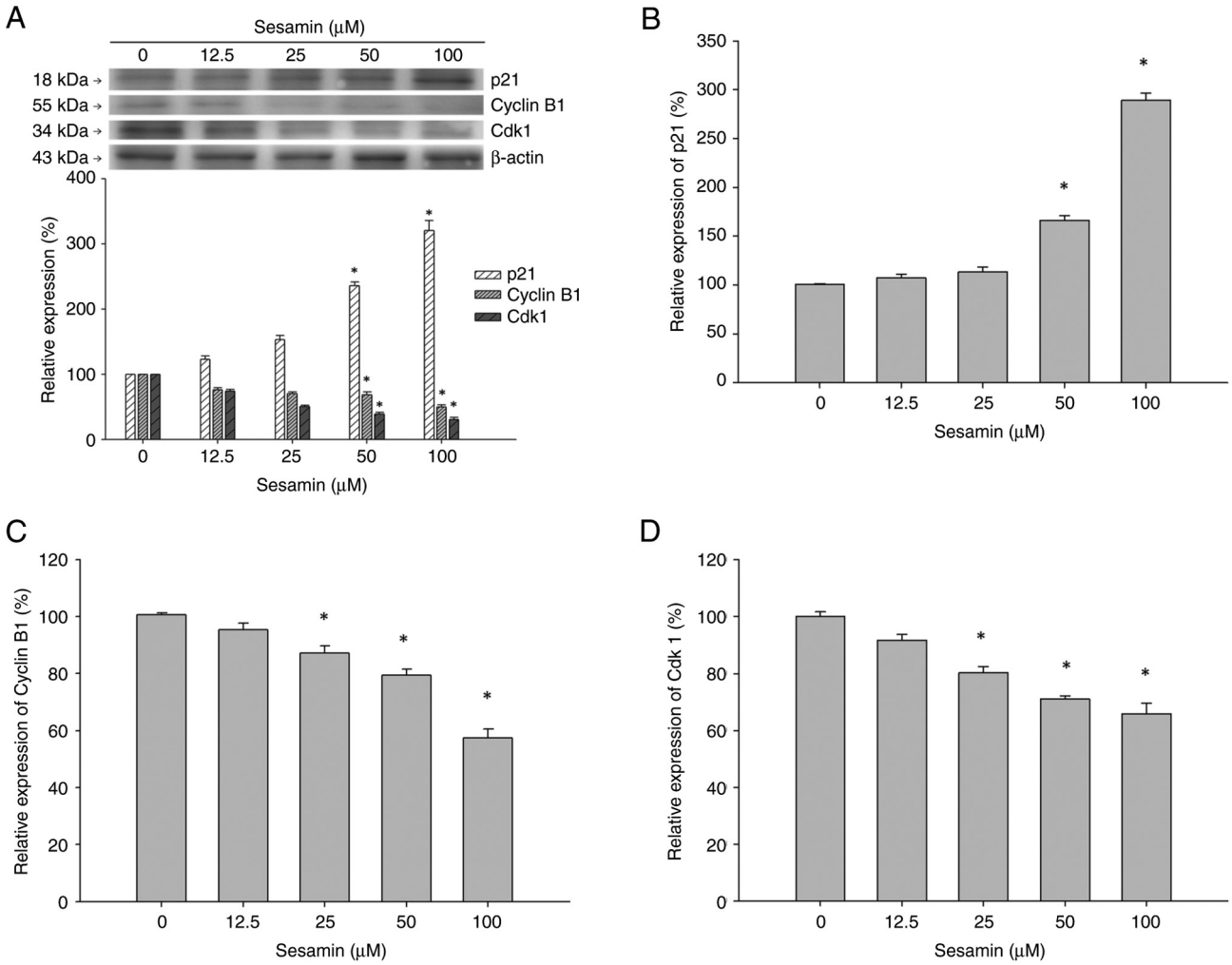

Figure 3. Effects of sesamin on the expression of cell cycle regulatory proteins in MG-63 osteosarcoma cells. MG-63 cells were treated with sesamin at indicated concentrations (0-100 μM) for 48 h. (A) Expression of cell cycle regulatory molecules at protein level in presence of sesamin were determined using western blot analysis. (B-D) mRNA levels of cell cycle regulatory molecules were measured by reverse transcription-quantitative PCR assay. Results were expressed as the mean of three independent experiments ± SD. *P<0.05 vs. 0 μM sesamin.

to the incubation with 50 and 100 μM of sesamin for 48 h, respectively (P<0.05) (Fig. 1). The doses of sesamin significantly inhibiting viability of MG-63 cells were chosen for subsequent experiments.

Sesamin induces cell cycle arrest at G_2/M phase in MG-63 osteosarcoma cells. The mechanism underlying sesamin-induced proliferation inhibition was investigated. Changes in cell cycle distribution in MG-63 cells treated with sesamin at designated concentrations were determined using flow cytometry. MG-63 cells exposed to sesamin at different concentrations for 24 h exhibited G_2/M cell cycle arrest (Fig. 2A). Treatment of MG-63 cells with 0, 12.5, 25, 50 and 100 μM sesamin for 48 h resulted in an increase in proportion of G_2/M phase cells from 12.8±3.5, 15.6±3.4, 19.2±3.2, 36.1±1.4 and 45.2±1.2%, respectively (Fig. 2B). The results revealed that sesamin induced G_2/M cell cycle arrest in a dose- and time-dependent manner.

To determine the molecular events involved in sesamin-induced cell cycle arrest, changes in expression of proteins involved in G_2/M phase including p21, Cdk1 and cyclin B1 in sesamin-treated MG-63 cells were assessed. The protein levels of p21, cyclinB1 and Cdk1 were detected using western blotting in MG-63 cells treated with 0, 12.5, 25, 50 and 100 μM sesamin for 48 h (Fig. 3A). Sesamin induced an increase in expression of protein p21, which is an inhibitor of Cdk/cyclin complex in MG-6 cells. The results revealed that sesamin inhibited the expression of cyclin B1 and Cdk1 at 48 h, which are involved in the G_2/M transition. The mRNA levels of p21, Cyclin B1 and Cdk 1 gene in MG-63 cells treated with sesamin for 48 h were validated using RT-qPCR. The mRNA expression of p21 was significantly upregulated in MG-63 cells treated with 50 and 100 μM sesamin (Fig. 3B). The mRNA levels of cyclin B1 and Cdk1 were significantly reduced in comparison with that of 0 μM MG-63 cells (Fig. 3C and D). The changes in p21, Cyclin B1 and Cdk 1 mRNA expression were parallel to that of the results of western blotting.

Sesamin increases NKG2D ligand expression in MG-63 osteosarcoma cells. As increased p21 expression has been revealed in association with expression of NKG2DLs, it was investigated whether sesamin has influences on the expression of the ligands for NKG2D in MG-63 cells. Expression of NKG2DLs in MG-63 cells treated with or without sesamin

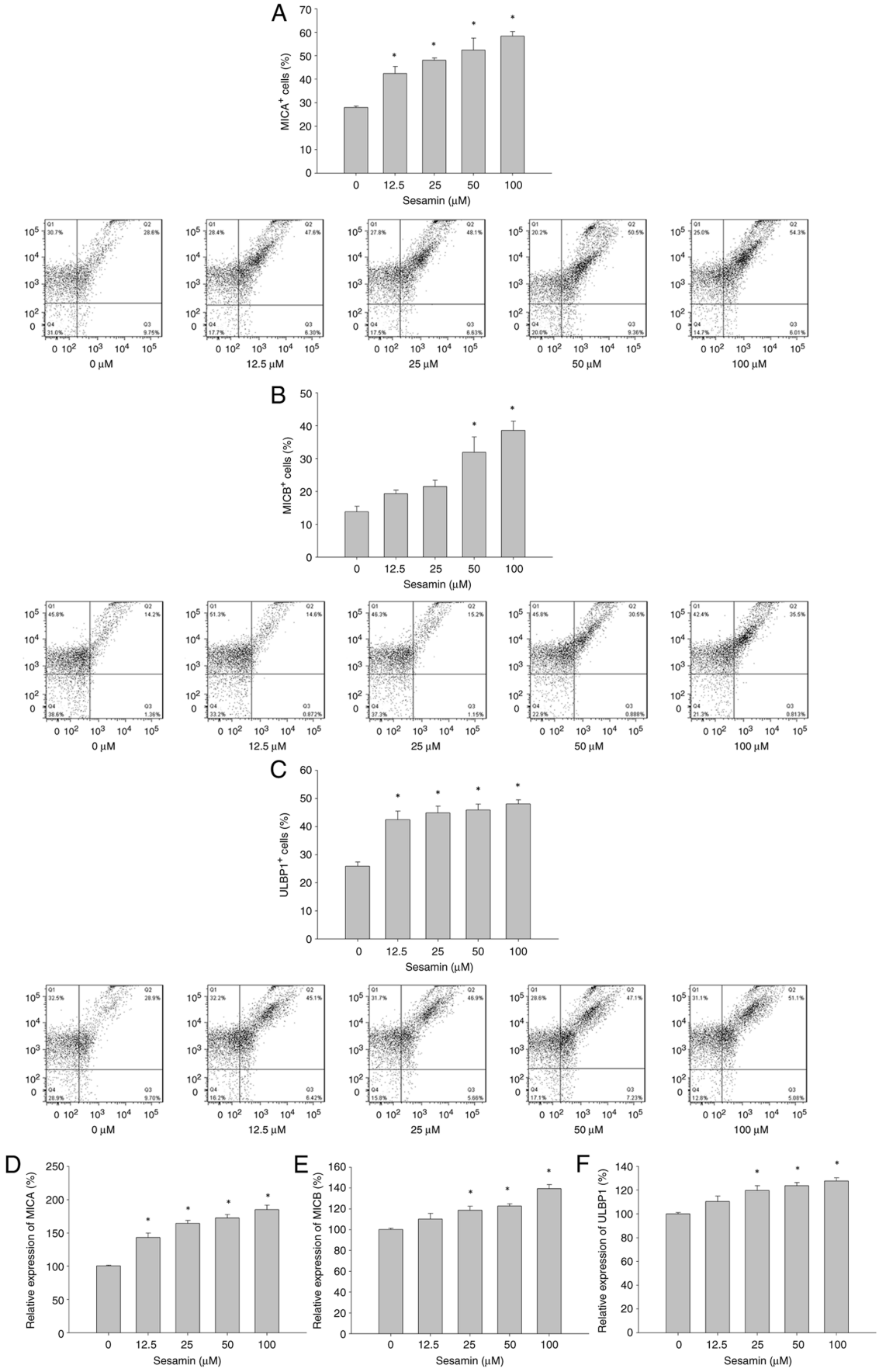

Figure 4. Effects of sesamin on the expression of NKG2D ligands in MG-63 osteosarcoma cells. MG-63 cells were treated with sesamin (0-100 μM) for 48 h. (A-C) The protein levels of MICA, MICB and ULBP1 on MG-63 cells were detected by flow cytometry. (D-F) mRNA levels of MICA, MICB and ULBP1 were measured by reverse transcription-quantitative PCR assay. Results were expressed as the mean of three independent experiments ± SD. *P<0.05 vs. 0 μM sesamin. NKG2DL, natural killer group 2, member D ligand; MICA, MHC class I chain related proteins A; MICB, MHC class I chain related proteins B; ULBP1, unique long 16 binding proteins 1.

were examined by flow cytometry and RT-qPCR. MG-63 cells constitutively express MICA, MICB and ULBP1 at different levels (Fig. 4A-C). Treatment with sesamin for 48 h resulted in increased percentage of MICA positive MG-63 cells in a dose-dependent manner. The percentage of MICB positive MG-63 cells was significantly increased at sesamin concentrations of 50 and 100 μM. Sesamin treatment increased the percentage of ULBP1 positive MG-63 cells in a dose-independent manner. Afterwards, the changes in mRNA levels of MICA, MICB and ULBP1 in MG-63 cells treated sesamin were investigated. The results of RT-qPCR revealed that mRNA expression of MICA, MICB and ULBP1 was significantly upregulated in MG-63 cells treated with sesamin for 48 h (Fig. 4D-F).

Sesamin enhances susceptibility of MG-63 osteosarcoma cells to NK cell-mediated cytotoxicity. Since sesamin upregulated the expression of NKG2DLs in MG-63 cells, it was next investigated whether sesamin primes MG-63-cells for NK cell-mediated antitumor activity by increasing NKG2DL expression. MG-63 cells were treated with sesamin at various concentration for 48 h and were subsequently subjected to co-culture with NK-92 cells. NK-92 cells inhibited the viability of untreated MG-63 cells up to 50% at an E:T ratio of 10:1 (Fig. 5). Treatment with sesamin enhanced MG-63 cell susceptibility to NK-92 cell elimination, resulting in a significant decrease in viability of MG-63 cells in a dose-dependent manner. Data obtained from cytotoxicity assay using E:T ratios of 1:1, 3:1 or 5:1 were used to calculate IC_{50} values, resulting in IC_{50} of 75.1, 51.1 or 16.8 μM, respectively.

The possible correlation between NKG2DL expression and NK cell-mediated cytotoxicity in MG-63 osteosarcoma cells was investigated. The correlation coefficient between MICA and NK cell-mediated cytotoxicity was significant at E:T ratios of 1:3 (P=0.04) and 1:1 (P=0.04) (Table II). The correlation between MICB levels in MG-63 cells and NK cell mediated elimination was significant at all E:T ratios. A significant correlation was detected between percentage of NKG2DL positive cells and NK-mediated cytotoxicity. There was no correlation between ULBP1 and NK-cell mediated killing at all E:T ratios tested.

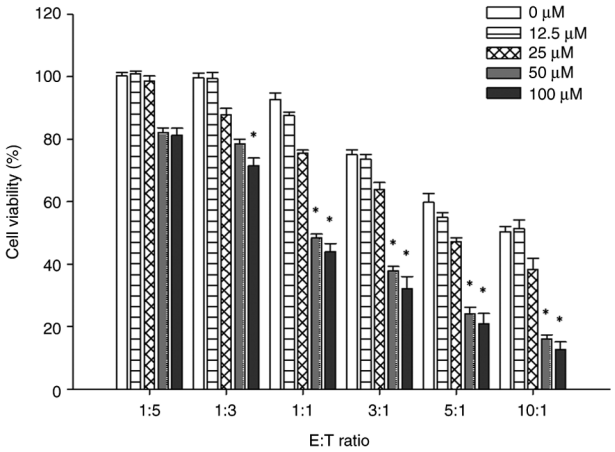

Figure 5. Effects of sesamin on susceptibility of MG-63 osteosarcoma cells to NK cell-mediated cytotoxicity. MG-63 cells were treated with sesamin (0-100 μM) for 48 h and co-cultured with NK-92 cells as effector cells at different effector-to target ratios for 4 h. Results were expressed as the mean of three independent experiments ± SD. *P<0.05 vs. 0 μM sesamin in each group. E:T ratio; effector/target ratio.

Discussion

In the present study, sesamin was revealed to have induced a G_2/M phase cell cycle arrest in MG-63 osteosarcoma cells. Sesamin was reported to have triggered upregulated expression of NKG2DLs and increased susceptibility of MG-63 cells to NK cell-mediated elimination. NK cell-mediated cytotoxicity was positively correlated with levels of NKG2DL expression.

Osteosarcoma is clinically treated with neoadjuvant chemotherapy followed by surgical resection and additional chemotherapy/radiotherapy. Considering the side effects of chemotherapy and functional impairments after surgical resection, a broad variety of natural compounds have been considered and examined for their therapeutic uses against osteosarcoma (19). Sesamin has been demonstrated to exert anti-neoplastic activities against several malignancies (20). Treatment with sesamin causes proliferation arrest at the G1 phase in cell cycle progression in the breast cancer cells (15). Siao *et al* (13) reported that sesamin inhibited the proliferation of MCF-7 cells through inducing Sub-G1 phase arrest and apoptosis. A previous study revealed that sesamin induced G1 cell cycle arrest and inhibited cyclin D1 and CDK2 expression in lung cancer (21). In the present study, it was found that sesamin at a high concentration inhibited proliferation of MG-63 osteosarcoma cells after long exposure time up to 48 h through arresting cell cycle at G_2/M phase. The data of the present study revealed that sesamin increased p21 expression in MG-63 cells. The findings are similar to a previous study revealing that sesamin induced G_2/M arrest in HepG2 cells through the p53/p21 signaling pathway (14). Several cellular stresses cause the activation of p53 and in turn cell cycle arrest through modulating downstream regulatory molecules such as p21, cyclins and CDKs (22-24). p53 protein is activated in response to DNA damage with results of G1 arrest (25). On the other hand, stress-induced increase of p21 and reduction of cyclins have been reported to be p53-independent (22,26,27). p21 appears to play a role in induction of G_2/M arrest in responses to DNA damages (28). In the present study, sesamin caused cell cycle arrest in p53-deficient MG-63 cells, suggesting that p53 may have no role in sesamin-induced cell cycle arrest and activation of p21 is p53-independent.

The cell cycle is regulated through a complex interaction over time between cyclins and Cdks. Cdks are activated by cyclins through forming cyclin/Cdk complexes. Interruption of cyclin B1/Cdk1 complex formation leads to G_2/M phase arrest (29). In the present study, sesamin arrested MG-63 cells at the G_2/M phase through a decreased formation of cyclin B1/Cdk1 complex. The results of the present study revealed that sesamin dose-dependently inhibited cyclin B1 and Cdk1 expression at the transcriptional and protein levels. Cyclin B1/Cdk1 complex is the primary regulator of transition from the G2 to M phase. It is suggested that sesamin induces G_2/M phase arrest in MG-63 cells by the downregulation of Cdk1 and cyclin B1.

Table II. Correlation between natural killer cell-mediated cytotoxicity and sesamin-induced NKG2DL expression.

Cell viability (effector: target ratio)	Ligand expression (% positive cells)	Correlation coefficient	P-value
1:5			
	MICA	-0.78	0.12
	MICB	-0.95	0.01
	ULBP1	-0.59	0.29
	NKG2DL	-0.81	0.10
1:3			
	MICA	-0.89	0.04
	MICB	-0.96	0.01
	ULBP1	-0.72	0.17
	NKG2DL	-0.90	0.04
1:1			
	MICA	-0.89	0.04
	MICB	-0.98	<0.001
	ULBP1	-0.73	0.16
	NKG2DL	-0.91	0.03
3:1			
	MICA	-0.86	0.06
	MICB	-0.98	<0.001
	ULBP1	-0.68	0.21
	NKG2DL	-0.88	0.05
5:1			
	MICA	-0.89	0.05
	MICB	-0.98	<0.001
	ULBP1	-0.73	0.16
	NKG2DL	-0.91	0.03
10:1			
	MICA	-0.85	0.07
	MICB	-0.96	0.01
	ULBP1	-0.67	0.21
	NKG2DL	-0.87	0.06

NKG2DL, natural killer group 2, member D ligand; MICA, MHC class I polypeptide-related sequence A; MICB, MHC class I polypeptide-related sequence B; UPBL1, unique long 16 binding protein 1.

NKG2D ligands are stress-inducible proteins, which are missing or present at very low levels on healthy cells. In addition to upregulated expression of NKG2DLs in infected cells, they are also widely expressed in several types of cancer and hematologic malignancies. Early tumorigenesis involves aberrant cell proliferation levels which are associated with DNA replication stress (30). The stress activates DNA damage response proteins including ataxia telangiectasia mutated (ATM) and/or ATM and Rad3-related (ATR) kinases, which in turn cause cell cycle arrest. In addition, NKG2DLs have been reported to be induced by DNA damage responses or oncogenes (31,32). Nevertheless, mutations in suppressive mechanisms and stress pathways contribute to tumor progression. In the present study, the data revealed that osteosarcoma MG-63 cells constitutively expressed certain levels of NKG2DLs. The present study gave substantial evidence that treatment of MG-63 cells with sesamin results in a concomitant upregulation of the already expressed NKG2DLs including MICA, MICB and ULBP1 both at the protein and mRNA levels. It was revealed that sesamin-induced NKG2DLs expression was associated with G_2/M cell cycle arrest in MG-63 cells. The findings are consistent with previous studies reporting that NKG2DL upregulation was associated with chemical-induced G_2/M cell cycle arrest in cancer cells (33,34). It is suggested that exposure to chemotherapeutics leads to upregulation of NKG2DLs through activation of DNA damage responses (35). The NKG2DL upregulation is a consequence of ATR/ATM-induced cell cycle arrest (31,33). The findings of the present study in p53-deficient MG-63 cells is supported by previous findings revealing that DNA stress induces G_2/M cell cycle arrest in p53-null cells through ATM/ATR checkpoint signaling (36).

Increasing evidence has highlighted the role of NKG2D/NKG2DL interaction in cancer immunotherapy. Strategies harnessing NKG2D/NKG2DL recognition to achieve cancer control have been developed with great interests (37,38).

Numerous anticancer drugs have been revealed to increase surface expression NKG2DL, which pharmacologically induced cell stresses (39) Upregulated NKG2GL expression has been reported in cancer cells exposed to ionizing radiation (40,41). However, current anticancer therapies have downside effects on NK cell-mediated antitumor immunity such as NKG2DL shedding leading to cancer immune evasion. In the present study, sesamin enhanced susceptibility of osteosarcoma MG-63 cells to NK cell-mediated cytotoxicity through inducing KNG2DL expression. The data of the present study revealed that sesamin increased sensitivity of MG-63 cells to NK cell killing at a low E:T ratio of 1:3. Among the NKG2DL analyzed, NK cell-mediated cytotoxicity was significantly correlated with expression of MICB in presence of sesamin. It is suggested that sesamin at low concentration may be adequate to achieve favorable effects of NK-cell immunotherapy against osteosarcoma MG-63 cells. Sesamin is a natural compound containing several pharmacological activities including anti-oxidative stress and anti-inflammation. The anti-inflammatory property of sesamin is suggested to contribute to addressing tumor microenvironment and ligand shedding. Oral ingestion of sesamin at doses up to 200 mg/day has been reported to be safe and tolerable (42). Sesamin is suggested to be a safe candidate for inducing NKG2DL expression, whereas the other chemotherapeutic drugs are markedly toxic. Tomimori *et al* (43) has reported that the highest concentration of sesamin in human plasma after ingestion of 50 mg of sesamin was 8 nM (43). This is substantially lower than the concentrations tested. Further studies are required to address bioavailability issue and assess NKG2D/NKG2DL axis *in vivo* with a consideration of complexity of tumor cells, tumor microenvironment and NK cells. The limitations of the present study include *in vitro* design, which inherently lacks the challenges of true clinical conditions such as tumor microenvironment and cell administration. In addition, one cell line was employed to study the correlation between the expression of NKG2D ligand and NK cell mediated elimination. *In vivo* studies are necessary to validate the findings of the present study.

In conclusion, within the limitations of the present study, sesamin induced cell cycle arrest at G_2/M phase in osteosarcoma MG-63 cells and in turn increased NKG2DL expression in osteosarcoma cells. Sesamin might further enhance the susceptibility of osteosarcoma cells to NK cell-mediated cytotoxicity through upregulated expression of NKG2DLs. Therefore, sesamin represents a potential candidate to improve the efficacy of NKG2DL-mediated anticancer therapy for osteosarcoma.

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Availability of data and materials

The data generated in the present study may be requested from the corresponding author.

Authors' contributions

SCC, CYK and YJL designed the study. SCC, CYK, HWK, PTH, CHL and LSW conducted the experiments and analyzed the data. SCC and CYK confirm the authenticity of all the raw data. SCC, CYK and YJL prepared and wrote the manuscript. All authors read and approved the final version of the manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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