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Please cite this article as https://doi.org/10.4097/kja.23323
The Effect of Sevoflurane on Metalloproteinase and Natural Killer Group2, Member D (NKG2D) Ligand Expression, and Natural Killer Cell-mediated Cytotoxicity in Breast Cancer: An In vitro Study

Running Title: Effects of sevoflurane on breast cancer

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Previous presentation in conferences: None.
Conflict of interest: None.

Financial support: This study was supported by Biomedical Research Institute Grant (20210033), Pusan National University Hospital.

Acknowledgments: None.

IRB number: Not applicable.

Clinical trial registration number: Not applicable.
The Effect of Sevoflurane on Metalloproteinase and Natural Killer Group2, Member D (NKG2D) Ligand Expression, and Natural Killer Cell-mediated Cytotoxicity in Breast Cancer: An In vitro Study

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Abstract

Background: We investigated the impacts of sevoflurane exposure on matrix metalloproteinase (MMP) expression, natural killer group 2, member D (NKG2D) ligands (UL16-binding proteins [ULBP] 1–3, and major histocompatibility complex class I chain-related molecules [MIC] A/B) expression and ablation, and natural killer (NK) cell-mediated cytotoxicity in breast cancer cells.

Methods: Three human breast cancer cell lines (MCF-7, MDA-MB-453, and HCC-70) were incubated with 0 (control), 600 (S6), or 1200 μM (S12) sevoflurane for 4 h. The gene expression of NKG2D ligands and their protein expression on cancer cell surfaces were measured using multiplex polymerase chain reaction (PCR) and flow cytometry, respectively. Protein expression of MMP-1 and 2 and concentration of soluble NKG2D ligands were analysed by western blot and enzyme-linked immunosorbent assays, respectively.

Results: Sevoflurane downregulated the mRNA and protein expression of the NKG2D ligand in a dose-dependent manner in MCF-7, MDA-MB-453, and HCC-70 cells. However, it did not affect the expression of MMP-1 and 2 or the concentration of soluble NKG2D ligands in MCF-7, MDA-MB-453, and HCC-70 cells. Sevoflurane attenuated NK cell-mediated cancer cell lysis in a dose-dependent manner in MCF-7, MDA-MB-453, and HCC-70 cells (P = 0.040, 0.040, and 0.040, respectively).

Conclusions: Our results demonstrated that sevoflurane exposure could attenuate the NK cell-mediated cytotoxicity of breast cancer cells in a dose-dependent manner. This could be
attributed to a sevoflurane-induced decrease in the transcription of NKG2D ligands rather than sevoflurane-induced changes in MMP expression and their proteolytic activity.

MeSH Keywords: Sevoflurane; breast neoplasms; matrix metalloproteinases; NKG2D ligands; killer cells, natural; anesthetics, Inhalation.
Introduction

In 2020, breast cancer surpassed lung cancer as the most common cancer globally, accounting for 12.5% of cancer diagnoses [1]. The incidence of breast cancer continues to rise, with a projected increase in new cases of over 40% and deaths of over 50% by 2040 [1]. Approximately 88% patients with breast cancer undergo at least one anesthetic and surgical treatment within one year of diagnosis [2]. However, surgery and anesthesia are associated with increased release of inflammatory mediators and angiogenic factors and cause postoperative immunosuppression, resulting in tumor progression [3].

The natural killer group 2, member D (NKG2D) ligands, UL16-binding proteins (ULBP) 1–3 and major histocompatibility complex class I chain-related molecules (MIC) A/B, expressed on the surface of cancer cells, bind to active receptors on natural killer cells (NK cells), transmitting signals and allowing NK cells to recognize and eliminate cancer cells [4]. A reduced expression of NKG2D ligands can impair NK cell cytotoxicity against cancer cells, leading to immune evasion and disruption of the cancer immunosurveillance system [5].

Matrix metalloproteinases (MMPs) are Zn$^{2+}$-dependent endopeptidases that play a critical role in tumor progression by promoting extracellular matrix and basement membrane degradation, leading to cell detachment and migration [5,6]. MMPs also promote neovascularization, contributing to tumor angiogenesis [5,6]. Moreover, recent cancer immunological studies have demonstrated that MMPs also cleave and remove NKG2D ligands [5–7]. MMP-induced shedding of NKG2D ligands
confers several advantages to cancer cells for immune evasion [8,9]. Firstly, it reduces the density of NKG2D ligands on the surface of cancer cells, thereby impairing their susceptibility to NK cells [8,9]. Moreover, the cleaved ligands (soluble NKG2D ligands) retain the ability to bind to the NKG2D receptors on NK cells [8,9]. This cleaved ligand-receptor engagement not only hinders the activation signalling of the receptor but also triggers the internalisation and downmodulation of the NKG2D receptor on NK cells [8,9].

Modulating surgery-related factors in clinical practise remains challenging and requires an enhanced understanding of the effects of anesthesia-related factors on cancer recurrence and survival rates. Therefore, elucidating the effects of anesthetics on the breast cancer microenvironment is essential for optimal anesthesia management and to improve postoperative outcomes.

In this study, we investigated the effect of sevoflurane, a commonly used general anesthetic, on MMP expression and NKG2D-mediated NK cell cytotoxicity in breast cancer cells. We evaluated the effects of sevoflurane exposure on MMP expression, NKG2D ligand expression and ablation, and NK cell-mediated cytotoxicity in breast cancer cells.
**Materials and Methods**

**Cell lines and reagents**

The study was conducted using three breast cancer cell lines: the estrogen receptor [ER]- and progesterone receptor [PR]-positive human breast cancer cell line MCF-7 (Korean Cell Line Bank, Seoul, Korea), the human epidermal growth factor receptor 2 [HER2]-positive human breast cancer cell line MDA-MB-453 (Korean Cell Line Bank), and the triple-negative human breast cancer cell line HCC-70 (Korean Cell Line Bank) [10]. All cell lines were maintained in the RPMI-1640 medium containing 10% fetal bovine serum and 1% penicillin (Welgene, Gyeongsan, Korea). As sevoflurane is a strong organic solvent capable of interacting with plastic materials to produce impurities [11], breast cancer cells were cultured in poly-L-lysine-coated (Sigma-Aldrich, St. Louis, MO, USA) glass culture dishes.

The human NK cell line NK92-MI (ATCC, Rockville, MD, USA) was maintained in an α-minimum essential medium containing fetal bovine serum (12.5%), horse serum (12.5%), 2 mercaptoethanol (0.1 mM), and L-glutamine (2 mM). All cell lines were cultured according to their specifications and incubated at 37 °C in humidified air containing 5% CO₂.

**Sevoflurane treatment**

As previously described [12–14], 100 μL sevoflurane (Sevoprane; Ilsung, Seoul, Korea) was diluted in 10 mL RPMI-1640 medium and stirred for a half-hour in an airtight, amber-colored glass bottle. The concentration of sevoflurane was determined using our preliminary data obtained from gas
chromatography-mass spectrometry analysis (GCMS-QP2010 Plus; Shimadzu, Kyoto, Japan) as
3.92 (1.26) mM (mean [SD]). Sevoflurane stock was serially diluted to 1200 and 600 µM (S12 and
S6) immediately before the experiments. Kharasch et al. [15] simultaneously measured the end-tidal
sevoflurane concentration and plasma concentration of sevoflurane during general anaesthesia and
revealed that the average peak plasma concentration of sevoflurane reached 772 µM at an end-tidal
sevoflurane concentration of 2.7% (equivalent to 1.3 minimum alveolar concentration [MAC]).
Based on this, we assumed clinically relevant concentrations of sevoflurane at 600 µM during
general anesthesia. Furthermore, to investigate the dose-response relationship, we administered an
additional dose of sevoflurane at a concentration of 1200 µM, which was higher than the
recommended dose. Corresponding concentration of distilled water in RPMI 1640 media were used
as controls (0 µM).
A four-hour sevoflurane exposure was administered to MCF-7, MDA-MB-453, and HCC-70 cells.
To account for evaporation-induced concentration reduction, both the sevoflurane solution and con-
trol group solution were replaced every hour (Fig. 1) [13,14]. Previous studies [12,13] have shown
that despite sevoflurane’s volatility, the concentration of sevoflurane solution dissolved in the cell
culture media remained stable, with less than a 10% loss over a one-hour period.
mRNA expression analysis was performed 18 hours after the completion of the treatment, whereas
the other experiments (cell viability test, flow cytometry assay for surface expression of NKG2D
ligands, western blotting analysis, enzyme-linked immunosorbent assay [ELISA] for soluble
NKG2D ligands, and flow cytometry assay for NK cell-mediated cytotoxicity) were performed 24 hours after the completion of the treatment (Fig. 1).

**Cell viability test**

The MTT (3-[4,5-dimethylthiazol-2-y1]-2,5-diphenyltetrazolium bromide) assay is based on the principle that mitochondrial activity in living cells converts MTT into formazan crystals. These crystals are dissolved following dimethyl sulfoxide (DMSO) addition and the detected spectrophotometrically at 540 nm; the absorbance is directly proportional to the cell viability [16]. As previously described [14], MCF-7, MDA-MB-453, and HCC-70 cells were plated in 96-well plates (1 x 10^4 cells/well) and incubated with the control or S12 solution for 4 h. After 24 h, cells were incubated with MTT solution (Sigma-Aldrich) for 4 h. The supernatant was discarded, and formazan crystals were dissolved using DMSO. Absorbance at 540 nm was determined using a microplate spectrophotometer (μQuant; Bio Tek, Winooski, VT, USA).

**mRNA expression analysis of NKG2D ligands**

mRNA expression analysis was performed by the method described in our previous research [14]. Briefly, after 4-hour treatment and an additional 18-hour incubation, cancer cells were harvested (Fig. 1). Total RNA was extracted from the cells using a RNeasy®Mini kit (Qiagen GmbH, Hilden, Germany). Then, reverse transcription PCR (RTPCR) and multiplex PCR were performed. For
denaturation, 3 μg extracted total RNA and 100 pmol random primers (Takara Shuzo, Kyoto, Japan) were incubated at 65 °C for 5 min and chilled at 4°C for 4 min. Then, 6 μL of the 5x reaction buffer, 4 μL deoxynucleotide triphosphate (10 mM; Promega Co., Madison, WI, USA), and 1.2 μL M-MLV RT (Promega Co.) were added and incubated at 37 °C for 60 minutes. Multiplex PCR was performed using the QIAGEN® Multiplex PCR kit (Qiagen GmbH). The primer sets used to evaluate NKG2D gene expression were as follows: 1) MICA: ribosomal protein L19 (RPL19), MICA, and β-actin genes; and 2) MICB and ULBP 1–3: RPL19, MICB, ULBP1–3, and β-actin genes. The primer sequences are listed in Table 1. All experiments were performed following the manufacturer’s protocol. PCR products were quantified using a microchip electrophoresis system MCE®-202MultiNA (Shimadzu, Kyoto, Japan). For normalisation, the mRNA band intensity of each NKG2D ligand was divided by that of the β-actin. The normalised mRNA band intensity of treated samples was divided by those of the controls to assess relative gene expression ratios.

Flow cytometry assay for surface expression of NKG2D ligands

Flow cytometry assay for surface expression of NKG2D ligands was performed by the method described in our previous research [14]. Briefly, after 4-hour treatment and an additional 24-hour incubation (Fig. 1), the cells were harvested and incubated with 10 μg/mL mouse anti-MICA/B and ULBP1–3, or the corresponding isotype controls (anti-IgG2a or anti-IgG2b; R&D Systems, Minneapolis, MN, USA). The samples were then incubated with a goat anti-mouse phycoerythrin
(PE)-conjugated antibody (BD Biosciences, San Jose, CA, USA). The mean fluorescence intensity (MFI) was measured using a FACSCanto™ II flow cytometer (BD Biosciences) and quantified using FlowJo (ver. 10.6.1; TreeStar, Ashland, OR, USA). The MFIs of the treated samples were divided by those of the controls to assess the relative expression ratios.

**Western blot analysis for determining protein expression of MMPs**

After 4-hour treatment and an additional 24-hour incubation (Fig. 1), western blot analysis was performed to evaluate the expression of MMP-1 and -2. The cells were washed three times with cold phosphate-buffered saline and lysed in a PRO-PREP protein extraction solution (Intron, Gyeonggi-do, Korea). Equal amounts of cell extracts were resolved by 4–20% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and analysed by western blotting. The separated proteins were transferred to polyvinylidene difluoride membranes (Millipore, Billerica, MA, USA). Next, the membranes were blocked with 3% BSA in Tris-buffered saline containing 0.1% Tween 20 at room temperature. Proteins of interest were detected using primary antibodies (MMP-1 and -2; Cell Signalling, Beverly, MA) and horseradish peroxidase-conjugated secondary antibodies (Enzo Life Sciences, Minneapolis, MN, USA) per manufacturer’s instructions and a chemiluminescence imaging system (AE-9150 Ez-capture II; Atto, Tokyo, Japan) was used for analysing the results. Each blot was probed with an anti-β-actin antibody (Sigma-Aldrich). The band intensity was quantified using ImageJ software (ver. 1.53; National Institutes of Health, Bethesda, MD, USA).
The protein expression in the treated cells was divided by the control cells to calculate the relative protein expression ratios.

Enzyme-linked immunosorbent assay (ELISA) for soluble NKG2D ligands

The breast cancer cells (4 × 10^6 cells) were plated on 60 mm glass culture dishes. After 4-hour treatment and an additional 24-hour incubation (Fig. 1), cell culture supernatants were centrifuged at 5000 rpm for 5 min at 4 °C, and aliquots were stored at −80 °C until further use. The levels of soluble NKG2D ligands (MICA for MCF-7 and MDA-MB-453; MICB for HCC-70) in the cell culture supernatant were measured using ELISA kits (MICA Human ELISA Kit, Invitrogen; Human MICB ELISA Kit, MyBioSource, San Diego, CA, USA), following the manufacturer’s protocol. Absorbance at 450 nm was measured using a microplate spectrophotometer (Synergy™ H1; BioTek). The samples were loaded in duplicate, and the mean soluble NKG2D ligand values were used for analysis.

Flow cytometry assay for NK cell-mediated cytotoxicity

After 4-hour treatment and an additional 24-hour incubation (Fig. 1), target cancer cells (MCF-7, MDA-MB-453, and HCC-70) were stained with carboxyfluorescein diacetate succinimidyl ester (CFSE; CellTrace™; Invitrogen) and co-cultured with NK92-MI cells, effector cells, for 4 h. Optimising the effector-to-target cell (E: T) ratio is crucial in cellular cytotoxicity assays to
maximise and distinguish cytotoxicity differences among treatment groups. Typically, higher E:T ratios result in enhanced NK cell cytotoxicity, as an increased number of effector cells relative to target cells leads to greater cytotoxicity [17]. The recommended E:T ratio for flow cytometry cytotoxicity assays is 10:1 or less [18]. As previously reported [14], we determined the E:T ratio to be between 1:1 (e.g., E: T = 1 × 10^5: 1 × 10^5) and 10:1 (e.g., E: T = 1 × 10^6: 1 × 10^5), with 10:1 considered the optimal ratio. The co-cultured cells were labelled with 1 μg/mL propidium iodide (PI; Invitrogen). FACSCanto™ II flow cytometer and BD FACSDiva™ Software (BD Biosciences) were used. The NK cell-mediated lysis (%) was calculated using the following equation:

\[
\frac{Q2}{Q2 + Q3} \times 100
\]

Where Q2 is CFSE positive and PI positive cells and Q3 is CFSE positive and PI negative cells.

**Statistical analysis**

MedCalc® (ver. 20; MedCalc Ltd., Ostend, Belgium) and IBM SPSS Statistics (ver. 25; IBM Corp., Armonk, NY, USA) were used; variables are presented as the median with the first and third quartiles (Q1, Q3). For comparison between groups, Mann-Whitney U tests or Kruskal-Wallis tests were performed. If the Kruskal-Wallis test was significant, post-hoc comparisons using the Conover method were conducted.

Our trial consisted of a zero-dose control group (C) and two sevoflurane treatment groups (S6, S12) that received escalating doses of sevoflurane in the following order: C S6 S12. As a secondary
endpoint, the dose-response relationship was evaluated using Jonckheere-Terpstra trend test.

Two-sided P values < 0.05 indicated statistical significance.
Results

Effect of sevoflurane on cell viability assessed by MTT assay

Sevoflurane did not affect the viability of MCF-7, MDA-MB-453, or HCC-70 cells. The relative cell viabilities (%) related to control measured by MTT assay were as follows (median [Q1, Q3]):

- MCF-7—S12: 100.2 (92.4, 118.9), P = 1.000;
- MDA-MB-453—S12: 101.4 (96.2, 109.0), P = 1.000;
- HCC-70—S12: 89.4 (87.3, 107.0), P = 0.305 (n=6 per each group).

Effect of sevoflurane on the mRNA expression of NKG2D ligands

The results are summarised in Fig. 2 (n = 6 per group). In MCF-7, MDA-MB-453, and HCC-70 cell lines, mRNA expression of MICA, MICB, ULBP2, and ULBP3 was observed, but the expression of ULBP1 was not observed.

In MCF-7 cells, the relative mRNA expression ratios of MICB at S6 and S12 were lower compared with those of the control (Kruskal–Wallis test: P = 0.013). In MDA-MB-453 cells, the relative mRNA expression ratios of MICA at S6 and S12 were lower than in the control (Kruskal–Wallis test: P = 0.013); the relative surface expression ratio of ULBP3 was downregulated at S12 compared with that of the control (Kruskal–Wallis test: P = 0.044). In HCC-70 cells, the relative mRNA expression ratios of ULBP2 and ULBP3 at S6 and S12 were decreased compared with those of the control (Kruskal–Wallis test: P=0.002 and 0.003, respectively).

In the secondary end point results, a dose-response relationship was observed in MCF-7 cells,
demonstrating significantly lower relative mRNA expression levels of MICA, MICB, ULBP2, and ULBP3 in response to increasing concentrations of sevoflurane (Jonckheere-Terpstra trend test; \( P = 0.015, 0.008, 0.036, \) and \( 0.043, \) respectively). Similarly, sevoflurane dose-dependently downregulated the mRNA expression of MICA and ULBP3 in MDA-MB-453 cells (Jonckheere-Terpstra trend test; \( P = 0.002 \) and \( 0.029, \) respectively). Likewise, sevoflurane exhibited a dose-dependent downregulation of the mRNA expression of MICA, MICB, ULBP2, and ULBP3 in HCC-70 cells (Jonckheere-Terpstra trend test; \( P = 0.043, 0.043, 0.006, \) and \( 0.012, \) respectively).

**Effect of sevoflurane on surface expressions of NKG2D ligands assessed by flow cytometry**

Fig. 3 summarises the outcomes of a flow cytometry analysis of the surface expressions of NKG2D ligands \( (n = 6 \) per group). Consistent with the mRNA expression, ULBP1 was rarely expressed on the surface of the MCF-7, MDA-MB-453, and HCC-70 cell lines. In MCF-7 cells, MICA and ULBP2 were expressed predominantly; the relative surface expression ratios of MICA and ULBP2 at S6 and S12 were downregulated compared with those of the control (Kruskal-Wallis test: \( P = 0.002 \) and \( < 0.001, \) respectively).

MDA-MB-453 exhibited a predominant surface expression of MICA and ULBP2. The relative surface expression ratios of MICA, ULBP2, and ULBP3 at S6 and S12 were lower than those of the control (Kruskal-Wallis test: \( P < 0.001, = 0.003, \) and \( = 0.004, \) respectively).

In HCC-70 cells, MICB, ULBP2, and ULBP3 were predominantly expressed. The relative surface
expression ratios of MICA, MICB, ULBP2, and ULBP3 at S6 and S12 were downregulated compared with those of the control (Kruskal-Wallis test: P = 0.002, = 0.017, = 0.002, and = 0.013, respectively).

In the dose-response analysis, we observed significantly lower relative surface expression ratios of MICA, MICB, and ULBP2 in MCF-7 cells with increasing concentrations of sevoflurane (Jonckheere-Terpstra trend test; P < 0.001, = 0.024, and < 0.001, respectively). Similarly, sevoflurane dose-dependently decreased the relative surface expression ratios of MICA, ULBP2, and ULBP3 in MDA-MB-453 cells (Jonckheere-Terpstra trend test; P < 0.001, = 0.002, and = 0.001, respectively). Likewise, sevoflurane dose-dependently downregulated the relative surface expression ratios of MICA, MICB, ULBP2, and ULBP3 in HCC-70 cells (Jonckheere-Terpstra trend test; P = 0.001, = 0.010, < 0.001, and = 0.029, respectively).

**Effect of sevoflurane on protein expression of MMP assessed by western blot analysis**

No changes in the protein expression of MMP-1 and -2 were observed between the control and sevoflurane treatment groups in MCF-7, MDA-MB-453, and HCC-70 cells through Western blot analysis (Fig. 4A; n = 6 per group).

In MCF-7 cells, the median (Q1, Q3) of the relative protein expression ratio of MMP-1 was as follows: control, 1.7 (0.9, 2.4); S6, 1.6 (0.8, 2.5); S12, 1.5 (0.7, 2.3); P values for Kruskal-Wallis and Jonckheere-Terpstra trend tests were 0.519 and 0.258, respectively; the median (Q1, Q3) of the
relative protein expression ratio of MMP-2 was as follows: control, 1.3 (0.8, 2.4); S6, 1.3 (1.1, 2.0); S12, 1.3 (0.8, 2.1); P values for Kruskal-Wallis and Jonckheere-Terpstra tend tests were 0.854 and 0.686, respectively.

In MDA-MB-453, the median (Q1, Q3) of the relative protein expression ratio of MMP-1 were: control, 1.3 (0.9, 1.5); S6, 2.0 (0.9, 5.3); S12, 1.5 (0.7, 3.9), P values for Kruskal-Wallis and Jonckheere-Terpstra tend tests were 0.653 and 0.628, respectively; the median (Q1, Q3) of the relative protein expression ratio of MMP-2 were: control, 0.9 (0.5, 1.1); S6, 0.8 (0.7, 0.9); S12, 0.8 (0.8, 1.3), P values for Kruskal-Wallis and Jonckheere-Terpstra tend tests were 0.778 and 0.746, respectively.

In HCC-70 cells, the median (Q1, Q3) of the relative protein expression ratio of MMP-1 were: control, 1.7 (1.0, 2.3); S6, 1.2 (0.8, 1.7); S12, 1.4 (0.8, 2.1), P values for Kruskal-Wallis and Jonckheere-Terpstra tend tests were 0.423 and 0.293, respectively; the median (Q1, Q3) of the relative protein expression ratio of MMP-2 were as follows: control, 0.8 (0.4, 1.0); S6, 0.7 (0.4, 1.0); S12, 0.9 (0.4, 1.0); and P values for Kruskal-Wallis and Jonckheere-Terpstra tend tests were 0.911 and 0.808, respectively.

**Sevoflurane did not affect the soluble NKG2D ligand concentration assessed by ELISA**

No significant changes were detected in the levels of soluble NKG2D ligands, as evaluated by ELISA, between the control group and the sevoflurane treatment group in MCF-7, MDA-MB-453,
and HCC-70 cells (Fig. 4B, n = 6 per group).

In the Jonckheere-Terpstra test, a dose-response relationship was not identified, and the P values for MCF-7, MDA-MB-453, and HCC-70 cells were 0.467, 0.125, and 0.686, respectively.

Effect of sevoflurane on NK cell-mediated cytotoxicity assessed by flow cytometry

The results are displayed in Fig. 5 (n = 4 per group). In all cell lines, NK cell-mediated cytotoxicity at S6 and S12 was decreased compared with that of the control, and P values compared with the controls were as follows: MCF-7—S6: 0.005, S12: 0.005; MDA-MB-453—S6: 0.005, S12: 0.005; HCC-70—S6: 0.005, S12: 0.005 (Fig. 5; effect cells: target cells = 10:1, n = 4 per group).

Analysing the results of secondary end points using the Jonckheere-Terpstra trend test revealed that sevoflurane dose-dependently reduced NK cell-mediated cytotoxicity in MCF-7, MDA-MB-453, and HCC-70 cells (effectors: target cells = 10:1; P = 0.040, 0.040, and 0.040, respectively).
Discussion

In this study, we demonstrated that sevoflurane exerts a dose-dependent downregulation in the mRNA and protein expression of NKG2D ligands in human breast cancer cell lines; however, it did not affect the expression of MMP-1 and 2, or the concentration of soluble NKG2D ligands, or proteolytically cleaved the surface NKG2D ligands. Furthermore, sevoflurane attenuated NK-cell-mediated cancer cell lysis in a dose-dependent manner.

The association of anesthetics with cancer recurrence was first reported in the 2000s. Since then, several preclinical and clinical studies have been conducted to identify the potential effects of anesthetics and anesthesia methods on breast cancer prognosis [19–21]. Although preclinical trials have suggested potential associations between anesthetic agents and the invasion and metastasis of breast cancer, the results of clinical research comparing these effects remain inconclusive [19]. A recent meta-analysis, in a subgroup analysis of breast cancer based on a prospective and three retrospective clinical studies, reported that recurrence-free survival and overall survival rates of breast cancers did not improve when total intravenous anesthesia was used compared with inhalation anesthesia (recurrence-free survival: hazard ratio [HR], 0.83; 95% confidence interval [95% CI], 0.59–1.15; overall survival: HR, 1.12, 95% CI, 0.90–1.39) [22]. Sessler et al. conducted a multi-center randomised controlled trial and demonstrated that sevoflurane-based general and regional anesthesia with propofol did not differ in recurrence-free survival (adjusted HR, 0.97; 95% CI, 0.74–1.28) [23].
Our results demonstrate that sevoflurane suppresses NK cell-mediated cancer cell lysis in a dose-dependent manner. Consistent with our results, previous studies have demonstrated a potential association between sevoflurane exposure and inhibition of NK cell activity and have suggested various underlying mechanisms in breast cancer patients [24,25]. In a pilot clinical trial conducted on ten breast cancer patients, sevoflurane-based general anesthesia reduced the expression of the NK cell-activating receptor (CD16) and their cytokines (interleukin (IL)-1β and IL-10) and decreased NK cell-mediated cytotoxicity [24]. Similarly, in another randomised controlled trial including 50 participants, NK cell-mediated cytotoxicity decreased after breast cancer resection under sevoflurane-based anesthesia with fentanyl analgesia [25].

In our previous study on a non-small cell lung cancer cell line, sevoflurane administered at an anaesthetic dose decreased the expression of NKG2D ligands and NK cell-mediated cytotoxicity. This effect was attributed to suppression of NKG2D ligand transcription and an increase in MMP expression [14]. However, our present study on breast cancer cell lines suggests a different mechanism for the reduction of NK cell-mediated cytotoxicity by sevoflurane. We propose that sevoflurane directly inhibits the transcription of NKG2D ligands rather than NKG2D ligand shedding induced through increased MMP expression.

Earlier studies examining the impact of sevoflurane on MMP expression in breast cancer have yielded conflicting results [26,27]. Deegan et al. reported that following primary breast cancer surgery, sevoflurane with opioid anesthesia increased the serum levels of MMP-3 and MMP-9, but
not MMP-1, compared to propofol with paravertebral block [26]. In contrast, Galos et al. demonstrated no difference in the serum levels of MMP-3 and MMP-9 before and after sevoflurane-based anesthesia in patients with breast cancer [27]. These discrepancies could be attributed to heterogeneity in cancer subtypes, patient characteristics, and anesthetic exposure regimens. However, only limited research studies have evaluated the effects of anesthetic agents on NK cell ligand expression in breast cancer, which requires a deeper understanding.

Our study has a few limitations. First, as an in vitro study, our results are not directly applicable to animals or humans. Second, the present study was not designed to elucidate the molecular mechanism of sevoflurane on the expression of NKG2D ligands and MMPs; further studies are warranted to understand the detailed mechanism. Third, sevoflurane was the only anesthetic agent used in this research. Consequently, whether the results presented in this study represent a universal phenomenon associated with higher concentrations of anesthetic, or if they are specific to sevoflurane remains unknown. To provide a comprehensive explanation for our findings, conducting additional research with other anaesthetic agents, such as propofol, is necessary.

In summary, sevoflurane attenuated NK cell-mediated cancer cell lysis in a dose-dependent manner, which could be attributed to the sevoflurane-induced decrease in transcription of NKG2D ligands rather than sevoflurane-induced changes in MMP expression and their proteolytic activity. Further research is essential to elucidate the effect of sevoflurane on immune escape and immunosurveillance in breast cancer.
References


17. Ozdemir O, Ravindranath Y, Savaşan S. Cell-mediated cytotoxicity evaluation using monoclonal antibody staining for target or effector cells with annexinV/propidium iodide co-labeling by fluorosphere-adjusted counts on three-color flow cytometry. Cytometry A


Table 1. List of primer used in multiplex RTPCR.

<table>
<thead>
<tr>
<th>Name</th>
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<th>Sequence (5’ → 3’)</th>
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Figure 1. The experimental protocol used in the study.

Treatment (1200 [S12], 600 [S6], and 0 [Control, C] µM) was administered for 4 hours, and each sevoflurane solution and control group solution were replaced on an hourly basis. mRNA expression analysis was performed 18 hours after the completion of the 4 hour-treatment, while other tests were performed 24 hours later.
**Figure 2.** Effect of sevoflurane on the mRNA expression of NKG2D ligands.

Results were analysed using Kruskal-Wallis tests with post-hoc Conover comparisons; variables are presented as the median with the first and third quartiles (n=6 per group). C: control group, S6: sevoflurane 600 µM group; S12: sevoflurane 1200 µM group; ULBP: UL16-binding proteins; MICA / B: major histocompatibility complex class I chain-related molecules A/B; RPL19: ribosomal protein L19. *p < 0.05 as compared to that of the C.
Figure 3. Effect of sevoflurane on surface expressions of NKG2D ligands assessed by flow cytometry.

Results were analysed using Kruskal-Wallis tests with post-hoc Conover comparisons; variables are presented as the median with the first and third quartiles (n=6 per group). C: control group; S6:
sevoflurane 600 μM group; S12: sevoflurane 1200 μM group; ULBP: UL16-binding proteins; MICA / B: major histocompatibility complex class I chain-related molecules A/B; PE: Phycoerythrin. * and † p < 0.05 as compared to that of the C and S6, respectively.
Figure 4. Effect of sevoflurane on MMPs expression and concentration of soluble NKG2D ligands

Variables are presented as the median with the first and third quartiles (n=6 per group). (A) Western blot analysis was performed to evaluate the expression of MMP-1 and -2. The protein expression of MMP-1 and -2 remained unchanged between the control and sevoflurane treatment groups in MCF-7, MDA-MB-453, and HCC-70 cells. (B) Enzyme-linked immunosorbent assay (ELISA) was performed to evaluate the concentration of soluble NKG2D ligands. No difference in the levels of soluble NKG2D ligands was observed between the control group and the sevoflurane treatment group in MCF-7, MDA-MB-453, and HCC-70 cells. MMP: matrix metalloproteinase; MICA / B: major histocompatibility complex class I chain-related molecules (MIC) A/B; C: control group; S6: sevoflurane 600 µM group, S12: sevoflurane 1200 µM group.
**Figure 5.** Effect of sevoflurane on NK cell-mediated cytotoxicity assessed by flow cytometry

Results were analysed using Kruskal-Wallis tests with post-hoc Conover comparisons; variables are presented as the median with the first and third quartiles (n = 4 per group). Target cancer cells (T; MCF-7, MDA-MB-453, and HCC-70) were stained with carboxyfluorescein diacetate succinimidyl ester (CFSE) and co-cultured with NK92-MI cells (E, effector cells) at a 1:1 (e.g., effector cells:
target cancer cells = $1 \times 10^5$: $1 \times 10^5$) or 10:1 (e.g., effector cells: target cancer cells = $1 \times 10^6$: $1 \times 10^5$). PI: propidium iodide, C: control group; S6: sevoflurane 600 µM group; S12: sevoflurane 1200 µM group. * $p < 0.05$ as compared to that of the C.