

Effects of KIR2DL4-Stimulated NK-92 Cells on the Apoptotic Pathways of HER2+/HER2- Breast Cancer Cells

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Abstract

Natural killer (NK) cells are immune cells that have attracted significant attention due to their cytotoxic properties. They are believed to be highly effective in cancer therapy. In this study, anti-KIR2DL4 (Killer cell Immunoglobulin like Receptor, 2 Ig Domains and Long cytoplasmic tail 4) was used to stimulate the NK-92 activator receptor to increase their cytotoxicity on breast cancer cell lines. Unstimulated and stimulated NK-92 cells (sNK-92) were cocultured with breast cancer (MCF-7 and SK-BR-3) and normal breast (MCF-12A) cell lines at 1:1, 1:5, and 1:10 (Target: Effector) ratios. The most effective cell cytotoxicity ratio (1:10) was used in the immunostaining and western blot assays to evaluate apoptosis pathway proteins. The sNK-92 cells showed higher cytotoxic activity on breast cancer cells than NK-92 cells. sNK-92 cells had a selective significant cytotoxicity effect on MCF-7 and SK-BR-3 cells but not MCF-12A cells. While sNK-92 cells were effective at all cell concentrations, they were most effective at a 1:10 ratio. Immunostaining and western blots showed significantly higher BAX, caspase 3, and caspase 9 protein levels in all breast cancer cell groups cocultured with sNK-92 than with NK-92 cells. NK-92 cells stimulated with KIR2DL4 showed elevated cytotoxic activity. The cytotoxic activity of sNK-92 cells on breast cancer cells is via apoptosis pathways. However, their effect on normal breast cells is limited. While the obtained data contains only basic information, additional clinical studies are needed to provide a basis for a new treatment model.

Introduction

Cancer is an aggressive disease in which the body's cells grow uncontrollably that is commonly seen in breast and other tissues [1–2]. Breast cancer is a highly complex, heterogeneous disease with different clinical and biological behaviors and is the leading cancer type among women [3–5]. In 2020, there were 2.3 million women diagnosed with breast cancer worldwide and 685,000 breast-cancer-related deaths. While traditional breast cancer treatment methods (surgery, chemotherapy, and radiotherapy) effectively eliminate cancer cells, they can damage healthy cells. Therefore, immunotherapy-based treatment approaches, considered targeted therapies, are crucial [6].

Cancer cells evade the immune system by expressing antigens that make them appear to be healthy cells [7]. Many studies have attempted to modulate the activator and inhibitor receptors of immune system cells using specific antibodies to overcome this.

Some of these antibodies have received approval from the US Food and Drug Administration (FDA) for use in clinical cancer therapy [8–11]. Immune system checkpoints are critical control steps in the immune response that can be used to detect cancer cells. Drug-candidate antibodies and molecules target these critical control points.

Natural killer (NK) cells play an important role in the immune system's fight against cancer cells. They detect and eliminate cancer cells without requiring activation. NK-92 cells have shown significant cytotoxic effects against cancer cells in preclinical studies. They are the only cell line approved by the

FDA for scientific studies [12]. They do not attack cells with normal major histocompatibility complex-I (MHC-I) expression but attack and eliminate cells that have decreased or absent MHC-I expression. They cause cancer cells to undergo apoptosis by interacting with fas ligand death receptors (FasL) and secreting lytic granules (perforin-granzyme B) [13 14]. They can regulate other immune system cells by secreting key cytokines such as interferon-gamma (IFN- γ) and tumor necrosis factor-alpha (TNF- α). In addition, they can arrest target cell proliferation [15–17].

Various inhibitors and activators of cell surface receptors mediate the cytotoxicity of NK cells. The balance between the signals of these receptors determines NK cell activation or inhibition [18]. The killer Ig-like receptors (KIR) receptor family is an important NK cell surface receptor group and includes both activator and inhibitor receptors [19].

The balance between activation and inhibition signals in NK cells determines their cytotoxicity against cancer cells. When this balance favors activation, NK cells directly or indirectly eliminate cancer cells. However, when the inhibition signal increases, NK cells arrest their cytotoxic effects [20]. Since NK cells induce cancer cell apoptosis without widespread inflammatory effects, they are preferable for therapeutic use.

Unlike other KIR receptors, the KIR2DL4 receptor has inhibitory and activator properties [21–23]. The KIR2DL4 receptor can be activated by recombinant HLA-G (major histocompatibility complex, class I, G) or agonistic antibodies, such as KIR2DL4 clones mAb 33 and mAb 181703 [24–25]. Stimulating the activator signals of NK cells increases their cytotoxicity. This activation can be through the stimulation of the KIR2DL4 receptor with an agonistic antibody. The cytotoxic effect of NK-92 cells stimulated with anti-KIR2DL4 #33 clone on MCF-7 and SK-BR-3 breast cancer cell lines remains to be investigated.

This study investigates the anti-cancer effect of NK-92 cells against MCF-7 and SK-BR-3 breast cancer cell lines under *in vitro* conditions by stimulating their KIR2DL4 receptors with the anti-KIR2DL4 mAb #33 agonistic antibody, which stimulates NK-92 cells to direct breast cancer cells to undergo apoptosis without harming normal breast cells. The apoptotic effect was examined at different target (breast cancer) to effector (NK-92 or stimulated NK-92 [sNK-92]) ratios (1:1, 1:5, and 1:10). This effect evaluated whether apoptosis occurs in cancer cells through intrinsic or extrinsic pathways. In this study, for the first time, sNK-92 cells showed apoptotic and cytotoxic effects on two different breast cancer cells (MCF-7, SK-BR3) without affecting normal breast cells (MCF-12A).

Materials And Methods

Cell culture

Breast cancer cell lines MCF-7 (American Type Culture Collection [ATCC]: HTB-22) and SK-BR-3 (ATCC: HTB-30) were maintained in Dulbecco's modified eagle medium (DMEM; BI, 01-355-1A) and McCoy's 5A modified medium (BI, 01-075-1A) supplemented with 10% fetal bovine serum (FBS; Stem Cell, 04F11678)

and 1% penicillin/streptomycin (Biowest, S12204L0018) in an incubator at 37°C with 5% CO₂. MCF-12A (ATCC: CRL-10782) normal breast cells were maintained in DMEM supplemented with Ham's F12 nutrient mixture, 10% FBS (Stem Cell, 04F11678), 1% penicillin/streptomycin (Biowest, S12204L0018), 20 ng/mL of epidermal growth factor (Genotropin, Pfizer), 0.01 mg/mL of insulin (Sigma, 16634), and 500 ng/mL of hydrocortisone (Gadexon 8 mg, MSpharma) in an incubator at 37°C with 5% CO₂. NK-92 (ATCC: CRL-2407) cells were cultured in TexMACS GMP medium (Miltenyi Biotec, 170-076-309) supplemented with 10% FBS (Stem Cell, 04F11678), 1% penicillin/streptomycin (Biowest, S12204L0018), and 500 IU of interleukin (IL)-2 (Proleukin, Novartis) in an incubator at 37°C with 5% CO₂.

Nk-92 Cells Stimulation By Anti-kir2dl4

NK-92 cells were incubated with anti-KIR2DL4 mAb #33 at 1 µg/mL for 20 hours in an incubator at 37°C with 5% CO₂ [24]. The cells were centrifuged in 200 g and room temperature, and the supernatant was removed. Cells were resuspended in phosphate-buffered saline (PBS) and recentrifuged to eliminate the residue of unstable antibodies, and the supernatant was removed. The immunostaining method was used to detect this antibody's binding to the KIR2DL4 receptor. The cells were resuspended and placed on a coverslip in a 24 well-plate, then incubated for 30 min at 37°C with 5% CO₂. After the supernatant was gently removed, 250 µL of 3.5% paraformaldehyde (PFA) was added to each well to fix the cells. Fluorescein isothiocyanate (FITC) anti-mouse was used as a secondary antibody to view this adhesion. It was incubated for 2 hours at 37°C in a CO₂ environment. Cells on the coverslip were washed with PBS to remove secondary antibody residues. Then, they were incubated with 7-aminoactinomycin D (7-AAD) as a DNA dye (1/500 concentration) for 30 min at 37°C in a CO₂ environment and washed with PBS. Samples covered on a slide with mounting media were examined under a confocal microscope (Carl Zeiss).

Coculturing

Cocultures were performed for cytotoxic and immunostaining experiments (MCF-12A, MCF-7, SK-BR-3: NK-92). For the cytotoxic tests, the experimental plan was performed in seven groups, with six replicates of different cell ratios (1:1, 1:5, 1:10 Target: Effector) for each cell line.

For cytotoxicity tests, MCF-12A, MCF-7, and SK-BR-3 cells were seeded in 96-well sterile cell culture plates at 5×10^3 , 5×10^3 , and 6×10^3 cells/well, respectively, and incubated for 24 hours at 37°C in a CO₂ environment. Cultured NK-92 and sNK-92 cells were centrifuged, and the supernatant was discarded. Then, they were resuspended in the prepared common medium (1:1 cancer cell line medium: NK cell line medium). NK-92 and sNK-92 cells were cocultured with cancer and normal breast cells at ratios of 1:1, 1:5, and 1:10 (Target: Effector cells) for 4 hours at 37°C with 5% CO₂. Then, the supernatant (containing NK-92 cells) was removed from all wells. The wells were washed with PBS, had fresh medium added, and were prepared for the cytotoxicity test.

The cytotoxic test data showed that a 1:10 (Target: Effector) ratio provided the greatest cytotoxicity. Therefore, a 1:10 cell ratio was used in immunostaining experiments. Immunostaining experiments were performed for each cell in two groups and six replicates. For these experiments, MCF-12A, MCF-7, and SK-BR-3 cells were seeded into 24-well cell culture plates as 2.5×10^4 , 2.5×10^4 , and 3×10^4 cells/well, respectively, and incubated for 24 hours at 37°C with 5% CO₂. The supernatant was removed, and cells were washed with PBS. NK-92 and sNK-92 cells were cocultured in a 1:10 (Target: Effector) cell ratio with a common medium (1:1 cancer cell line medium:NK cell line medium) for 4 hours at 37°C in a 5% CO₂ incubator. The supernatant was then removed and washed with PBS. Next, 250 µL of 3.5% PFA (Sigma, 158127) was added to each well and incubated for 25 minutes at room temperature to achieve fixation. The PFA was removed, and cells were washed with PBS. After adding fresh PBS to each well, the samples were stored at + 4°C until immunostaining.

Cytotoxicity Assay

For the cytotoxicity experiments, 10 µl of 2-(4-Iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium sodium salt (WST-1; Takara, MK400) solution was added to each well in 96 well plates. Next, this plate was incubated at 37°C for 4 hours. Then, readings were taken for each cell line at 450 nm in a spectrophotometer (Tecan Infinite 200 PRO). The values were recorded and exported to a Microsoft Excel file.

Granzyme B Assay

MCF-7 and SK-BR-3 cells were inoculated in a 24-well plate in amounts of 25×10^3 . The 24-well plate was incubated in a CO₂ environment for 24 hours at 37°C. The supernatant was drawn off and discarded. NK-92 (sNK-92) cells and NK-92 cells stimulated with the KIR2DL4 antibody were co-cultured at 1:10 (Target: Effector) for 4 hours. After 4 hours the cells were removed and the supernatant was obtained. Lysate of cells remaining on the plate was taken and added to the supernatant. The protocol stated in the Human granzymes B ELISA kit was applied. The specified standards were prepared and the samples were measured spectrophotometrically at 450 nm according to this standard.

Immunostaining

Cells that were ready for immunostaining and fixed on a coverslip were incubated separately with primary antibodies against BAX (Abcam, ab32503, rabbit), caspase 3 (Abcam, ab13847, rabbit), and caspase 9 (Abcam, ab202068, rabbit) for 24 hours at + 4°C. Samples were washed twice with PBS to remove antibody residues. Then the samples were incubated with a FITC-labeled anti-rabbit secondary antibody (Sigma, F9887) for 2 hours at 37°C. After washing twice with PBS, the samples were incubated with the 7-AAD DNA dye at 37°C for 45 minutes. After washing thrice with PBS, the slides were covered with a mounting medium containing Hoechst and examined under a fluorescent microscope. In the

immunostaining experiments, negative controls stained only with the secondary antibody were used to eliminate non-specific interactions.

Western Blot

The MCF-12A, MCF-7, and SK-BR3 cell lines were cocultured with NK-92 and sNK-92 cells at a 1:10 (Target: Effector) ratio for 4 hours. Following coculturing, the NK-92 cells were removed, and the remaining cells were harvested with a scraper. RIPA buffer was used to lyse cells. Proteins were quantified using the Bradford method. Proteins were separated on a gradient gel, loading 10 µg of protein per well. The separated proteins were wet transferred from the gel to a membrane, which was then blocked in 5% bovine serum albumin. The membranes were incubated with primary antibody at + 4°C overnight. Next, they were washed thrice for 10 minutes with Tris-buffered saline containing 0.1% Tween 20 (TBS-T). Then, the membranes were incubated with secondary antibodies for 1.5 hours at room temperature and washed thrice for 10 minutes with TBS-T. Finally, the membranes were visualized using an LI-COR imaging system with an enhanced chemiluminescence substrate. βTubulin was used as the reference protein in all groups.

Statistical analysis

Cytotoxic text optical densities (ODs) were evaluated using a one-way analysis of variance (ANOVA) in GraphPad Prism 9. Shapiro–Wilk and Kolmogorov–Smirnov tests were used to assess the normality of the data distributions. Data were analyzed using one-way ANOVA with Tukey’s multiple comparisons post hoc test. All results with $p < 0.05$ were considered statistically significant.

Result

NK-92 cells stimulated by anti-KIR2DL4

The interaction between NK-92 cells and anti-KIR2DL4 mAb #33 was visualized with a confocal microscope (Fig. 1). The green signal (FITC) observed across the cell membrane shows the sites where the antibody binds to the receptor. Figure 1 shows that the binding was successfully achieved.

Cytotoxicity Assay Result

The effects of sNK-92 and NK-92 cells in cytotoxicity tests were analyzed for MCF-12A, MCF-7, and SK-BR-3 cell lines.

MCF-12A

The cytotoxic effect of NK-92 and sNK-92 cells on the MCF-12A cell line was analyzed with OD values obtained from the WST-1 experimental results. Based on the statistical analyses performed, NK-92 and

sNK-92 cells applied at different concentrations did not have a significant cytotoxic effect on MCF-12A cells compared to the control cells (untreated MCF-12A cells; Fig. C).

MCF-7

A cytotoxic effect was detected for all sNK-92 and NK-92 cell ratios cocultured with MCF-7 breast cancer cells compared to control cells (untreated MCF-7 cells). Cell viability was significantly lower in all treatment groups of sNK-92 cells than of NK-92 cells (Fig. D).

SK-BR-3

A decrease in cell viability was observed for all sNK-92 and NK-92 cell ratios except 1:1 cocultured with SK-BR-3 breast cancer cells compared to the control cells (untreated SK-BR-3 cells). sNK-92 cells showed significantly lower cell viability compared to NK-92 cells at 1:10 ($p < 0.01$) and 1:5 ($p < 0.0001$) ratios. However, NK-92 and sNK-92 cells did not have any effect on SK-BR-3 cells at a 1:1 ratio (Fig. E).

Granzyme B Expression Levels

Our result demonstrated that granzyme B expression levels with sNK-92 were significantly more than NK-92 cells against SK-BR-3 and MCF7 cells. On the other hand, granzyme B expression levels from NK-92 and sNK-92 have no significant difference against MCF-12A. Additionally, NK-92 cells expressed more granzyme against both breast cancer cell lines than normal breast cells. Also, sNK-92 cells express more granzyme against cancer cells compared to normal breast cells. (Fig. 2A and B)

Immunostaining

BAX

MCF-12A, MCF-7, and SK-BR-3 cells were cocultured with sNK-92 and NK-92 cells, and the synthesis of the BAX protein involved in the intrinsic apoptosis pathway was assessed by immunostaining. In normal breast MCF-12A cells, BAX protein synthesis was altered in NK-92 and sNK-92 groups (Fig. 3). BAX protein synthesis was higher in the sNK-92 group than in the NK-92 cell group with the MCF-7 cancer cell line (Fig. 3). Similarly, BAX protein synthesis was higher in the sNK-92 group than in the NK-92 group with the SK-BR3 cancer cell line (Fig. 3).

Caspase 3

Based on fluorescent microscope signals, caspase 3 protein synthesis decreased in MCF-12A cells cocultured with NK-92 and sNK-92 cells (Fig. 4). In MCF-7 cells, caspase 3 protein synthesis was higher in the sNK-92 group than in the NK-92 group (Fig. 4). In SK-BR3 cells, caspase3 protein synthesis was significantly higher in the sNK-92 group than in the NK-92 group (Fig. 4).

Caspase 9

Considering MCF-12A normal breast cells cocultured with NK-92 and sNK-92 cells, increased caspase 9 protein synthesis was not observed in either group compared to the control group (Fig. 5). In MCF-7 cells, caspase 9 protein synthesis was higher in the group cocultured with sNK-92 cells than with NK-92 cells (Fig. 5). In SK-BR3 cells, caspase 9 protein synthesis was higher in the sNK-92 group than in the NK-92 group (Fig. 5).

Western Blots

BAX

BAX protein synthesis was investigated by coculturing NK-92 and sNK-92 cells with normal breast cell MCF-12A cells and breast cancer SK-BR-3 and MCF-7 cells using β -tubulin as the reference protein in all cell groups. In MCF-12A cells cocultured with NK-92 and sNK-92, no difference in BAX protein synthesis was observed in either group. However, in SK-BR-3 and MCF-7 cells cocultured with NK-92 and sNK-92, BAX protein synthesis was elevated in groups cocultured with sNK-92 cells. These results indicate that the synthesis of apoptosis pathway protein BAX was not activated in sNK-92 cells cocultured with normal breast cells, but it was in sNK-92 cells cocultured with cancer cells (Fig. 6a-b).

Caspase 3

Apoptosis pathway protein caspase 3 levels did not increase in MCF-12A cells cocultured with sNK-92 cells compared to NK-92 cells. In addition, coculturing of NK-92 and sNK-92 cells with SK-BR-3 and MCF-7 cancer cells, caspase 3 protein synthesis was elevated in both cell lines' sNK-92 group (Fig. 6a, c).

Caspase 9

Synthesis of the caspase 9 protein, which is involved in the apoptosis pathway, was investigated in cocultures of NK-92 and sNK-92 cells with normal breast cells MCF-12A and breast cancer SK-BR-3 and MCF-7 cells by western blot. In MCF-12 cells, compared to the reference protein β -tubulin, caspase 9 protein synthesis did not differ significantly between the sNK-92 and NK-92 groups. In SK-BR-3 cells, caspase 9 protein synthesis was significantly higher in the sNK-92 group than in the NK-92 group. In MCF-7 cells, caspase 9 protein synthesis did not differ significantly between the sNK-92 and NK-92 groups (Fig. 6a, d).

Discussion

NK cells belong to the innate immune system. They participate as a first defense mechanism by detecting cancer cells [26]. NK cells must activate inhibitory receptors on their surface [27], and the balance between these inhibitory and activating receptors determines NK cell cytotoxicity. The NK cells'

mechanism to detect and destroy cancer cells is critical in immune surveillance. Therefore, the interaction between these cells is important for therapeutic use in NK cell-based cancer immunotherapy.

Activating NK cells with agonist antibodies and increasing their cytotoxicity is a promising approach in cancer immunotherapy [28–30]. NK cells stimulated against target cells are a new approach in immunotherapy. The cytotoxic effect of NK cells on cancer cells after inducing their activating KIR2DL4 receptor had not previously been studied. Therefore, this study enhanced NK-92 cell cytotoxicity on breast cancer cell lines by inducing their KIR2DL4 receptor with an agonistic antibody.

The receptor-antibody interaction is the main focus of our research. In the first step, the antibody's binding to the receptor was shown by immunohistochemistry (Fig. 1). In 2006, Rajagopalan et al. used immunohistochemistry to show that the anti-KIR2DL4 antibody binds to the corresponding activating receptor [22]. This study used the same antibody to activate NK-92 cell activity against breast cancer cells.

The second step investigated the cytotoxic effects of NK-92 and sNK-92 cells on two breast cancer cell lines (SK-BR-3 and MCF-7) and one normal breast cell line. sNK-92 cells had a greater cytotoxic effect on breast cancer cells than NK-92 cells. Moreover, NK-92 cells were non-cytotoxic to normal breast cells at all examined ratios (1:1, 1:5, and 1:10). There was a significant effect with all ratios except 1:1 in the SK-BR-3 cells (Fig. 2C, D and E).

The sNK-92 groups showed greater cytotoxic effects than the NK-92 groups at examined ratios. Their greatest cytotoxic effect in both cancer cell lines was with a 1:10 ratio. The cytotoxic effects of NK-92 cells on cancer cells increased in parallel with their proportion. Inducing the NK-92 cells' KIR2DL4 receptor to enhance their cytotoxicity against cancer cells had not been previously investigated. In addition, previous studies used different receptors to regulate NK cell activity, focusing primarily on stimulating NK cells.

In 2021, Karlitepe et al. investigated the cytotoxic effect of NK cells from umbilical cord blood without stimulation [31]. The results of our study are similar to the cytotoxicity tests of Wang et al. They used NK cells obtained from umbilical cord blood and, unlike our study, used cytokines instead of antibodies to stimulate NK cells. They found that the cytotoxic effects of sNK-92 cells were higher than NK-92 cells [32].

The results of our cytotoxic experiments were similar to those of Turaj et al. Their study showed that NK cells stimulated with CD134 agonist antibodies increased IFN- γ production and cell cytotoxicity. Unlike our study, their experiments were performed on lymphoma cells [33]. In 2012, Kohert et al. studied the cytotoxic effects of NK cells stimulated by CD137 agnostic antibodies on breast cancer cells, showing that using this antibody increased trastuzumab's effectiveness [34].

According to the data obtained from granzyme expression analysis, the amount of granzyme expressed by sNK-92 cells against both cancer cells is significantly higher than the amount of granzyme produced

by NK-92 cells against MCF-7 and SK-BR-3 cancer cell lines. The data obtained show that the cytotoxicity of NK cells increases when they are stimulated. also, both NK-92 and sNK-93 express granzyme to normal breast cells equally. These results indicate that stimulation of NK-92 cells with KIR2DL4 antibody had no cytotoxic effect on normal breast cells. (Fig. 2A and B)

Our immunostaining experiments showed that BAX, caspase 3, and caspase 9 protein quantities increased when MCF-7 and SK-BR-3 cancer cells were cocultured with sNK-92 cells. The higher cytotoxic effect of sNK-92 cells compared to NK-92 cells occurs through apoptotic pathways. In addition, quantities of apoptotic proteins BAX, caspase 3, and caspase 9 did not change when normal breast MCF-12A cells were cocultured with sNK-92 and NK-92 cells. The fluorescence microscope images from the immunostaining experiments showed that sNK-92 cells increased apoptosis protein quantities in cancer cells more than NK-92 cells. The synthesis of these proteins increased with received signals. These results are consistent with our cytotoxic experiments. In 2021, Zhang et al. showed that methionine enkephalin (MENK) pentapeptide activated NK cells against lung cancer. Our study achieved activation using the agonistic anti-KIR2DL4 #33 antibody. They examined the increase in granzyme B and IFN- γ release using enzyme-linked immunosorbent assays. Granzyme B and IFN- γ target the tumor cells' apoptosis pathway. They determined the quantity of apoptosis pathway protein (BAX, BCL2, and caspase 3) in lung cancer cells using a western blot. They showed that the synthesis of these proteins was elevated in cells cocultured with sNK-92 cells but not NK-92 cells. Stimulated NK cells are highly effective against lung cancer cells [35]. Their results are similar to our protein analysis experiment results.

Targeting the receptors on NK-92 cells to increase their cytotoxicity and ensuring their effectiveness against target cells has been the focus of current studies. This study focused on the induction of NK-92 cells with agonistic antibody anti-KIR2DL4 against target cells. Its results are significant and should lead to further studies and shed light on in vivo and phase studies. The sNK-92 cells did not show any cytotoxic effect on normal breast cells (MCF-12A). Therefore, the autologous and allogeneic use of sNK-92 cells has the potential to be translated into the clinic after the completion of preliminary studies.

Conclusions

This study showed that NK-92 cells stimulated with anti-KIR2DL4 mAb#33 had higher cytotoxicity than NK-92 cells on HER2+/HER – breast cancer cells. Furthermore, sNK-92 cells acted specifically on cancer cells without significant cytotoxic effects on normal breast cells (MCF-12A). Our results showed that MCF-7 and SK-BR-3 cell death was achieved by increasing the synthesis of proteins BAX, caspase 3, and caspase 9 in the apoptosis pathway.

Declarations

Authors' contributions

MD and NK designed the experiments, developed the methodology analyzed results, prepared data presentations, and wrote and reviewed the draft manuscript. EY and IK analyzed results and reviewed the draft manuscript; IK was responsible for project administration and provision of study materials and equipment. MD was the project supervisor.

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Data availability

All datasets used or analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests, The authors do not declare any conflict of interest.

Ethical approval Not applicable

Consent to participate Authors agreed to participate in this research.

Consent for publication Authors has approved the last version of the manuscript for its submission.

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Figures

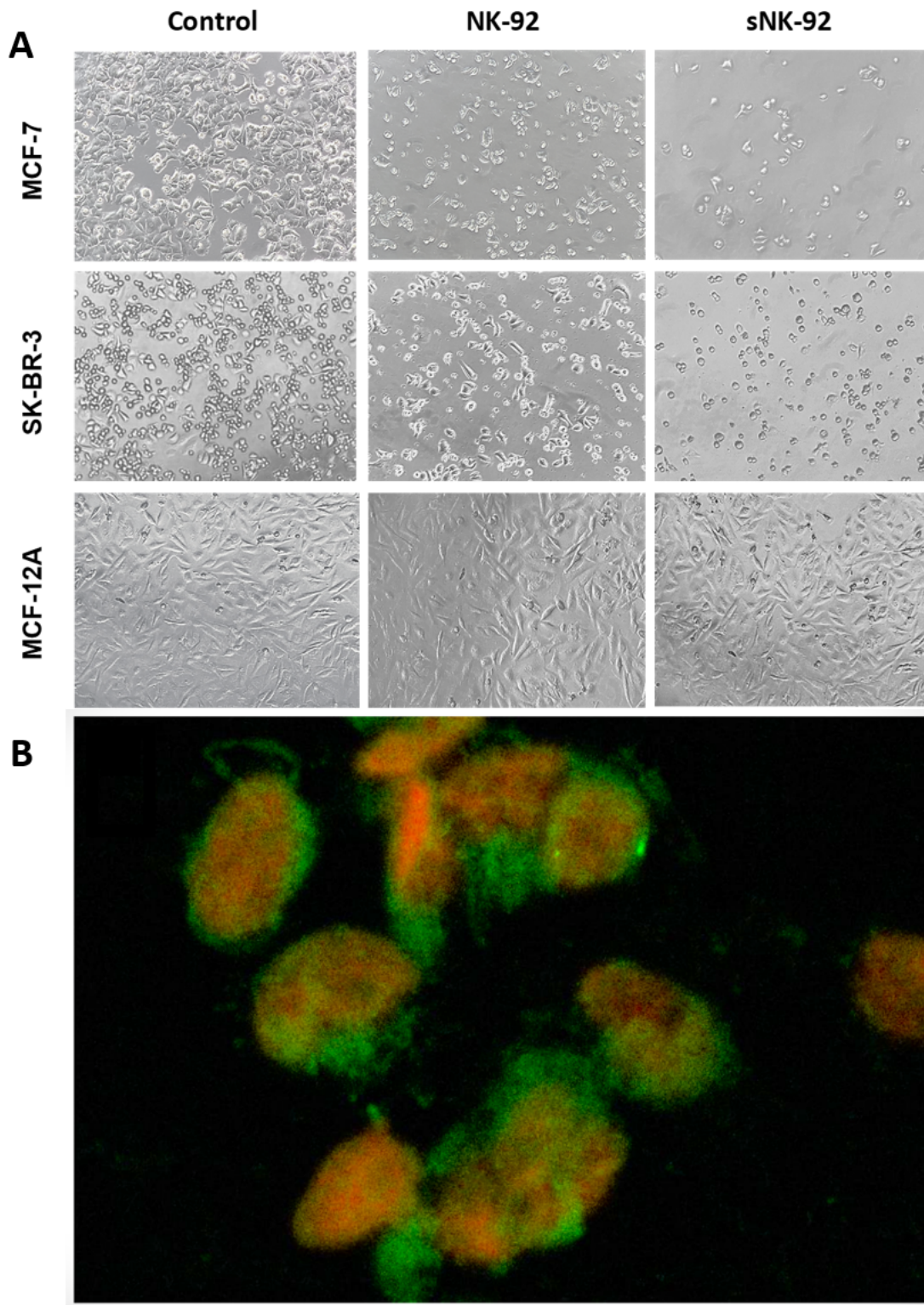


Figure 1

DIC cell line images. MCF-7, SK-BR-3 and MCF-12A as a control and coculture with Nk-92 and sNK-92 cells with 1:10 (Target: Effector) ratio (10×). (e) NK-92 cells were incubated with an anti-KIR2DL4 antibody for 20 hours. After fixation, the binding of the antibody to the KIR2DL4 receptor on the cell surface was visualized with a confocal microscope (63×). A nuclear DNA stain (7-AAD) and a secondary

FITC antibody were used to show the KIR2DL4 antibody. The green signal received indicates the antibody's adhesion to the relevant membrane receptor, while the red signal indicates nuclear localization.

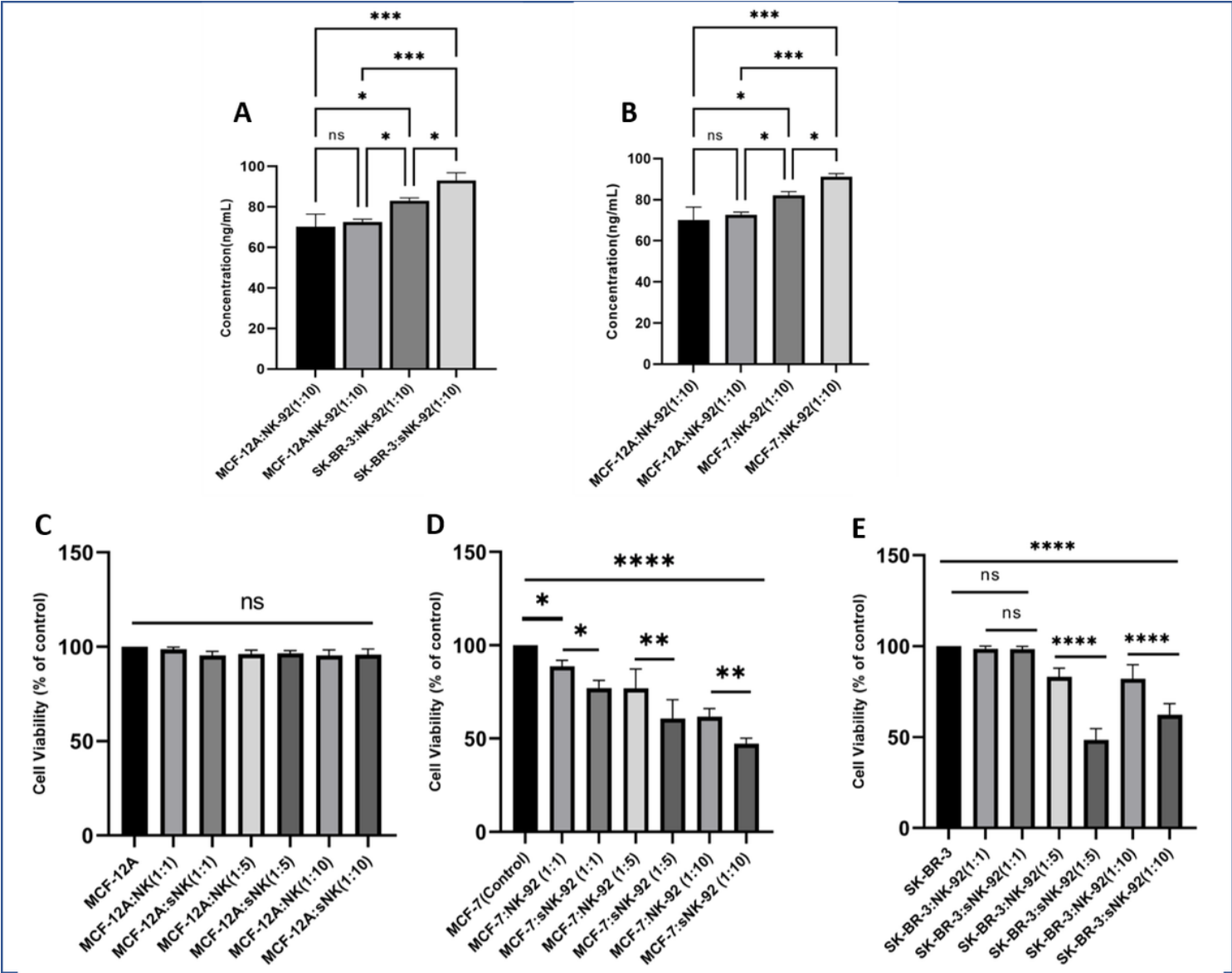


Figure 2

(a) MCF-12A. (b) MCF-7. (c) SK-BR-3. Cell line viability percentages after coculture with NK-92 and sNK-92 cells were determined with the WST-1 cytotoxicity assay. Cancer cells and NK-92 or sNK-92 cells were cocultured at ratios of 1:1,1:5, or 1:10. Key: *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$; ****, $p<0.0001$.

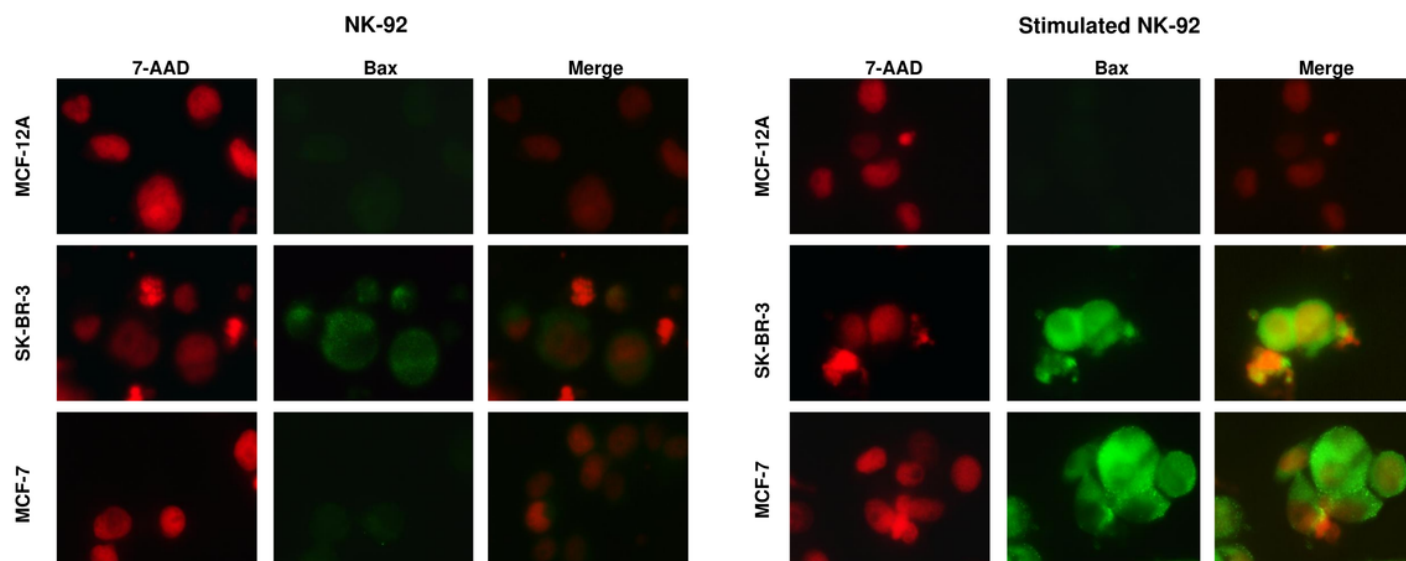


Figure 3

Fluorescent microscope images (100×) of BAX protein synthesis in normal breast and breast cancer cells after coculturing with NK-92 and sNK-92 cells. The nuclear DNA dye (7-AAD) is shown in red, and the FITC-labeled secondary antibody is shown in green.

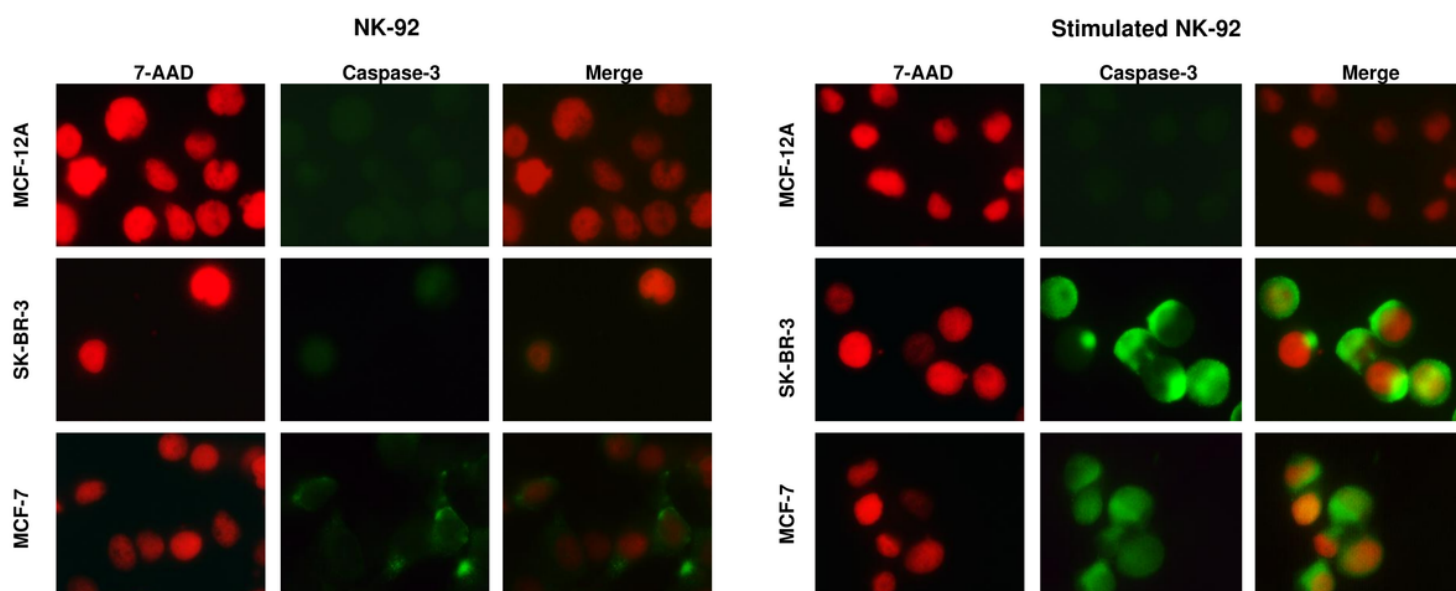


Figure 4

Fluorescent microscope images (100×) of caspase 3 protein synthesis in normal breast and breast cancer cells after coculturing with NK-92 and sNK-92 cells. The nuclear DNA dye (7-AAD) is shown in red, and the FITC-labeled secondary antibody is shown in green signal.

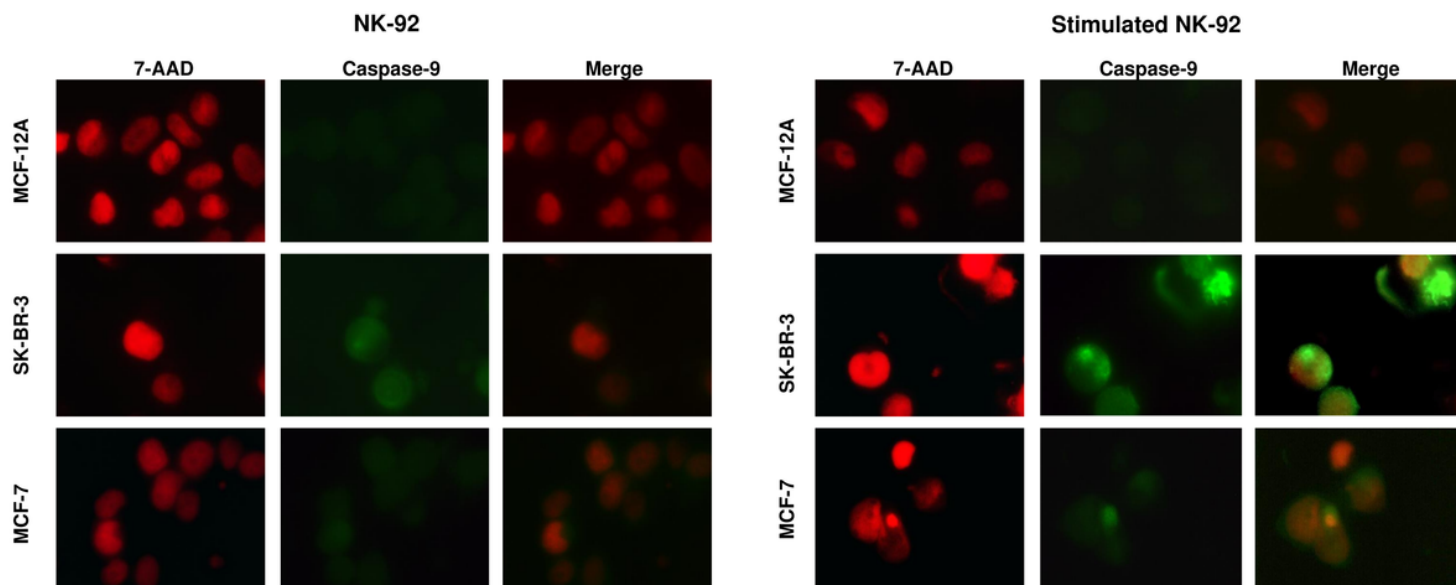


Figure 5

Fluorescent microscopy images (100×) of caspase 9 protein synthesis in breast cancer and normal breast cells after coculturing with NK-92 and sNK-92 cells. The nuclear DNA dye (7-AAD) is shown in red, and the FITC-labeled secondary antibody is shown in green.

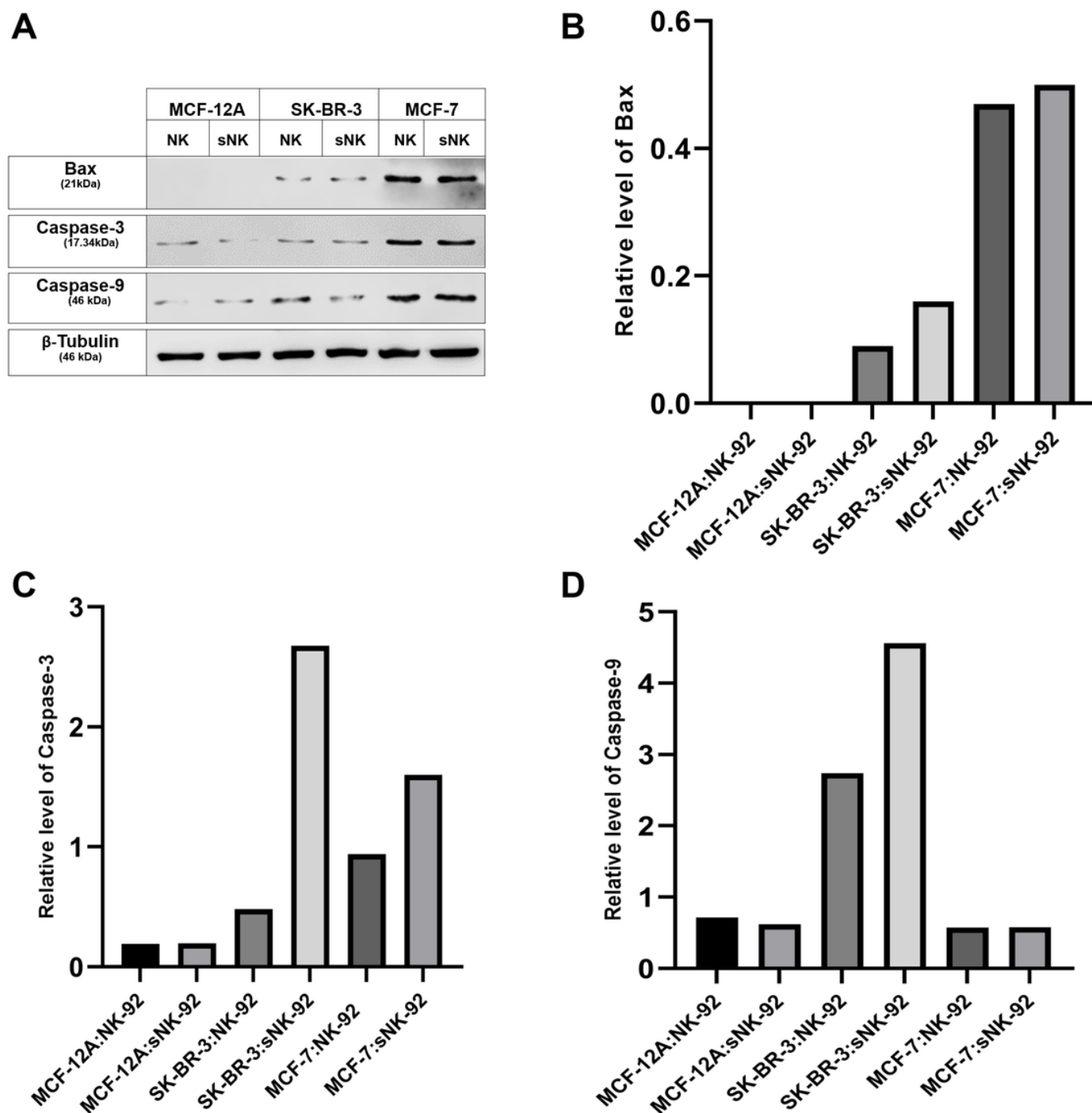


Figure 6

Western blot analyses. (a) Western blot analysis of the effect of NK-92 and sNK-92 cells on BAX, caspase 3, and caspase 9 protein synthesis in breast cancer SK-BR-3 and MCF-7 cell lines and the normal breast MCF-12A cell line. (b) BAX, (c) caspase 3, and (d) caspase 9 amounts relative to β -tubulin in different groups