



Ketamine used as an acesodyne in human breast cancer therapy causes an undesirable side effect, upregulating anti-apoptosis protein Bcl-2 expression

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ABSTRACT. Ketamine is a dissociative anesthetic agent that has been widely used in surgery and for relieving pain in chronic cancer patients. We applied ketamine to breast cancer cell line MDA-MB-231 to detect the effect of treatment and molecular mechanisms involved. We found that ketamine can upregulate the level of anti-apoptosis protein Bcl-2, which promotes breast cancer cell invasion and proliferation. Knockdown of Bcl-2 could inhibit the increase of Bcl-2 and reduce the invasion and proliferation caused by ketamine in human breast cancer cells. Our findings provide new insight into the effects of ketamine in cancer treatment; we suggest that ketamine, which has been widely used in cancer operations and for relieving pain in chronic cancer patients, may be not the best choice because it can worsen the cancer through promotion of anti-apoptosis.

Key words: Ketamine; Bcl-2; Breast cancer

INTRODUCTION

Breast cancer is one of the main causes of female cancer death and the most common form of cancer in the world. Invasive growth, metastatic capability, and rapid proliferation are the key reasons for breast cancer being so refractory and stubborn (Adams and Cory, 1998). Curing breast cancer requires the clarification of the mechanisms of breast tumor invasion, metastasis, and proliferation and establishment of appropriate blocking channels based on the previous research. The B-cell lymphoma 2 (Bcl-2) protein is a conserved member of the Bcl-2 family and plays an important role in regulating apoptosis (Borner, 2003). Bcl-2 activity is associated with γ -radiation-induced cell death (Sentman et al., 1991). Bcl-2 may also function in an antioxidant pathway that inhibits lipid peroxidation (Hockenbery et al., 1993). An association between the ras gene and Bcl-2 has been reported, suggesting a possible role of the ras gene family in regulating cell death (Haldar et al., 1989; Fernandez-Sarabia and Bischoff, 1993). Members of the Bcl-2 family are critical death regulators that reside immediately upstream of the mitochondria (Adams and Cory, 1998). Bcl-2 is closely related to tumor generation. High Bcl-2 levels are correlated with a worse survival rate in leukemia (Robertson et al., 1996). In high-grade gliomas, the Bcl-2 gene family shows upregulated expression in tumors exhibiting recurrence and progression (Krajewski et al., 1997; Strik et al., 1999). The anti-apoptotic effect of Bcl-2 may shift tumors to anaplastic phenotype-necrosis, a cardinal feature of high-grade gliomas (Stegh et al., 2008).

Ketamine is a dissociative anesthetic agent that has been widely used in a variety of surgical procedures. The utility of ketamine as an anesthetic has been hampered by its troublesome psychomimetic effects. Previous studies have shown that analgesia can be produced with subhypnotic doses of intravenous ketamine with a lower frequency of psychomimetic reactions to provide relief for patients experiencing intractable cancer pain (Backonja et al., 1994; Felsby et al., 1996; Ilkjaer et al., 1996; Warncke et al., 1997; Bell et al., 2003; Slatkin and Rhiner, 2003).

As an anesthetic agent or anodyne, ketamine and its side effects have been studied thoroughly, but insufficient research has been carried out to explore the effect of ketamine on cancer therapy. Our results indicated that ketamine might reduce apoptosis in the breast cancer cell line MDA-MB-231. Ketamine promoted the invasion and proliferation of cancer cells, which mediated the upregulation of Bcl-2 expression that resisted cell-programmed apoptosis. To determine whether the side effect of ketamine is caused by Bcl-2, we performed a rescue experiment. We found that knockdown of Bcl-2 rescued the increase in Bcl-2 caused by ketamine, inhibiting invasion and proliferation in MDA-MB-231. Our study provides a new insight into ketamine side effects in cancer therapy. These results highlight a need to determine whether other anesthetic agents or anodynes have this side effect so that safer pain relievers can be chosen for intractable cancer pain.

MATERIAL AND METHODS

Cell line and culture

MDA-MB-231 cells were purchased from the Cell Bank Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China) and cultured in 1640 medium supplemented with 10% fetal bovine serum (HyClone, Logan, UT, USA), 100 U/mL penicillin (Gibco BRL, Paisely, Scotland), and 100 μ g/mL streptomycin (Gibco BRL), and grown in an incubator at 37°C in a humidified atmosphere containing 5% CO₂.

Transwell assay

Invasion assays were carried out using a modified transwell chamber system as follows: cells (2×10^5) were seeded on 500 ng/mL Matrigel-coated (BD Bioscience-Laware, Bedford, MA, USA) membrane inserts with a pore size of 8 mm (BD Bioscience, San Jose, CA, USA). The same medium was placed in the lower well. The cells were then incubated for 24 h in the presence of ketamine dissolved in 0.9% NaCl or 0.9% NaCl without ketamine as a negative control. The cells on the upper side were scraped off. The cells that had migrated into the lower compartment were fixed (4% paraformaldehyde in phosphate-buffered saline), stained with 4',6-diamidino-2-phenylindole, and counted in 5 random high power fields at 200X magnification in each well.

Methyl thiazolyl tetrazolium (MTT) assay

Cells were cultured on 96-well plates for varying periods of time and exposed to fresh media every other day. The cells were treated with MTT (50 μ g per well; Sigma, St. Louis, MO, USA). The generated formazan was dissolved with dimethyl sulfoxide and measured at 570 nm to assess cell viability.

Vector constructs

The details of the creation of RCAS-Bcl-2 are described elsewhere (Ikegaki et al., 1994).

Western blotting analysis

Cells were lysed with lysis buffer (0.5 mL 0.5 M Tris-HCl, pH 6.8, 0.1 g dithiothreitol, 0.1 g sodium dodecyl sulfate, 0.005 g bromophenol blue, and 0.5 mL glycerine). Equal amounts of protein were loaded on 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis and then transferred to a nitrocellulose membrane. The membrane was incubated with primary antibodies for 12 h at 4°C against Bcl-2 and β -actin (Cell Signaling). After the membrane was incubated with appropriate secondary antibodies, signals were visualized with enhanced chemiluminescence.

Transfection assays

For transfection, cells were grown to 80% confluency and transfected with Bcl-2 small interfering RNA (siRNA; Santa Cruz, Bcl-2 siRNA (h): sc-29214) using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA), according to manufacturer recommendations.

Quantitative real-time polymerase chain reaction (PCR)

Total RNA isolated using Trizol (Sigma) was subsequently reverse transcribed to complementary DNA using Oligo(dT) 12-18 Primer from 2 μ g total RNA. The quantitative real-time PCR primer sequences were as follows: Bcl-1 (forward: 5'-CTGCACCTGACGCC TTCACC-3'; reverse: 5'-CACATGACCCCACCGAACTCAAAGA-3'); β -actin (forward: 5'-AGCGAGCATCCCCAAAGTT-3'; reverse: 5'-GGGCACGAAGGCTCATCATT-3'). The

reactions were placed on a 96-well plate using an Mx3000P system (Stratagene). The amount of target gene expression ($2^{-\Delta\Delta Ct}$) was normalized using the endogenous β -actin reference.

Statistical analysis

The results were evaluated using the Student *t*-test for unpaired data. Statistical significance was defined as values of $P < 0.05$, $P < 0.01$, and $P < 0.001$. Values are reported as means \pm SD.

RESULTS

Ketamine promotes the invasiveness of MDA-MB-231 cells

To study the effect of ketamine on the invasion capability of MDA-MB-231 cells, we performed a transwell invasion assay in a dosage experiment. We prepared the ketamine solution using 0.9% NaCl solution. MDA-MB-231 cells treated with ketamine (1, 10, and 100 μ M) for 24 h were plated in the upper chamber, and the cells that moved to the underside of the coated membrane were counted. The results showed that the number of cells that invaded the lower chamber was significantly increased approximately 1-fold by the 24-h ketamine treatment. Such a significant increase was treated with the concentration of 100 μ M ketamine (Figure 1). We found that ketamine may be harmful for breast cancer therapy owing to its promotion of MDA-MB-231 cell invasiveness.

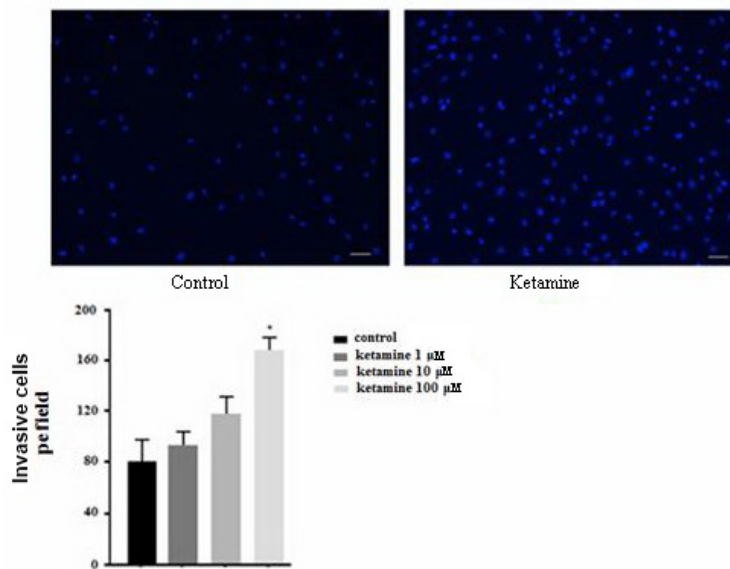


Figure 1. Ketamine promotes the invasiveness of MDA-MB-231 cells. MDA-MB-231 cells were treated with ketamine (1, 10, 100 μ M) for 24 h and detected by transwell invasion assays. Representative photomicrographs of ketamine (100 μ M, top) showing membrane-associated cells assayed using DAPI staining (200X). Quantitative analysis (bottom) of the invasion (1, 10, 100 μ M) in MDA-MB-231 cells. All data are reported as means \pm SD for 3 independent experiments. * $P < 0.05$. Control = cells treated with only 0.9% NaCl without ketamine.

Ketamine can promote the proliferation of MDA-MB-231 cells

Our MTT assay also detected the effect of ketamine on MDA-MB-231 cell proliferation, and we found that ketamine promoted proliferation (Figure 2). We also studied whether ketamine affected the migration of MDA-MB-231 cells and found that it did not promote migration significantly (data not show). These results indicated that ketamine may enhance the proliferation and invasion of breast cancer cells significantly. According to these results, we concluded that ketamine might be an inappropriate choice to relieve pain because of these side effects.

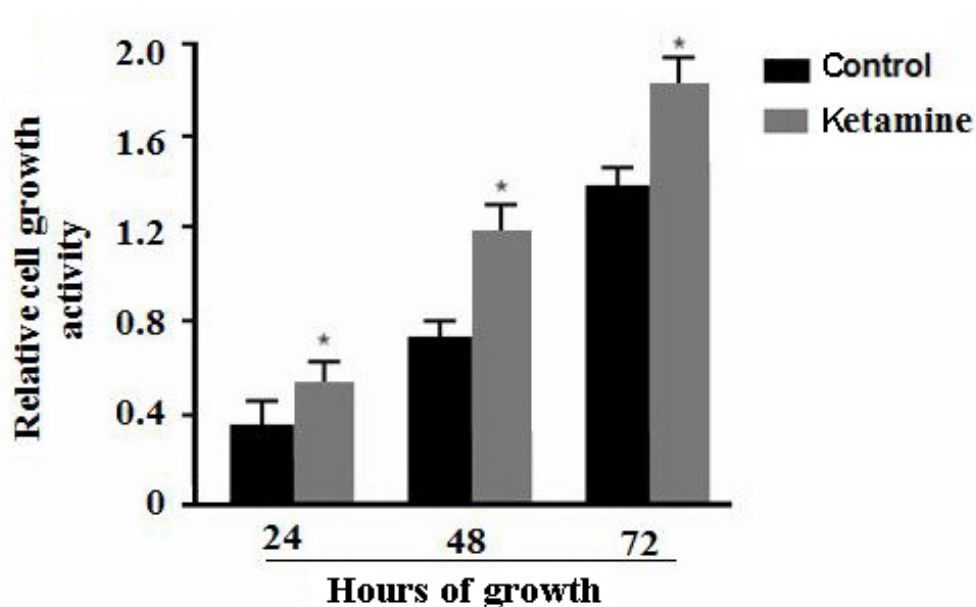


Figure 2. Effect of ketamine on MDA-MB-231 cell proliferation. Cell growth activity of MDA-MB-231 cells at the indicated times as determined by the methyl thiazolyl tetrazolium assay. MDA-MB-231 cells were treated with ketamine (100 μ M) for 24 h and detected on 24, 48, 72 h. Relative cell growth activity is shown. All data are reported as means \pm SD for 3 independent experiments. * $P < 0.05$. Control = cells treated with 0.9% NaCl without ketamine.

Ketamine upregulates expression of Bcl-2 in MDA-MB-231 cells

We sought the mechanism of this side effect and found that Bcl-2 mRNA and protein levels can be consistently upregulated in MDA-MB-231 cells treated with ketamine compared with effects in negative controls (0.9% NaCl alone; Figure 3A). Overexpression of Bcl-2 promoted MDA-MB-231 cell invasion (Figure 3B). Bcl-2 also increased the proliferation of cancer cells, which has been demonstrated by previous research (Fanidi et al., 1992) (Figure 3C).

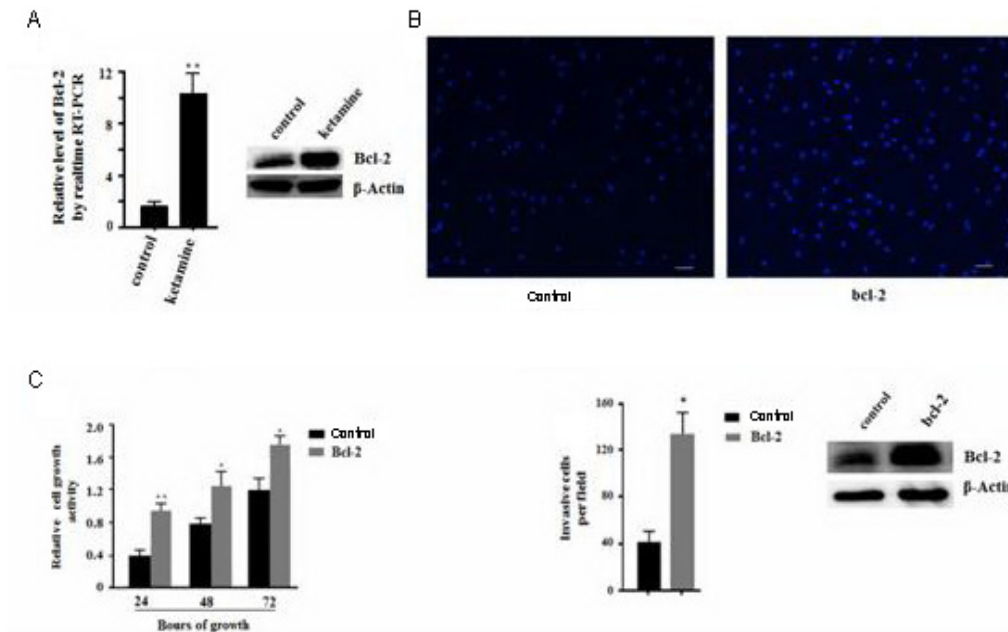


Figure 3. Ketamine upregulates expression of Bcl-2 in MDA-MB-231 cells. **A.** Analysis of Bcl-2 expression in cells treated with ketamine (100 μ M) and harvested at 48 h. The protein level of Bcl-2 is upregulated (shown on the right), and the mRNA level is shown on the left. The quantification of 3 independent experiments is shown at the bottom. All data are reported as means \pm SD for 3 independent experiments. ** $P < 0.01$. Control = 0.9% NaCl without ketamine. **B.** Bcl-2 can promote the invasion of MDA-MB-231 cells. Representative photomicrographs showing membrane-associated cells assayed using DAPI staining (200X, top). Quantitative analysis of the invasion of MDA-MB-231 cells is shown at the bottom. Control = empty vector. **C.** Detection of the effect of Bcl-2 on proliferation of MDA-MB-231 cells. Relative cell growth activity is shown. All data are reported as means \pm SD for 3 independent experiments. * $P < 0.05$, ** $P < 0.01$. Control = empty vector.

Ketamine enhances the invasion and proliferation capabilities of MDA-MB-231 cells by upregulating Bcl-2

To determine whether ketamine enhanced the invasion and proliferation of MDA-MB-231 cells by upregulating Bcl-2, we transfected Bcl-2 siRNA into MDA-MB-231 cells treated with ketamine to downregulate the increase of Bcl-2 caused by ketamine. We found that the invasion capability of the cancer cells was similar to that of cells treated with negative siRNA control and 0.9% NaCl without ketamine (Figure 4A). We also found that the proliferation of the cancer cells was similar to that of cells treated with negative siRNA control and 0.9% NaCl without ketamine (Figure 4B). The rescue experiments showed that ketamine enhanced the invasion and proliferation capabilities of MDA-MB-231 cells by upregulating Bcl-2.

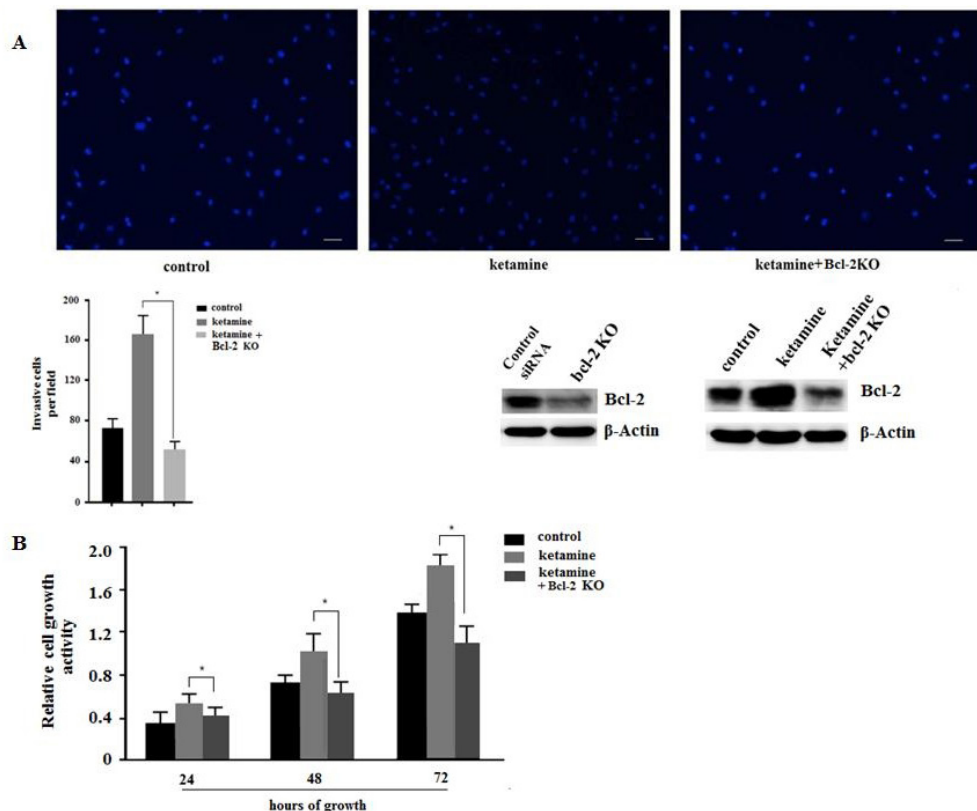


Figure 4. Knockdown of Bcl-2 can reverse the effect of ketamine on invasion and proliferation of MDA-MB-231 cells. **A.** Representative images are shown on the top, and the quantification is shown at the left bottom. Expression of Bcl-2 on protein level is shown at the right bottom. Bcl-2 KO = knockdown of Bcl-2 by transfecting Bcl-2 siRNA. Control siRNA means negative siRNA control. All data are reported as means \pm SD for 3 independent experiments. * $P < 0.05$, ** $P < 0.01$. **B.** Cell proliferation was analyzed by MTT. Control = cells transfected with siRNA negative and treated with 0.9% NaCl without ketamine. All data are reported as means \pm SD for 3 independent experiments. * $P < 0.05$.

DISCUSSION

Breast cancer incidence is high in women and is the second most frequent cause of mortality in women worldwide. The mechanism of breast cancer generation is complex and remains the subject of debate. It may involve multiple factors. Bcl-2 is regarded as a crucial oncogene that is closely related to tumor generation. Bcl-2 is an anti-apoptosis gene that allows tumor cell escape from programmed apoptosis. Although independent expression of Bcl-2 does not result in tumor formation, overexpression of the Bcl-2 gene has been found in a variety of human malignancies (Ikegaki et al., 1994; Monni et al., 1997; Andrews et al., 2004).

Ketamine has been used for more than 30 years to produce anesthesia. For early experience, ketamine can produce analgesia that sometimes well outlasts its anesthetic effects. The mechanisms of the analgesic effects of ketamine are debated (Felsby et al., 1996; Ilkjaer

et al., 1996; Warncke et al., 1997). Ketamine can be used as a preemptive analgesic (Wong et al., 1997; McCall et al., 2007) for cancer-related pain (Roytblat et al., 1993; Clark and Kalan, 1995; Mercadante et al., 2000) or the diffuse pain of fibromyalgia (Sorensen et al., 1995). Any chemical may have more than one kind of function. Ketamine not only has anesthetic properties but also exerts other effects through action in the cell signaling pathway.

This study revealed that ketamine can promote breast cancer cell line MDA-MB-231 invasion and proliferation by upregulating the expression of Bcl-2. We also found that Bcl-2 can affect the invasion capabilities of MDA-MB-231. Knockdown of Bcl-2 reduced the increase of Bcl-2 caused by ketamine and inhibited the invasion and proliferation of MDA-MB-231 cells. We concluded that the proliferation may be caused by a reduction in apoptosis induced by ketamine. The invasion capability might occur through several other pathways related to Bcl-2. Therefore, using ketamine to relieve the pain of chronic cancer patients may be harmful, because ketamine can upregulate Bcl-2 expression, reducing apoptosis and promoting the growth of cancer cells. The mechanism through which ketamine affects expression of Bcl-2 is unknown. It may occur through limitation of inhibitors of Bcl-2 or activation of the transcription of Bcl-2. All evidence currently comes from *in vitro* studies, so the effects in patients must be examined.

Our study provides a new insight into the side effects of ketamine in cancer therapy. We should not only study these side effects in relation to the promotion of cancer growth but also investigate whether other anesthetics have this side effect or can inhibit cancer generation. More studies are also needed to elucidate the mechanism of this effect and discover anesthetic agents and anodynes that are safer than ketamine.

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