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Ketamine inhibits colorectal cancer cells malignant potential via blockage of NMDA receptor

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ABSTRACT

Ketamine, a common *N*-methyl-D-aspartate receptor (NMDAR) antagonist, is an option for cancer pain treatment in clinical practice. Ketamine has been shown to have the capacity to attenuate cancer cells malignancy. However, the underlying mechanism remains elusive. In the present study, we reported that ketamine inhibited the malignant potential of colorectal cancer cells and investigated the possible mechanisms involved. Ketamine suppressed the expression of VEGF, HIF-1α, p-AKT, p-ERK, and p-CaMK II, and reduced intracellular Ca^{2+} level in a concentration dependent manner (1, 5, 10 μg/ml). Furthermore, AP5 and MK801 (NMDAR inhibitors), and KN93 (CaMK II inhibitor), decreased the expression of VEGF, HIF-1a, p-AKT, p-ERK, and p-CaMK II, which were similar to the effect of ketamine. Further, the anti-tumor effect of ketamine was reversed by D-serine (NMDAR activator). Ketamine did not affect NMDA receptor expression, however knockdown of NMDA receptor using siRNA attenuated the effect of ketamine on cell migration. Collectively, these findings demonstrated that ketamine attenuated the expression of VEGF and cell migration ability in colorectal cancer cells, probably via blockage of NMDA receptor.

1. Introduction

Cancer is one of the leading causes of death globally. At present surgery remains to be the primary selection for the treatment of most solid tumors. However, a plethora of evidences indicated that surgical stress could impact the body's immune and neuroendocrine systems (Boomsma et al., 2010; Tsuchiya et al., 2003), resulting in inadvertent seeding of cancer cells intra-operatively (Camara et al., 2006; Mori et al., 1996), and thus influencing the outcome of cancer patients. Colorectal cancer is one of the most common gastrointestinal malignancies and is the third cause for cancer related death worldwide (Siegel et al., 2016). Surgery is the prior option for the treatment of regional and liver metastasis from colorectal cancers (Brenner et al., 2014; Ferlay et al., 2015). However, postoperative local recurrence and distant metastases are still serious problems to be urgently solved (Caricato et al., 2006).

Hypoxic inducible factor 1α (HIF1-α) was found to be aberrantly expressed in various human cancers (Talks et al., 2000). Moreover, upregulation of HIF1-α level is closely related to tumor cell proliferation (Terraneo et al., 2010; Wang et al., 2011), vascularization (Ban et al., 2010) and migration (Burrows et al., 2011), thus leading to a poorer prognosis for cancer patients. It was reported that ERK and AKT are involved in the activation of HIF-1α (Huang et al., 2014; Liu et al., 2010).

Ketamine, one of the most common *N*-methyl-D-aspartate receptor (NMDAR) antagonists, is an option for cancer pain treatment in clinical practice (Zgaia et al., 2015). Recently it has been indicated that ketamine affects the proliferation and apoptosis of many cancers, including pancreatic cancer (Malsy et al., 2015), lung adenocarcinoma (Zhou et al., 2018) and hepatocellular carcinoma (Yamaguchi et al., 2013). However, the anti-tumor mechanism of ketamine is still poorly understood. In the present study, we mainly focus on the mechanism by which ketamine inhibits the malignant potential in colorectal cancer cells.

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2. Materials and methods

2.1. Cell culture and reagent

Human HT29 (ATCC®HTB-38TM) and SW480 (ATCC®CCL-228TM) colorectal cancer cells, purchased from the American Type Culture Collection (ATCC), were cultured in DMEM with 10% fetal bovine serum (FBS) at 37 °C in a humidified 5% CO_2 incubator. Cells were subcultured when reaching 90% confluence.

KN93, an inhibitor of Ca^{2+}/calmodulin dependent protein kinases (CaMK II), AP5 and MK801, inhibitors of NMDAR, and D-serine, an activator of NMDAR, were purchased form Sigma (St. Louis, MO). The substances employed in the present study were diluted by DMSO (St. Louis, MO).

2.2. Western blot analysis

Whole cell protein extracts were obtained by cell lysis buffer (Cell Signaling Technology, Danvers, MA). Same amounts of proteins obtained from different groups of cells were loaded and separated by 8 or 10% SDS-PAGE and transferred to PVDF membranes. After being incubated in 5% non-fat dry milk solution to block the non-specific binding, the membranes were incubated with specific primary antibody at 4 °C overnight. The primary antibodies used were monoclonal antibodies against β-actin (Cell Signaling Technology, Danvers, MA), extracellular regulated protein kinases (ERK) (Santa Cruz Biotechnology, Santa Cruz, CA), p-ERK (Santa Cruz Biotechnology, Santa Cruz, CA), Ca^{2+}/calmodulin dependent protein kinases II (CaMK II) (Cell Signaling Technology, Danvers, MA), p-CaMK II (Santa Cruz Biotechnology, Santa Cruz, CA), vascular endothelial growth factor (VEGF) (Abcam, Cambridge, UK), HIF-1α (Cell Signaling Technology, Danvers, MA), and NMDAR (Cell Signaling Technology, Danvers, MA). Thereafter, the primary antibodies were washed away with tris-buffered saline containing Tween 20, and the membranes were incubated with corresponding secondary antibodies for 1 h at room temperature. Subsequently, the membranes were washed and the specific protein bands were detected by the ECL system. The respective densities of the protein bands were analyzed by Scan-gel-it software. In the present study, β-actin was used as a loading control in whole cell extracts.

2.3. Real-time PCR analysis

Total RNA was extracted with TRNzol (Tiangen, Beijing, China). cDNA was synthesized by reverse transcription with a TaKaRa PrimeScript RT Regent Kit. Real-time PCR reactions were carried out using a QuantStudio 7 Flex Real-Time PCR System (Applied Biosystems) and the following primers: NMDAR: 5′-AGCCCCATCATTCTTCTTTACT GTACCAAG-3′ (forward) and 5′-CCTTTCCCACTTCCTCTCCTTGTT CAG-3′ (reverse); β-actin: 5′-CTAACTTGCGCAGAAAACAAGAT-3′ (forward) and 5′-TTCCTGTAACAACGCATCTCATA-3′ (reverse).

2.4. Small interfering RNAs transfection

Small interfering RNAs (siRNAs) against human NMDAR were purchased from GenePharma (Shanghai, China). The sequences of siRNAs against NMDAR were as follows: si-NMDAR-1, 5′-CCTCTATGATAATGG CAGATACTCGAGTATCTGCCATTATCATAGAGG-3′; si-NMDAR-2 5′-GCA CCCGAAACTGGTGATAATCTCGAGATTATCACCAGTTTCGGGTGC3′.
The sequence of control siRNA (NC) was 5′-GATCTGGACACCCATG ACT-3′. siRNA transfection was conducted using lipofectamine 3000 regent (Thermo Fisher Scientific, Waltham, USA) according to the manufacturer's instructions.

2.5. Calcium (Ca^{2+}) concentration assay

Intracellular free calcium concentration was detected by Fluo-3 AM (Beyotime biotechnology, Shanghai, China) according to the manufacturer's instructions. Briefly, after treatment, cells were harvested by scraping and washed twice with PBS. Then cells were suspended with 5 μM Fluo-3 AM for 30 min in the dark to allow de-esterification. Intracellular calcium concentration was detected by flow cytometer, via calculating the ratio of 510 nm emission, in response to 340/380 nm excitation. Data were expressed as fluorescence intensity.

2.6. Migration assay

Migration assay was determined with the use of a filter of transwell system (8.0 μm pore size; BD Biosciences, SanDiego, CA, USA). After corresponding treatment, HT29 (10×10^4 viable cells) and SW480 (8×10^4 viable cells) colorectal cancer cells with 200 μl FBS-free DMEM were joined in the upper chamber of transwell inserts and 800 μl of DMEM with 10% FBS was joined in the lower chamber. Then cells were incubated with the standard culture conditions for 16 h for migration assay. Residual cells on the top of membrane were cleared away and the migrated cancer cells on the bottom of membrane were stained by 0.1% crystal violet (Sigma, St. Louis, MO) for calculating analysis.

2.7. Statistical analysis

In the present study, results were obtained from 5 independent experiments and expressed as mean ± S.D. Sample size (N) represents the times of repeated experiments. Statistical comparison was performed with one-way analysis of variance followed with Bonferroni-corrected pairwise comparisons. $P < .05$ was considered significant.

3. Results

3.1. Ketamine attenuated the expression of VEGF and cell migration in HT29 and SW480 colorectal cancer cells

In HT29 and SW480 cells, ketamine attenuated VEGF expression (Fig. 1A, B) and migration (Fig. 1C) in a concentration dependent manner. Compared with control group, incubation of cells with 5 μg/ml ketamine significantly inhibited VEGF expression and cell migration in HT29 and SW480 cells (Fig. 1).

3.2. Ketamine attenuated the expression of HIF-1α, the phosphorylation of ERK and AKT in HT29 and SW480 cells

In HT29 and SW480 cells, ketamine suppressed the expression of HIF-1α (Fig. 2A, B), and phosphorylation of ERK and AKT (Fig. 2A, C, D) in a concentration dependent manner. Compared with control group, incubation of cells with 5 μg/ml ketamine significantly inhibited the expression of HIF-1α, and phosphorylation of ERK and AKT (Fig. 2).

3.3. Ketamine attenuated the phosphorylation of CaMK II and Ca^{2+} level in HT29 and SW480 cells

In HT29 and SW480 cells, ketamine attenuated the phosphorylation of CaMK II (Fig. 3A, B) and reduced intracellular Ca^{2+} level (Fig. 3C) in a concentration dependent manner. Compared with control group, incubation of cells with 5 μg/ml ketamine significantly inhibited the phosphorylation of CaMK II and intracellular Ca^{2+} level (Fig. 3). Thereafter, 5 μg/ml ketamine was employed in the next experiments to explore the potential signaling pathways involved in the antitumor effects of ketamine.

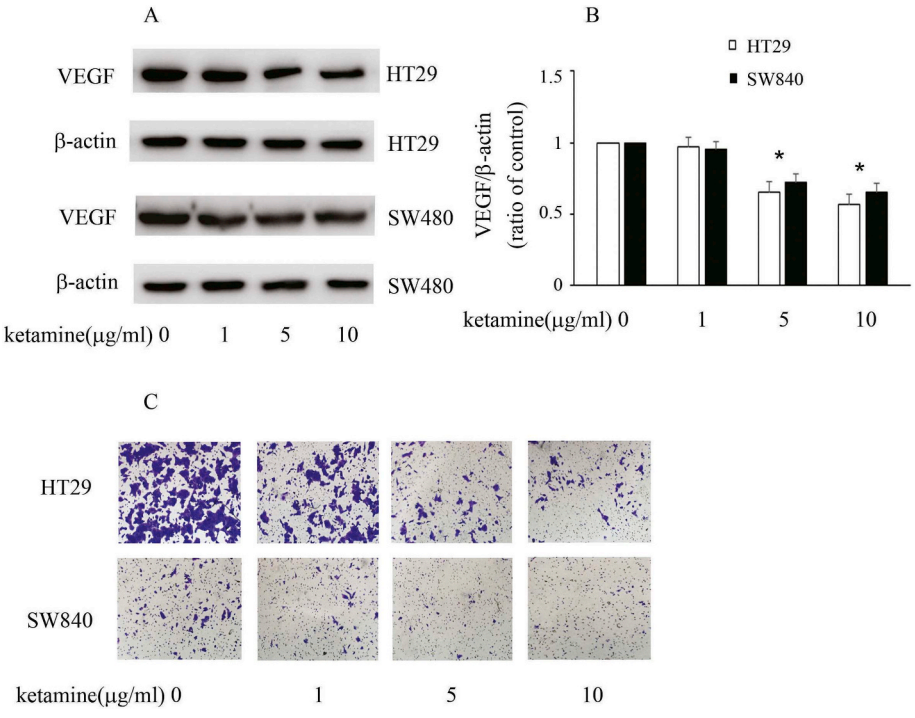

Fig. 1. Ketamine attenuates the expression of VEGF and cell migration in HT29 and SW480 cells. (A) Cells were incubated in different concentrations of ketamine (1, 5, 10 μg/ml) for 4 h. Equal amounts of proteins were separated by SDS-PAGE and immunoblotted with antibody against VEGF. (B) The protein expression ratio of VEGF and β-actin. The ratio in the control group was set as 1. (C) Ketamine attenuated the migratory capability of HT29 and SW480 cells in a concentration-depend manner. *P < .05 vs. control (n = 5). Data are shown as mean ± S.D.

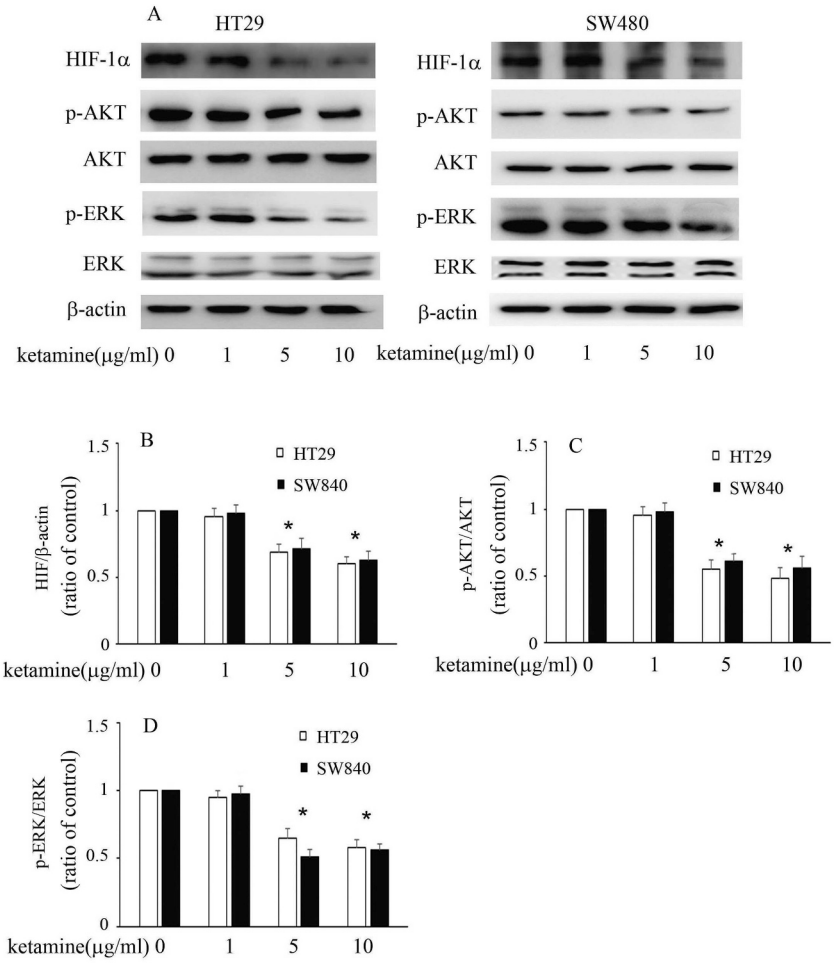

Fig. 2. Ketamine attenuates the of expression HIF-1α, the phosphorylation of ERK and AKT in HT29 and SW480 cells. (A) Cells were incubated in different concentrations of ketamine (1, 5, 10 μg/ml) for 4 h. Equal amounts of proteins were separated by SDS-PAGE and immunoblotted with antibodies against HIF-1α, p-ERK, ERK, p-AKT, AKT. (B, C, D) The protein expression ratio of specific protein and β-actin, ERK or AKT. The ratio in the control group was set as 1. *P < .05 vs. control (n = 5). Data are shown as mean ± S.D.

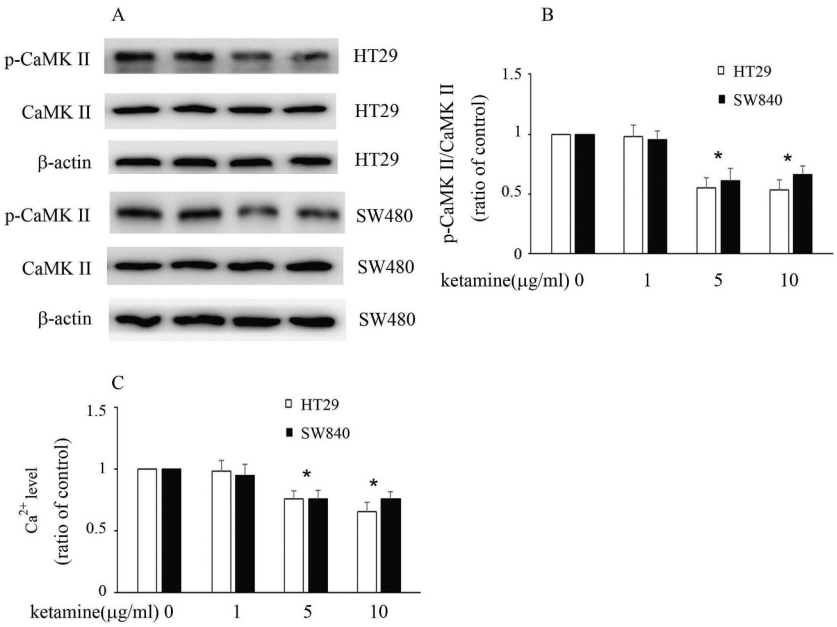

Fig. 3. Ketamine attenuates the phosphorylation of CaMK II and intracellular Ca^{2+} level in HT29 and SW480 cells. (A) Cells were incubated in different concentrations of ketamine (1, 5, 10 μg/ml) for 4 h. Equal amounts of proteins were separated by SDS-PAGE and immunoblotted with antibodies against CaMK II and p-CaMK II. (B) The protein expression ratio of p-CaMK II and CaMK II. The ratio in the control group was set as 1. (C) Ketamine attenuated intracellular Ca^{2+} level in a concentration-depend manner. *P < .05 vs. control (n = 5). Data are shown as mean ± S.D.

3.4. Phosphorylation of CaMK II and intracellular Ca^{2+} level were modulated by ketamine, KN93, AP5, MK801, and D-serine in HT29 and SW480 cells

KN93, which is an inhibitor of CaMK II, inhibited the phosphorylation of CaMK II. Moreover, the effect of KN93 was similar to that of ketamine treatment (Fig. 4A, B). However, KN93 had no effect on intracellular Ca^{2+} level in HT29 and SW480 cells (Fig. 4C). AP5 and MK801, which are two inhibitors of NMDAR, attenuated the phosphorylation of CaMK II and reduced intracellular Ca^{2+} level (Fig. 4A–C). Moreover, the effect of AP5 and MK801 was similar to that of ketamine treatment. Further, the anti-tumor effect of ketamine was counteracted by D-serine, a NMDAR activator (Fig. 4A-C).

3.5. Phosphorylation of AKT, ERK and the expression of HIF-1α were modulated by ketamine, KN93, AP5, MK801, and D-serine in HT29 and SW480 cells

KN93, AP5 and MK801 all inhibited the phosphorylation of ERK, AKT and the expression of HIF-1α. The effect of KN93, AP5 and MK801 was similar to that of ketamine treatment (Fig. 5A–D). Moreover, these effects of ketamine were counteracted by D-serine (Fig. 5A–D).

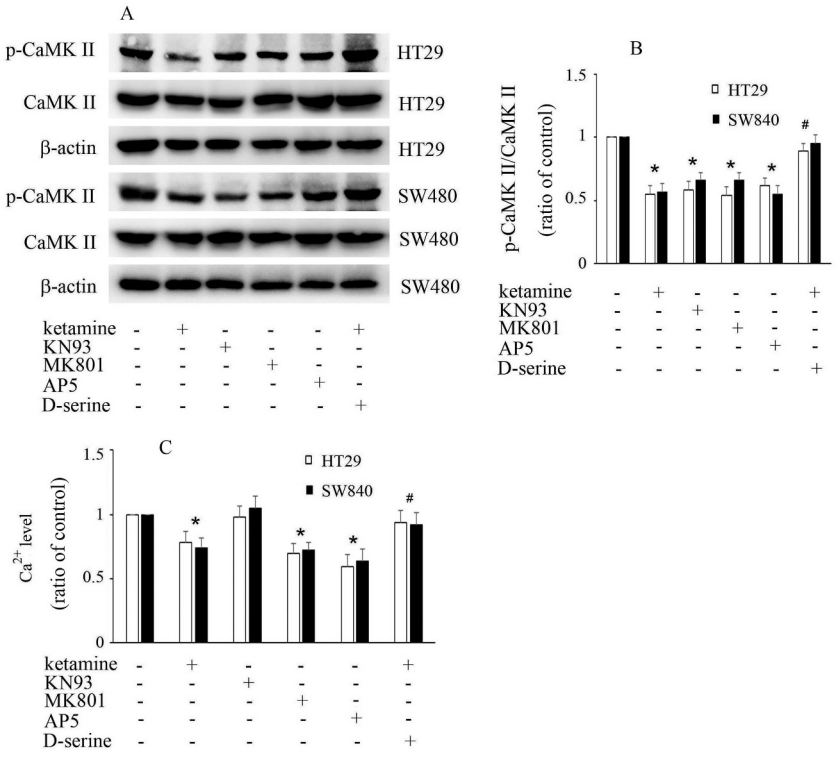

Fig. 4. The phosphorylation of CaMK II and intracellular Ca^{2+} level were modified by ketamine, KN93, AP5, MK801, and D-serine in HT29 and SW480 cells. (A) Cells were cultured with ketamine (5 μg/ml), KN93 (10 μM), AP5 (100 μM), MK801 (200 μM) or ketamine (5 μg/ml) plus D-serine (2 mM) for 4 h. Equal amounts of proteins were separated by SDS-PAGE and immunoblotted with antibodies against CaMK II and p-CaMK II. (B) The protein expression ratio of p-CaMK II and CaMK II. The ratio in the control group was set as 1. (C) Ketamine attenuated intracellular Ca^{2+} concentration. The effect of ketamine was similar to that of AP5 and MK801. Moreover, the effect of ketamine was reversed by D-serine. KN93 did not affect intracellular Ca^{2+} level. *P < .05 vs. control (n = 5). #P < .05 vs. ketamine treatment (n = 5). Data are shown as mean ± S.D.

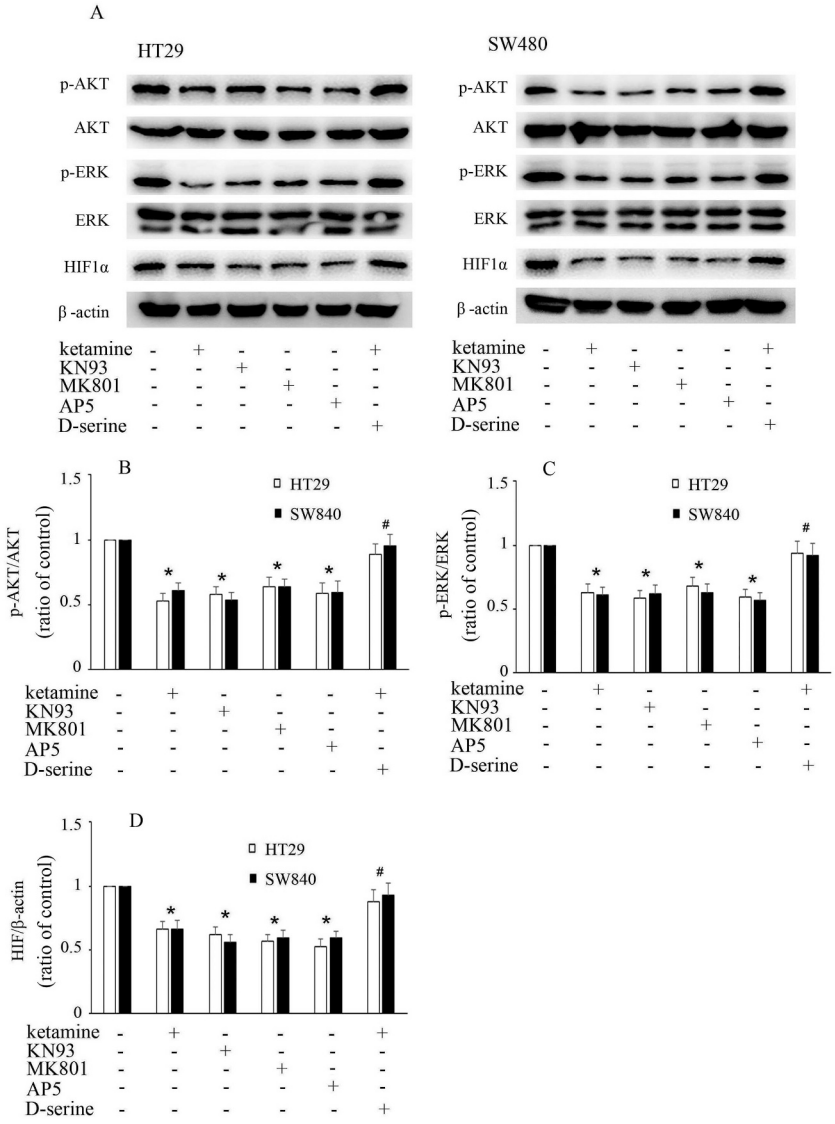

Fig. 5. The phosphorylation of AKT, ERK and the expression of HIF-1α were modulated by ketamine, KN93, AP5, MK801, and D-serine in HT29 and SW480 cells. (A) Cells were incubated with ketamine (5 μg/ml), KN93 (10 μM), AP5 (100 μM), MK801 (200 μM) or ketamine (5 μg/ml) plus D-serine (2 mM) for 4 h. Equal amounts of proteins were separated by SDS-PAGE and immunoblotted with antibodies against HIF-1α, p-ERK, ERK, p-AKT, AKT. (B, C, D) The protein expression ratio of specific protein and β-actin, ERK or AKT. The ratio in the control group was set as 1. $^{*}P < .05$ vs. control (n = 5). $^{\#}P < .05$ vs. ketamine treatment (n = 5). Data are shown as mean ± S.D.

3.6. The expression of VEGF and cell migration were modulated by ketamine, KN93, AP5, MK801 and D-serine in HT29 and SW480 cells

KN93, AP5 and MK801 all attenuated the expression of VEGF (Fig. 6A, B) and cell migration (Fig. 6C). The effect of KN93, AP5 and MK801 was similar to that of ketamine treatment. Moreover, the anti-tumor effect of ketamine was reversed by D-serine (Fig. 6A–C).

3.7. Knockdown of NMDAR using siRNA attenuated the effect of ketamine on cell migration

Knocking down of NMDAR using siRNAs were confirmed by western blot and Real-time PCR analysis (Fig. 7A, B, C). Ketamine had no effect on NMDAR expression (Fig. 7A, B, C), however, the effect of ketamine on cell migration was abolished in NMDAR silencing cells (Fig. 7D).

4. Discussion

In the present study we indicated that ketamine via inhibiting NMDAR, decreased intracellular Ca^{2+} level, down-regulated the phosphorylation of CaMK II, ERK, AKT and the expression of HIF-1α, thus inhibiting VEGF expression and cell migration in colorectal cancer cells.

Accumulating evidence suggest that HIF-1α activates a series of downstream genes to facilitate angiogenesis (Ban et al., 2010) and migration (Burrows et al., 2011). Elevated of HIF-1α expression in cancer tissues was also associated with poor outcomes in cancer patients. Therefore, HIF-1α has been regarded as a potential therapeutic target in cancer treatment (Semenza, 2003). Interestingly, it has been reported that the expression of HIF-1α was regulated by ERK and AKT (Huang et al., 2014; Liu et al., 2010). Our studies also suggested that ketamine could inhibit the phosphorylation of ERK and AKT, and down-regulate the expression of HIF-1α, leading to a decreased cell migration ability and VEGF expression in colorectal cancer cells. This line of investigation is likely to have significant implication as reported by several other studies that ketamine also exerted inhibitory role on the malignant potential in lung adenocarcinoma cells (Zhou et al., 2018), pancreatic cancer cells (Malsy et al., 2015) and hepatocellular carcinoma cells (Yamaguchi et al., 2013).

The CaMKs, whose activity are modulated by Ca^{2+} signaling, are

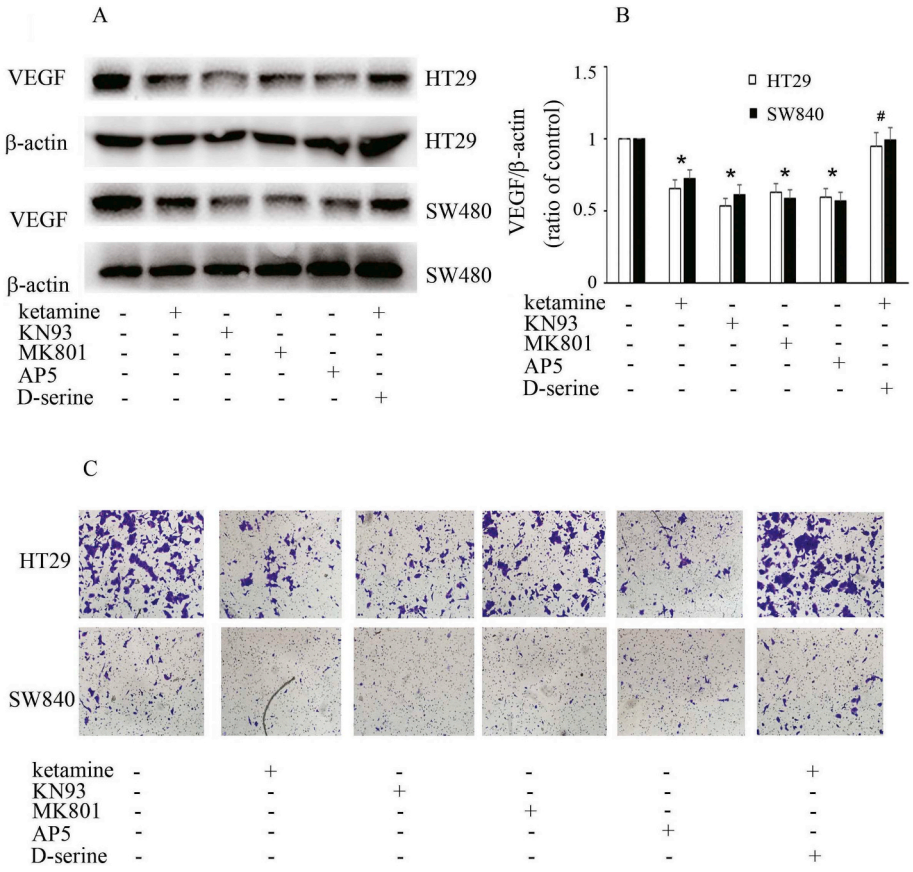

Fig. 6. The expression of VEGF and cell migration were modulated by ketamine, KN93, AP5, MK801, and D-serine in HT29 and SW480 cells. (A) Cells were cultured with ketamine (5 μg/ml), KN93 (10 μM), AP5 (100 μM), MK801 (200 μM) or ketamine (5 μg/ml) plus D-serine (2 mM) for 4 h. Equal amounts of proteins were separated by SDS-PAGE and immunoblotted with antibody against VEGF. (B) The protein expression ratio of VEGF and β-actin. The ratio in the control group was set as 1. (C) Ketamine attenuated cell migration. The effect of ketamine was similar to KN93 AP5, and MK801. Moreover, the effect of ketamine was reversed by D-serine. $^{*}P < .05$ vs. control (n = 5). $^{\#}P < .05$ vs. ketamine treatment (n = 5). Data are shown as mean ± S.D.

multifunctional serine/threonine kinases (Hook and Means, 2001). Amount of studies have indicated that tumor tissues expressed high levels of CaMK II (Britschgi et al., 2013) (Chai et al., 2014; Wang et al., 2008). CaMKII modulates the activity of different proteins (Erickson, 2014; Lisman et al., 2012) and plays a pivotal role in modifying cell proliferation, differentiation and migration of tumor cells (Britschgi et al., 2013; Chai et al., 2014; Wang et al., 2008; Wang et al., 2014). It was reported that augment of intracellular Ca^{2+} level could induce the phosphorylation of CaMKII, which in turn activated HIF-1α in colon cancer cells (Riganti et al., 2009). In the present study, we found ketamine could inhibit CaMK II, AKT and ERK phosphorylation, and HIF-1α expression. Moreover, the effect of ketamine is quite similar to that of KN93, an inhibitor of CaMK II. These data revealed that ketamine, via inhibiting CaMK II activity, attenuated AKT, ERK phosphorylation and HIF-1α expression, thus attenuating the malignant potential of colorectal cancer cell.

The expression of NMDAR was mainly related to the central nervous system. However, recent studies have demonstrated that functional NMDAR were also expressed in cancer cells (Luksch et al., 2011). Indeed, in our experiments, we found NMDAR was also expressed in colorectal cancer cells (Fig. 7). Emerging data suggested that NMDAR play an important role in tumor development, proliferation, and metastasis (Kalariti et al., 2005; Watanabe et al., 2008). NMDAR activation results in an augmentation of intracellular Ca^{2+} level (MacDermott et al., 1986; Mayer and Westbrook, 1987; Wahl et al., 1989), thus upregulating of CaMK II phosphorylation in cancer cells, and facilitating malignant potential of cancer cells. Several clinical experiments indicated that inhibition of NMDAR could attenuate the proliferation of cancer cells, such as lung cancer and esophagus cancer (Kim et al., 2006; Stepulak et al., 2005). Similarly, in the present study, we observed that blocking NMDAR or knockdown of NMDAR using siRNA could attenuate VEGF expression and migration in colorectal cancer cells.

Ketamine is one of the most commonly used NMDAR antagonists, and is used for cancer pain treatment in clinical practice (Zgaia et al., 2015). Colorectal cancer surgery (Kuo et al., 2006) plus anesthesia (Zhu et al., 2018) duration is usually about 4 h. In order to simulate clinical conditions, in the present study, we treated colorectal cancer cells with ketamine for 4 h. The present study indicated that ketamine could decrease intracellular Ca^{2+} level and the phosphorylation of CaMK II, which is similar to AP5 and MK801, two inhibitors of NMDAR. Several lines of evidences revealed that MK801 could inhibit proliferation (Malsy et al., 2015), migration (Chen et al., 2017) and aerobic glycolysis (Chen et al., 2018) in pancreatic and colorectal cancer cells. Similarly, our data indicated that AP5 and MK801 inhibited the expression of VEGF and migration in colorectal cancer cells. Furthermore, the anti-tumor effect of ketamine could be reversed by D-serine, an activator of NMDAR. Moreover, knockdown of NMDAR using siRNA attenuated the effect of ketamine against cell migration. Our data supported the concept that the anti-tumor effect of ketamine may be achieved by blocking NMDAR, thus resulting in decreased Ca^{2+} level and CaMK II phosphorylation in HT29 and SW480 cells.

The current study has some limitations. First, we determined that ketamine, via blockage of NMDAR, attenuates colorectal cancer cell malignant potential. However, other biochemical experiments are essential to draw such a conclusion. Second, the study was implemented in HT29 and SW480 colorectal cells, and situations may be different in in vivo systems. Further studies in in vivo models are essential to confirm the present results.

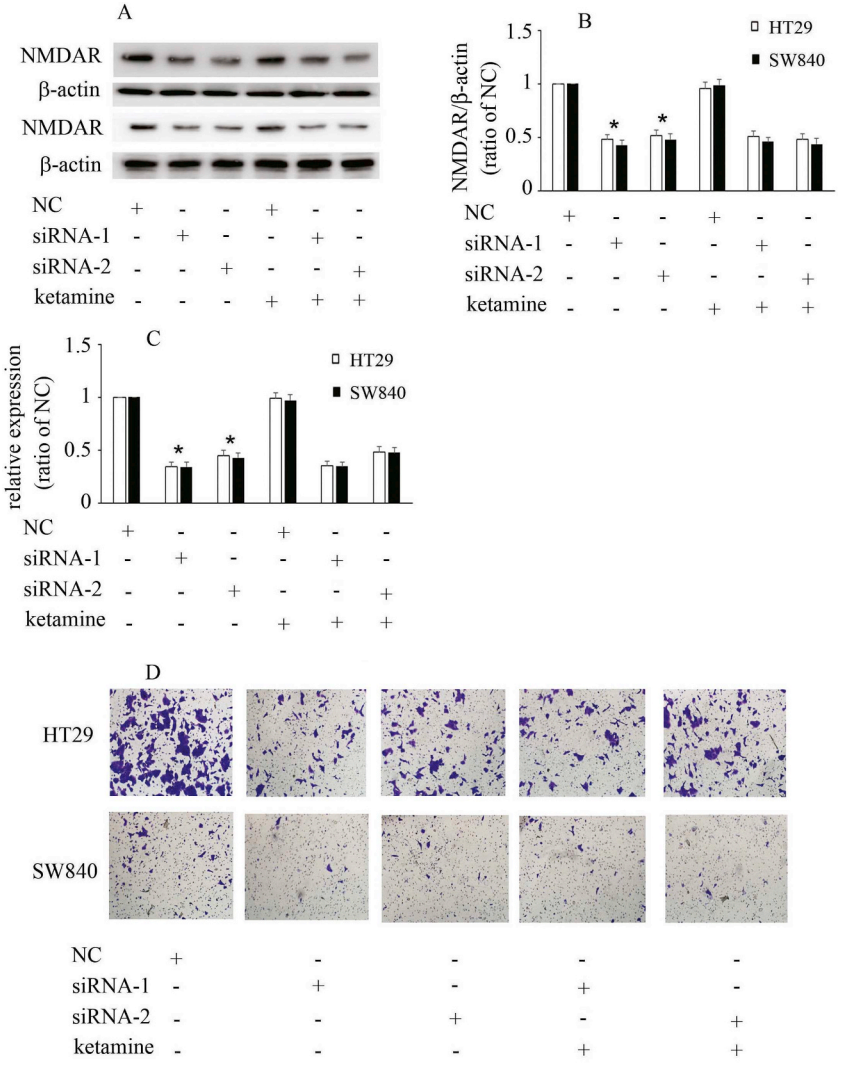

Fig. 7. Knockdown of NMDAR using siRNA attenuated the effect of ketamine on cell migration. (A, B, C) The expression of NMDAR was detected with western blot and Real-time PCR analysis. Ketamine had no effect on the expression of NMDAR. (D) Ketamine had little effect on cell migration in NMDAR silencing cells. *P < .05 vs. NC (n = 5). Data are shown as mean ± S.D.

5. Conclusion

In summary, this study demonstrated that ketamine decreased intracellular Ca^{2+} level, attenuated the phosphorylation of CaMK II, ERK and AKT and inhibited HIF-1α expression, leading to reduced VEGF expression and cell migration. The anti-tumor effect of ketamine may be achieved via NMDAR blockage.

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