

Original Article

Metabotropic glutamate 2/3 receptor agonist LY404039 inhibits gastric cancer cell proliferation via regulating PI3K/Akt signaling pathway

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Abstract: Gastric cancer, the second leading cause of cancer-related deaths around the world, is still with limited effective treatment options. LY-404039 is a highly selective agonist for mGluR2 and mGluR3 (G protein-coupled membrane receptor). Here, we investigated the effect of LY-404039 on gastric cancer cell MKN28 in order to explore the potential role of mGluR2 and mGluR3 in gastric cancer. The proliferation, invasion, migration and apoptosis of MKN28 cells were examined by CCK8 assay, transwell invasion and migration assay, and flow cytometry after treated with LY-404039. The involvement of proteins in PI3K/Akt pathway was tested by western blot. We found that LY-404039 showed inhibitory effects on the proliferation, invasion and migration of MKN28 cells and exhibited a promotion in MKN28 cell apoptosis. From the western blot results, we found that the expression levels of Bax and Active Caspase3 were up-regulated and Bcl-2 was down-regulated after treated with LY-404039. Moreover, the levels of p-AKT and p-mTOR were obviously declined in the LY-404039 treated group compared with the control group. These observations demonstrated that LY-404039 might inhibit cell growth and induce apoptosis by suppressing PI3K/Akt pathway, indicating the involvement of mGluR2 and mGluR3 in gastric cancer development.

Keywords: LY404039, gastric cancer, proliferation, PI3K/Akt signaling pathway

Introduction

Gastric cancer, the most common gastric tumor, is the second leading cause of cancer-related deaths around the world [1]. Due to the complex pathogenesis of gastric cancer and its correlation with various factors, the molecular mechanism of gastric cancer still remains largely unclear [2]. Although the current treatment of gastric cancer has made great progress, the options for effective treatment are still very limited [3, 4] and over half advanced stage patients die of recurrence despite undergoing gastrectomy [5]. Hence, a detailed understanding on the mechanism of gastric tumorigenesis and the development of more effective treatments are urgently needed.

Glutamate (Glu) is one of the major excitatory neurotransmitters in the central nervous system, which plays a role in synaptic excitation

conduction regulation [6]. Metabotropic glutamate receptors (mGluRs) belong to the G protein-coupled membrane receptor, which exert their effects on second messengers or ion channels by activating GTP-binding proteins [7]. Many researchers have reported the role and involvement of mGluRs on the pathobiology of neural injury and neuropsychiatric disorders in the central nervous system. Except its widespread in the brain, it has also been found in most peripheral tissues, including adrenal, bone, bone marrow, stomach, heart, liver and so on [6]. Besides, it was detected in tumor cells and nuclear membrane where Glu was inaccessible as well [8]. Hence, mGluRs may be involved in the development of cancer cells. mGluR2 and mGluR3 are two members of mGluRs, which inhibits cyclic adenosine monophosphate formation, voltage-sensitive Ca²⁺ channels and activates K⁺ channels [6]. LY-404039 is a highly selective agonist for mGluR2 and mGluR3, which

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aims to treat schizophrenia and anxiety disorders [9]. Until now, whether mGluR2 and mGluR3 involved in the development of gastric cancer remains unintelligible. Here, we explored the effect of LY-404039 on gastric cancer cell proliferation, migration, invasion and apoptosis, and investigated its potential mechanism, attempting to reveal the role of mGluR2 and mGluR3 in gastric cancer.

Methods

Cell culture and treatment

The gastric cancer cell line MKN28 was obtained from Cell Bank of the Chinese Academy of Sciences (Shanghai, China) and cultured in RPMI-1640 medium containing 10% (v/v) serum (Gibco, Thermo Fisher Scientific, Waltham, Massachusetts, USA), 100 U/ml penicillin (sigma) and 0.1 mg/ml streptomycin (sigma) at 37°C with 5% CO₂. When the cells entered the logarithmic growth phase, they were washed by PBS for three times and then digested by trypsin. After the cells become rounded, the medium was re-added to stop digestion. The single cell suspension was prepared and inoculated in a six-well plate for next experiments. When the cell density in the well plate reaches about 80%, cells were treated with 10 μM LY404039 (experiment group) or DMSO (1:1000) (negative control group, NC) for 24 h.

CCK8 assay

The proliferation of MKN28 cells was tested using a Cell Counting Kit-8 (Solarbio solarbio science & technology co., ltd, Beijing). MKN28 cell suspension was prepared and plated into 96-well plates with 1000 cells for each well. Cells were then incubated with 10 μM LY404039 or DMSO (0.1%, NC) under standard conditions. At every 24 h, the optical density (OD) at 450 nm was measured by a microplate reader after adding 10 μl CCK8 chemical to each well and being incubated at 37°C for 1.5 h. Proliferation curve of MKN28 cells was drawn using Graph Pad Prism 5.

Transwell invasion and migration assay

Transwell (Millipore, Billerica, MA, USA) assays were performed to exam the invasion and migration of MKN28 cells. For invasion assay, the upper chamber was added with 100 μl matrigel (diluted with serum-free medium at a ratio of

1:6) (Becton Dickinson, USA) and kept static at 37°C for 4 h to make matrigel polymerize into a gel. MKN28 cell suspensions were prepared after treated with LY404039 or DMSO for 24 h. Then the cell suspensions were added to the upper chamber (1 × 10⁵ cells/well) and 500 μl culture medium were added to the lower chamber. After 24 h incubation, the remaining cells in the upper chamber were wiped with cotton swab. The invaded cells were fixed with formaldehyde and stained by 0.1% crystal violet dye. The number of the invaded cells was counted under a light microscope and five field of visions were randomly selected for each experiment. Migration assay was similar to invasion assay, but matrigel is not required. A number of 5000 MKN28 cells were added to each well. All assays were performed in triplicate.

Flow cytometry

MKN28 cells treated with 10 μM LY404039 or DMSO (1:1000) were collected and digested with trypsin. After washing by PBS, cell suspensions were prepared using binding buffer and the cell density was adjusted to 1-5 × 10⁶ cells/ml. Annexin V-FITC/PI Apoptosis Detection Kit was used to stain the cells following manufacturer's instructions. After staining, the effect of LY404039 on MKN28 cells was detected by a flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA). The results were analyzed by Flowjo 10 software.

Western blot

Total protein of MKN28 cells in each group were extracted using RIPA lysate (Com Win Biotech Co, Ltd, Beijing, China) containing protease inhibitor. The concentration of the protein was measured by BCA Protein Assay Kit (Com Win Biotech Co, Ltd, Beijing, China). Then the proteins were heated and separated by 10% SDS polyacrylamide gel electrophoresis. Subsequently, the proteins on the gel were transferred to polyvinylidene fluoride (PVDF) membranes (Millipore, Bedford, MA). Next, the membranes were blocked by 5% skimmed milk for 1 h at room temperature and incubated with the rabbit anti-human primary antibodies: AKT (GST, USA), p-AKT(GST, USA), mTOR (GST, USA), p-mTOR (GST, USA), Bcl2 (PTG), Bax (PTG), active-caspase3 (PTG), p70 (PTG) and GAPDH (PTG), at 4°C overnight. GAPDH was used as the internal reference. All the primary antibodies were diluted at 1:1000, except anti-body against to GAPDH (1:5000). Then the membr-

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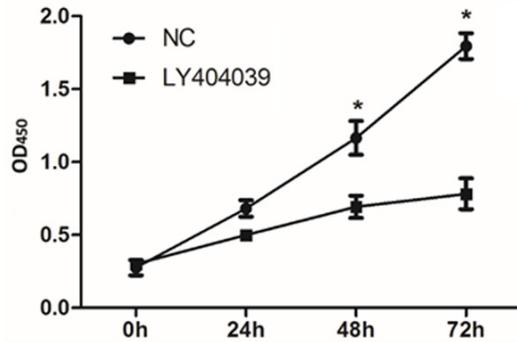


Figure 1. The inhibitory effect of LY-404039 on MKN28 cell proliferation examined by CCK8 assay as described in methods. The cell proliferation was tested at every 24 h. The data are presented as means \pm SD. $n = 6$, * $P < 0.05$, vs. negative control group (NC). 1% DMSO was used as negative control.

anes were washed by TBST and incubated with the secondary anti-body conjugated to HRP (PTG, goat anti-rabbit, 1:5000) for 1 h at room temperature followed by being washed with TBST again. Enhanced chemiluminescence (ECL) plus detection kit (Thermo Fisher Scientific, Inc.) was used to detect the signals. Quantity One software was used for density analysis.

Statistical analysis

SPSS18.0 software was used to perform the statistical analysis. Data were shown as mean \pm SD. Student's t-test was used to compare the experimental and control group. $P < 0.05$ was considered as statistical significant.

Results

LY404039 inhibited MKN28 cell proliferation

The effect of LY404039 on MKN28 cell proliferation was examined by CCK8 assay. We can easily observe that the OD₄₅₀ value in the LY404039 treated group was lower than that in the NC group after 24 h treatment and the differences between these two groups were significant at 48 h and 72 h ($P < 0.05$, **Figure 1**). These phenomena indicated that the proliferation of MKN28 and AGS cells were dramatically suppressed by LY404039 in a time-dependent manner.

LY404039 inhibited MKN28 cell invasion and migration

To explore the effect of LY404039 on MKN28 cell invasion and migration, transwell assays

were performed. In transwell invasion assay, we found that the number of crystal violet staining positive cells decreased significantly in LY404039 treated group compared with the NC group ($P < 0.05$). This result suggested that LY404039 played an inhibitory role in MKN28 cell invasion (**Figure 2A, 2C**). In transwell migration test, the observation was similar to the invasion assay. The number of crystal violet staining positive cells is obviously lower in LY404039 treated group than that in NC group ($P < 0.05$), indicating that LY404039 also could suppress the migration of MKN28 cells (**Figure 2B, 2D**).

LY404039 induced MKN28 cell apoptosis

Next we investigated whether LY404039 induced MKN28 cell apoptosis using flow cytometry. The result of flow cytometry analysis was shown in **Figure 3**. The proportion of early apoptotic cells in the LY404039 treated group was $17.50 \pm 0.41\%$, which was higher than that in the NC group ($2.87 \pm 0.16\%$). The number of late apoptotic cells in LY404039 treated group ($4.80 \pm 0.23\%$) was also increased compared to the NC group ($3.96 \pm 0.13\%$). Compared with the total proportion of apoptotic cells in NC group ($6.83 \pm 0.32\%$), the total proportion of apoptotic cells in LY404039 treated group ($22.30 \pm 0.26\%$) increased significantly (**Figure 3A**). These observations implied that LY404039 may induce apoptosis in MKN28 cells.

Subsequently, we examined the expression level of apoptosis-related proteins by western blot to further investigate the possible mechanism of LY404039 induced apoptosis. The results showed that the expression level of anti-apoptotic protein Bcl2 in MKN28 cells declined significantly after treated with LY404039 for 24 h compared with the NC (**Figure 3C**, $P < 0.05$). On the contrary, the expression levels of pro-apoptotic protein Bax and Active-Caspase 3 were all statistically significantly increased in the LY404039 group compared to the NC group (**Figure 3D, 3E**, $P < 0.05$). Hence, LY404039 might promote apoptosis of MKN28 cells by suppressing the expression of Bcl2 and up-regulating the expression of Bax and Active-Caspase 3. Furthermore, this observed apoptosis induced by LY404039 was consistent with the decrease in cell proliferation observed previously.

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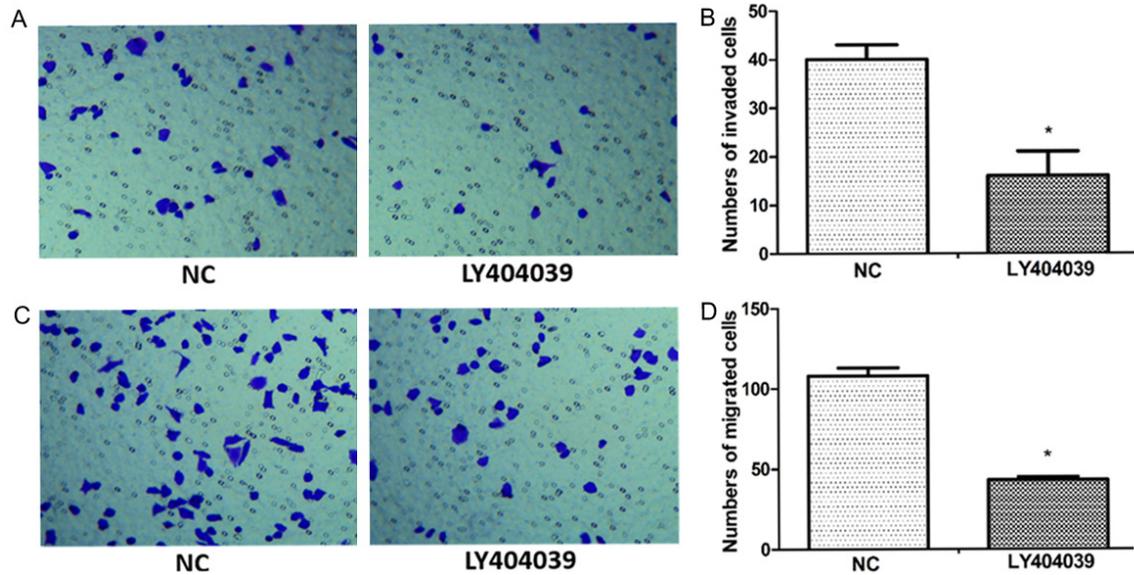


Figure 2. LY-404039 suppressed the invasion and migration of MKN28 cells. (A) A representative image of Transwell invasion assays (A) and Transwell migration assays (C) for each group (10 × 10), respectively. (B) Quantification of invaded cells and (D) Quantification of the migrated cells. N = 5, *P < 0.05, vs. negative control group (NC). The data are presented as means ± SD.

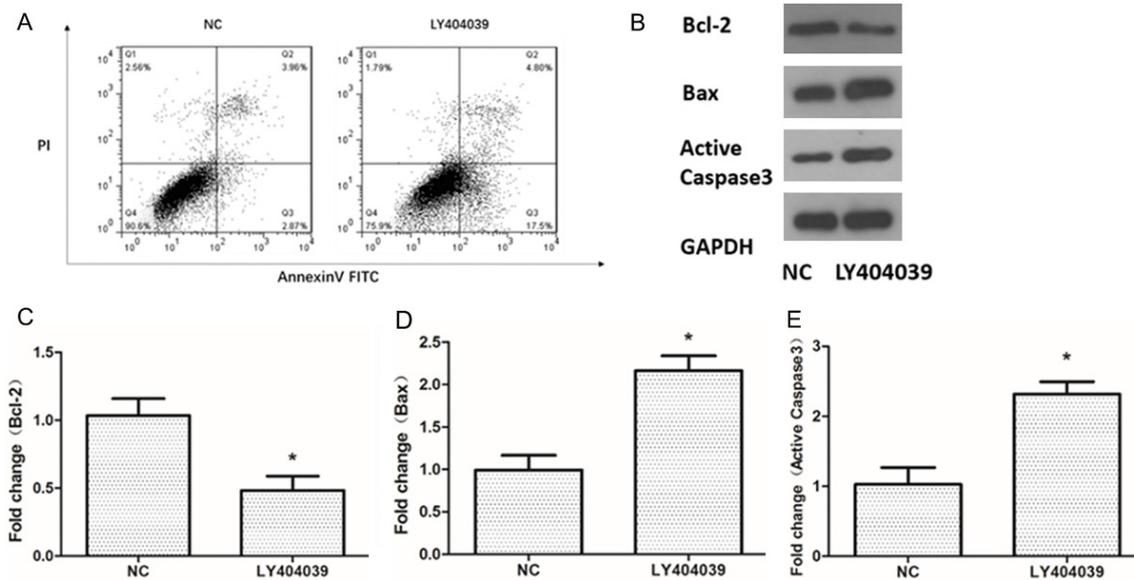


Figure 3. (A) Fluorescence-activated cell sorting (FACS) analysis of MKN28 cells treated with LY-404039 or DMSO (0.1%) for 24 h. (B) The expression of Bcl2, Bax and Active Caspase 3 detected by western blot. Quantification of (C) Bcl2, (D) Bax and (E) Active-Caspase3 expression levels. n = 6, *P < 0.05, vs. negative control group (NC). The data are presented as means ± SD.

LY404039 suppressed PI3K/Akt signaling pathway

PI3K/Akt signaling pathway is known as a very important signal pathway in tumor and the key proteins in this pathway play an important role

in the proliferation, apoptosis and metastasis of tumor cells. Therefore, we further investigated whether PI3K/Akt signaling pathway is involved in LY404039-induced MKN28 cell apoptosis by western blotting (Figure 4). We identified that the level of AKT and mTOR almost not

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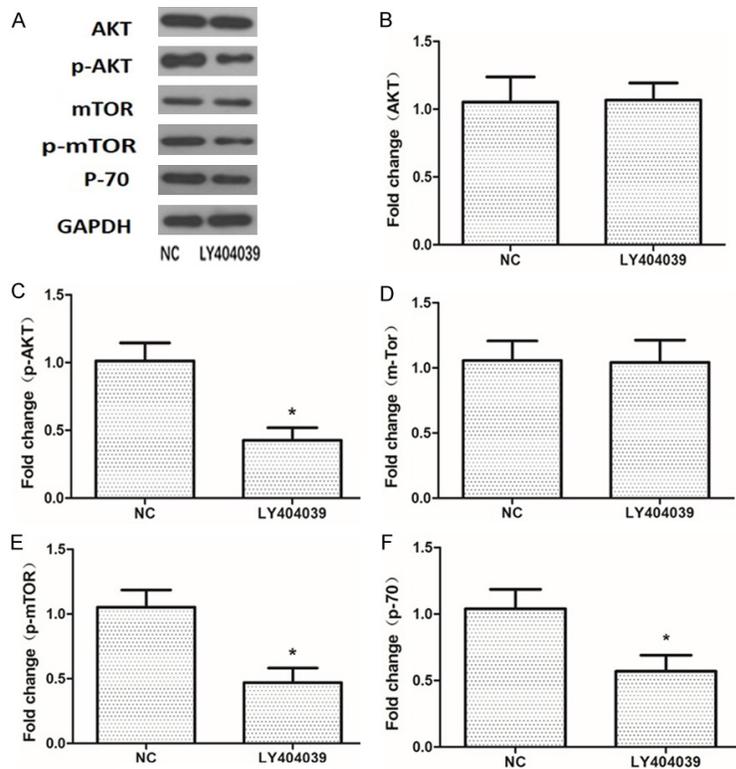


Figure 4. Expression of AKT, p-AKT, mTOR, p-mTOR and p70 in LY-404039 treated MKN28 cells. A. Western blot analysis of AKT, p-AKT, mTOR, p-mTOR and p70 expression. B-F. Relative expressions of AKT, p-AKT, mTOR, p-mTOR and p70, respectively. Bars, SD. n = 6, *P < 0.05, vs. negative control group (NC).

changed, while the expression of p-AKT and p-mTOR showed an obviously decrease after treated with LY404039 for 24 h. The protein expression level of cell proliferation associated protein P-70 was down-regulated as well. These phenomena suggested that LY404039 probably suppressed proliferation and induced apoptosis by inhibiting the phosphorylation of AKT.

Discussion

Gastric cancer is one of the leading causes of cancer-related deaths worldwide. It is an urgent need to develop effective treatment approaches for gastric cancer [3]. In this paper, we observed that the mGluR2 and mGluR3 agonist LY404039 could inhibit MKN28 cell proliferation, migration and invasion and induce MKN28 cell apoptosis. Moreover, the effect of LY404039 on MKN28 cell apoptosis and proliferation maybe mediated by PI3K/Akt signaling pathway.

Glutamate (Glu) as the main excitatory neurotransmitters in the central nervous system, has been currently implied that it may be also associated with the growth of cancer cells by novel receptor-mediated auto-crine/paracrine signal transduction pathways [10]. It has been demonstrated that peripheral glutamate receptors play important role in human histiocytic lymphoma-derived U9-37 cell proliferation and the exact role depends on the culture conditions [10]. In addition, previously reports have identified that pharmacological blockade of mGluR2/3 resulted in a decline in glioma cells growth [11]. In the present study, we identified that the proliferation of MKN28 cells treated with mGluR2/3 agonist LY-404039 were significantly suppressed compared with the negative control group (Figure 1), prompting the involvement of mGluR2/3 in gastric cancer cell proliferation.

Cell apoptosis plays an important role in the initiation and development of tumors. The promotion of SP 2/O cell apoptosis has been observed when metabotropic glutamate receptor 3 was overexpressed [12]. Here, the flow cytometry results showed that LY404039 could promote apoptosis of MKN28 cells (Figure 3). Apoptosis is controlled by various proteins and it is well known that Bcl-2 family are proteins that play important regulatory role in cell apoptosis. Bcl-2 and Bcl-XL proteins are anti-apoptotic protein members, while Bak and Bax are pro-apoptotic protein members of Bcl-2 family [13]. Caspase3 is a protein which plays a vital role in the apoptotic signal transduction and is the main performer of apoptosis [14]. Many anti-cancer drugs such as Isoliquiritigenin and Berberine have been reported to promote cell apoptosis by down-regulating the level of Bcl-2 and up-regulating the expression of Bax and Active Caspase3 [15, 16]. While whether mGluR2/3 agonist LY404039 also induced MKN28 cell apoptosis through regulating apoptosis-

related proteins has not been reported yet. In this study, we found that the expression levels of Bax and Active Caspase3 were significantly increased and the expression level of Bcl-2 was declined obviously in MKN28 cells after treated with LY404039 for 24 h. Our results suggested the involvement of Bcl-2, Bax and Active Caspase3 in LY404039 induced apoptosis.

The closely association between PI3K/Akt signaling pathway and the proliferation and apoptosis of tumor cells has been reported by many researchers [17, 18]. Furthermore, several studies have proposed that PI3K/Akt system is an intracellular target of mGluR3 [19, 20]. As shown in **Figure 4**, we found that LY-404039 treatment made the expression levels of p-AKT and p-mTOR decreased obviously whereas the levels of AKT and mTOR almost not changed. These observations implied that mGluR2/3 agonist may play an inhibitory role in cell proliferation and a promoting role in cell apoptosis via blocking PI3K/Akt signaling pathway. However, our results are not consistent with the potential role of mGluR2/3 agonist in central nervous system.

It has been found that mGluR3 agonists LY-404039 showed an anti-apoptotic effect in cultured astrocytes by regulating cAMP/Akt/p65-c-Rel pathway [21]. Moreover, the effect of mGluRs on PI3K activation in Drosophila Motor Neurons has been illustrated previously as well [22]. This difference between our results and the previous reports may be due to the differences between central nervous system and peripheral tissue.

In summary, our results indicated that LY-404039 exerts anti-proliferative, anti-migration, anti-invasion and pro-apoptotic role in MKN28 cells, which may via suppressing PI3K/Akt signaling pathway. These results suggested the involvement of mGluR2/3 in the development of gastric cancer and the potential mechanisms need further investigation.

Disclosure of conflict of interest

None.

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