mGluR5 promotes the progression of multiple myeloma in vitro via Ras–MAPK signaling pathway

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Abstract

Background. Multiple myeloma (MM) is a malignant plasma cancer which remains difficult to be cured. Recently, numerous research studies have appeared, exploring MM from molecular level. However, there is no study about the impact of metabotropic glutamate receptors (mGluRs), especially *mGluR5*, on MM progression. Thus, the present research was dedicated to the exploration of the influence of mGluR5 on MM.

Objectives. In this research, we used quantitative real-time polymerase chain reaction (qRT-PCR) to check the gene expression in MM, western blot assay to check the protein expression of the gene, MTT assay to quantify the cell viability, and flow cytometry (FCM) apoptosis method to evaluate cell apoptosis in order to acquire the results. The purpose was to assess the role of *mGluR5* in MM cells.

Materials and methods. The qRT-PCR was used and it was found that *mGluR5* was overexpressed in MM cell lines and MM tissues compared to normal ones. To better observe the function of *mGluR5* in MM, cell viability and apoptosis were checked using MTT and FCM apoptosis assays after the treatment with agonists and antagonists.

Results. Agonist-induced *mGluR5* upregulation could promote MM cell viability and inhibit apoptosis. The same results were obtained through MTT and FCM apoptosis assays after upregulation and down-regulation of *mGluR5* by transfection. To further investigate the inner mechanism, the effect of *mGluR5* on Ras–MAPK pathway was checked using western blot. It was found that the upregulation of *mGluR5* could activate the Ras–MAPK pathway.

Conclusions. The *mGluR5* might be involved in promoting cell proliferation and inhibiting cell apoptosis in MM. It can be an essential biomarker in the screening for MM and a potential part of future MM therapies.

Key words: apoptosis, multiple myeloma, viability, *mGluR5*

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Background

Multiple myeloma (MM) is recognized as plasma cell myeloma, myelomatosis or Kahler's disease.[1] It is an age-related plasma cell cancer and a clonal plasma cell malignancy that is responsible for approx. 10% of hematologic malignancies.[2] The latest drug discoveries could lead to greatly improved survival percentages.[3] Even though previous research on therapies such as immunomodulatory drugs (IMiDs) and proteasome inhibitors have benefited the MM patients, the development mechanism of this disease still remains to be explored.[4,5] Until now, the frequently discussed genes in MM include *KRAS*, *NRAS*, *TP53*, *BRAF*, and *CCND1*.[6] It has been widely reported that the mutations in *KRAS* and *NRAS* could regulate the cell apoptosis of MM cells.[6,7] The *TP53*, a classic tumor suppressor, is also correlated with cellular apoptosis in MM.[8]

Metabotropic glutamate receptors (mGluRs), as G-protein coupling receptors, have been extensively studied in correlation with cell survival and death, and Group I consists of *mGluR1* and *mGluR5* subtypes (*mGluR1/5*), which are coupled to $G\alpha_{q/11}$ proteins.[9] Both *mGluR1* and *mGluR5* have been reported to be expressed in a variety of tumors, including lung cancer[10] and osteosarcoma,[11] among others. It has been confirmed that *mGluR5* is involved in the osteosarcoma cell proliferation and its inhibition reduces cell proliferation.[11] Similarly, *mGluR5* antagonist was found to suppress cell proliferation in laryngeal cancer.[12] Apart from that, *mGluR5* was also discovered to be expressed in MM.

However, there has been no further research on MM regarding *mGluR5*. Oncomine (https://www.oncomine.org) online analysis showed that *mGluR5* ranked the top 10% of the significantly upregulated genes in MM tissues compared to normal ones, suggesting that *mGluR5* might play an oncogenic part in MM. Therefore, we hypothesized that *mGluR5* might be involved in the occurrence and progression of MM and tried to explore whether *mGluR5* regulates the cell apoptosis and proliferation and also the functional mechanism beneath MM.

Objectives

The aim of this study was to investigate the role of *mGluR5* in MM and also explore its regulatory mechanism.

Materials and methods

Cell culture

The MM cell lines (MM1S, OPM-2, NCI-H929, U266, and RPMI-8226) and normal human umbilical vein endothelial cells (HUVECs) were bought from the American Type Cell Culture (ATCC; Manassas, USA). The MM1S, OPM-2, NCI-H929, U266, and RPMI-8226 are different B lymphocytes derived from MM patients. The cells were cultured in RPMI-1640 medium (Thermo Fisher Scientific, Waltham, USA) supplemented with 10% fetal bovine serum (FBS; Invitrogen, Shanghai, China) and 1% penicillin/streptomycin at 37°C in 5% CO_2.

qRT-PCR

Total RNA was extracted from cells using Triazole reagent (Thermo Fisher Scientific). The cDNA was reversely transcribed as a template. A Roche LC480 real-time polymerase chain reaction (RT-PCR) machine and a SYBR Green kit (both from Qiagen, Düsseldorf, Germany) were utilized to perform the quantitative RT-PCR (qRT-PCR) analysis. A total of 10 µL of the SYBR Green PCR master mix, 50 ng cDNA and 250 nM of each primer were mixed and diluted to a total volume of 20 µL. Each group was analyzed in triplicate and the process was repeated 3 times. The conditions for thermal cycling were as follows: 10 min at 95°C, followed by 40 cycles of 95°C for 15 s and 60°C for 60 s. The $2^{-\Delta\Delta Cq}$ method was used. Primer sequences were as follows: *mGluR5*: forward, 5'-TGAGGGTTGTGCCTTCAGAT-3', reverse, 5'-AAGAGTGGGCGATGCAAATC-3'. The *GAPDH* was used as an internal control and its primer sequences were as follows, forward, 5'-GTCGGAGTCAACGGATTTGG-3', reverse, 5'-TGACGGTGCCATGGAATTTG-3'.

Cell treatment

After qRT-PCR detected the differential expression of *mGluR5* in the 4 MM cell lines, we selected the cell line with the highest *mGluR5* expression to knock down the gene expression through its antagonist or inhibitor (OPM2) and the cell line with the lowest *mGluR5* expression (MM1S) to upregulate the expression of *mGluR5* by treating it with its agonist or oe-mGluR5. Therefore, *mGluR5* siRNA plasmid, lentivirus plasmid and respective empty vectors (Santa Cruz Biotechnology, Santa Cruz, USA) were used to regulate the expression levels of *mGluR5* in MM1S and OPM2 cells. The MM1S cells were treated with 50 nmol/L agonist (S)-3,5-dihydroxyphenylglycine (DHPG) (Santa Cruz Biotechnology), with the group treated with dimethyl sulfoxide (DMSO, 10 ng/mL; Santa Cruz Biotechnology) as a positive control. The OPM2 cells were treated with *mGluR5* antagonist 1 (2-methyl-6-(phenylethynyl)-pyridine – MPEP; 50 nmol/L; Santa Cruz Biotechnology) and the other group of OPM2 cells – with *mGluR5* antagonist 2 (3-[(2-methyl-1,3-thiazol-4-yl) ethynyl]pyridine – MTEP, 50 nmol/L, ab 144307; Abcam, Cambridge, UK), with the DMSO-treated (10 ng/mL) group as a positive control.

Western blot assay

The separation of protein was conducted with ProteoJET Mammalian Cell Lysis Reagent (Thermo Fisher Scientific).

This was completed step by step according to the manufacturer's guidelines. Then, the centrifugation (15 min, 4°C) was performed. The proteins were denatured and forced to undergo sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The *mGluR5* primary antibodies – Rabbit Anti-Metabotropic Glutamate Receptor 5 antibodies (ab27190, 1:2000) were purchased from Abcam. The antibodies related to the rest proteins were also bought from Abcam: RAS primary antibody – Rabbit Anti-Ras antibody (ab69747, 1.0 µg/mL); Raf1 primary antibody – Rabbit Anti-Raf1 antibody (ab230850, 1:1000); Rabbit Anti-beta Actin antibody (ab8227, 1:2000); and secondary antibody Goat Anti-Rabbit IgG H&L (HRP) (ab205718, 1:2000). The resulting bands (RAS, Raf1 and b-actin) were analyzed with electrochemiluminescence (ECL) (Thermo Fisher Scientific) and densitometry analysis using ImageJ software (Bharti Airtel Ltd., New Delhi, India).

MTT assay

The MTT assay was utilized to evaluate the cell viability. Shortly thereafter, MM1S and OPM2 cells were placed in 96-well plates and treated with actinomycin for 0 h, 2 h, 4 h, and 8 h; then, 20 µL of MTT reagent (5 mg/mL; Abcam) was applied to each plate, after which they were incubated for 3–4 h, and the absorbance was measured at 495 nm using a microplate reader (Thermo Fisher Scientific).

Flow cytometry method for apoptosis assay

The cell apoptosis was evaluated with the flow cytometry (FCM) method. Annexin V PE and 7AAD (eBioscience, San Diego, USA) were utilized to stain cells in accordance with the instructions of the manufacturers. The percentages of apoptotic cells (annexin V PE+/7AAD–) were evaluated using Cell Quest software in a FACScan cytometer (Becton Dickinson Bioscience, Franklin Lakes, USA).

Statistical analyses

The Graphpad Prism v. 8.2 (GraphPad Software, San Diego, USA) was applied for statistical analysis. The Kruskal–Wallis test (K–W) followed by Dunn's post hoc and Kolmogorov–Smirnov test (K–S) were applied in the statistical analysis. The experiments were repeated 3 times independently.

Results

mGluR5 is upregulated in MM cell lines

The *mGluR5* expression in MM cells lines MM1S, OPM2, U266, NCI-H929, and RPMI-8226 and normal cell line HUVECs was measured using qRT-PCR (Fig. 1A). The results revealed that the *mGluR5* expression was higher in MM cells compared to the normal cells. Among the MM cell lines, *mGluR5* expression in MM1S cells was the highest and in OPM2 was the lowest, though still higher than in HUVECs. Therefore, we selected MM1S cell line for *mGluR5* downregulation and OPM2 for upregulation in the following assays. The MM1S cells treated with *mGluR5* agonist DHPG, and OPM2 cells treated with *mGluR5* antagonist MPEP and MTEP were measured using qRT-PCR with the DMSO groups as controls in both cell lines. The *mGluR5* was increased in DHPG agonist group compared to the DMSO group of the MM1S, while *mGluR5* had low expression in both the MPEP and MTEP antagonist groups (Fig. 1B,C). Furthermore, we measured the relative protein levels of *mGluR5* in all the groups of both cell lines. The results showed that *mGluR5* was downregulated in the MPEP and MTEP, and upregulated in the DHPG compared to the DMSO positive controls, which was consistent with the qRT-PCR findings (Fig. 1D,E).

Agonist-induced upregulation of *mGluR5* promotes cell viability in MM cells

The MTT assay revealed that the DHPG group had higher viability than the DMSO group in MM1S cells (Fig. 2A), while the MPEP and MTEP groups had lower cell viability than the control group in OPM2 cells (Fig. 2B) due to the regulation of the *mGluR5* expression.

Agonist-induced *mGluR5* upregulation inhibited apoptosis in MM cells

The cell apoptosis was evaluated in the groups of both MM1S and OPM2 cell lines using FCM apoptosis assays. The apoptosis rates in the *mGluR5* agonist group were lower than those of the control group in MM1S cells (Fig. 3A). Higher apoptosis was found in the groups of MTEP and MPEP in comparison with the DMSO control group (Fig. 3B). The results pointed out that the agonist-induced upregulation of *mGluR5* could decrease the cell apoptosis, while the antagonist-induced downregulation of *mGluR5* could elevate the cell apoptosis in MM cells.

Transfection-induced upregulation of *mGluR5* promotes cell viability and curbs cell apoptosis of MM cells

After MM1S cells were transfected with oe-mGluR5, oe-NC and OPM2, the cells were transfected with si-mGluR5 and si-NC, and qRT-PCR was used to confirm the regulation of *mGluR5* expression in different groups. It was verified that the oe-mGluR5 group had a high expression of *mGluR5*, while the si-mGluR5 group had a lower *mGluR5* expression compared to their control groups (Fig. 4A). The MTT assay revealed that the oe-mGluR5 induced an increase in cell viability, while si-mGluR5

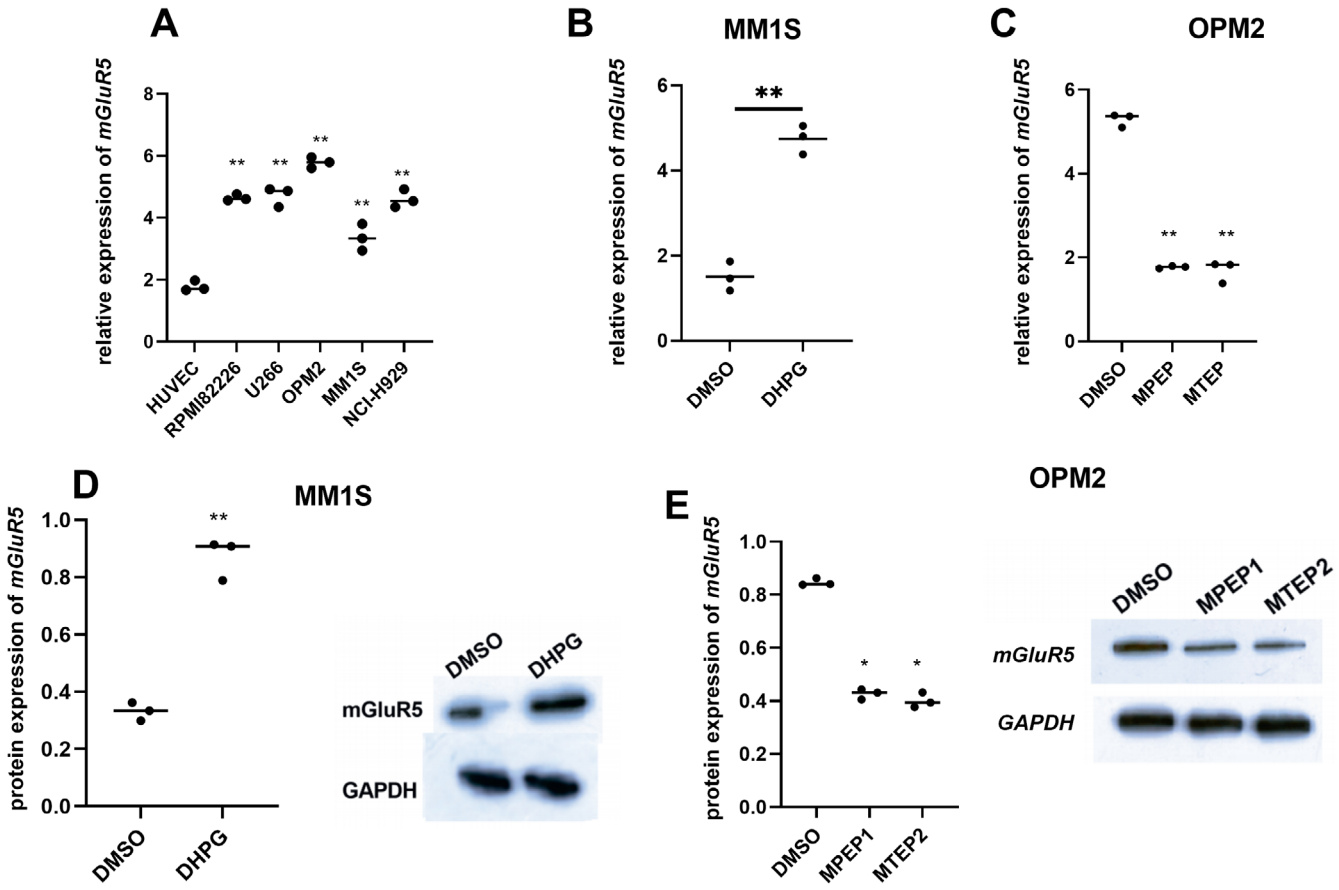

Fig. 1. *mGluR5* had higher expression in multiple myeloma (MM) cell lines. A. Quantitative real-time polymerase chain reaction (qRT-PCR) was applied to evaluate the *mGluR5* expression in MM cell lines and MM1S, OPM-2, U266, NCI-H929, and RPMI-8226, and normal cell line CD143; B,D. MM1S cell line was treated with dimethyl sulfoxide (DMSO) as a control group and 3,5-dihydroxyphenylglycine (DHPG) as an agonist group. The qRT-PCR was used to measure *mGluR5* RNA expression in each group in MM1S cell line. Western blot was used to measure *mGluR5* protein expression in each group in MM1S cell line; C,E. OPM2 cell line was treated with DMSO as a control group, 2-methyl-6-(phenylethynyl)-pyridine (MPEP) or 3-[(2-methyl-1,3- thiazol-4-yl)ethynyl]pyridine (MTEP) as antagonist groups. The qRT-PCR was used to measure *mGluR5* RNA expression in each group in the MM1S cell line. Western blot was used for *mGluR5* protein expression in each group in MM1S cell line. Each assay was performed thrice independently. Kruskal–Wallis test (K–W) with Dunn's post hoc test and Kolmogorov–Smirnov test (K–S) were applied in the statistical analysis (Fig. 1A. human umbilical vein endothelial cells (HUVECs) compared to RPMI82226, p = 0.0197; HUVECs compared to U266, p = 0.0324; HUVECs compared to OPM2, p = 0.0029; HUVECs compared to MM1S, p = 0.0452; HUVECs compared to NCI-H929, p = 0.0298; K–W, Dunn's post hoc. Fig. 1B. p = 0.0237, K–S; Fig. 1C. DMSO compared to MPEP, p = 0.0376; DMSO compared to MTEP, p = 0.0425; K–W, Dunn's post hoc; Fig. 1D. p = 0.0198, K–S; Fig. 1E. DMSO compared to MPEP1. p = 0.0487; DMSO compared to MTEP2, p = 0.0341; K–W, Dunn's post hoc)

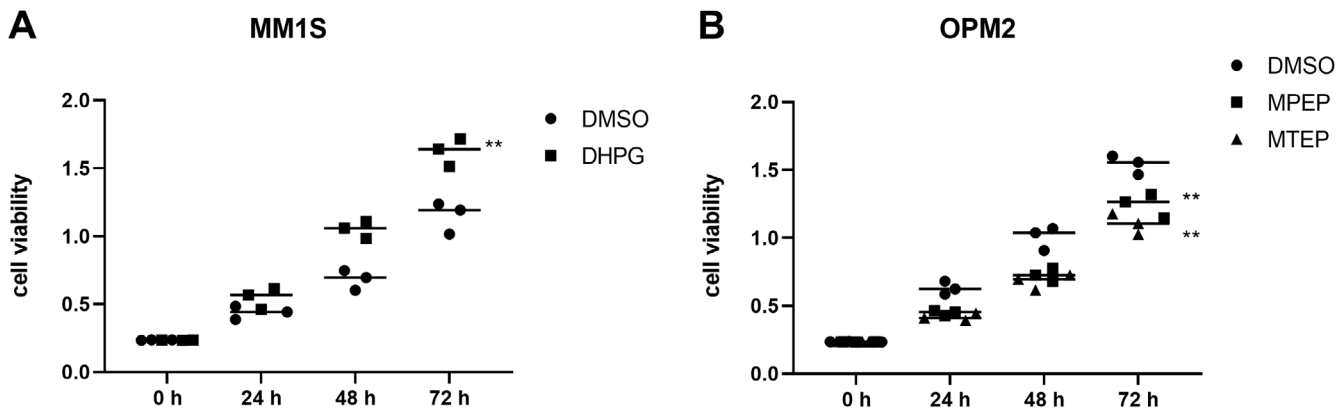

Fig. 2. Agonist-induced *mGluR5* upregulation promoted cell viability in multiple myeloma (MM) cells. A,B. The MTT assay measured the cell viability situation when MM1S cell line was treated with dimethyl sulfoxide (DMSO) as a control group and 3,5-dihydroxyphenylglycine (DHPG) as an agonist group, and OPM2 cell line was treated with DMSO as control group, with 2-methyl-6-(phenylethynyl)-pyridine (MPEP) or 3-[(2-methyl-1,3- thiazol-4-yl) ethynyl]pyridine (MTEP) as antagonist groups. Each assay was performed thrice independently. Kruskal–Wallis test (K–W) with Dunn's post hoc test and Kolmogorov–Smirnov test (K–S) were applied in the statistical analysis (Fig. 2A. p = 0.0032, K–S; Fig. 2B. DMSO compared to MPEP, p = 0.0356; DMSO compared to MTEP, p = 0.0225; K–W, Dunn's post hoc)

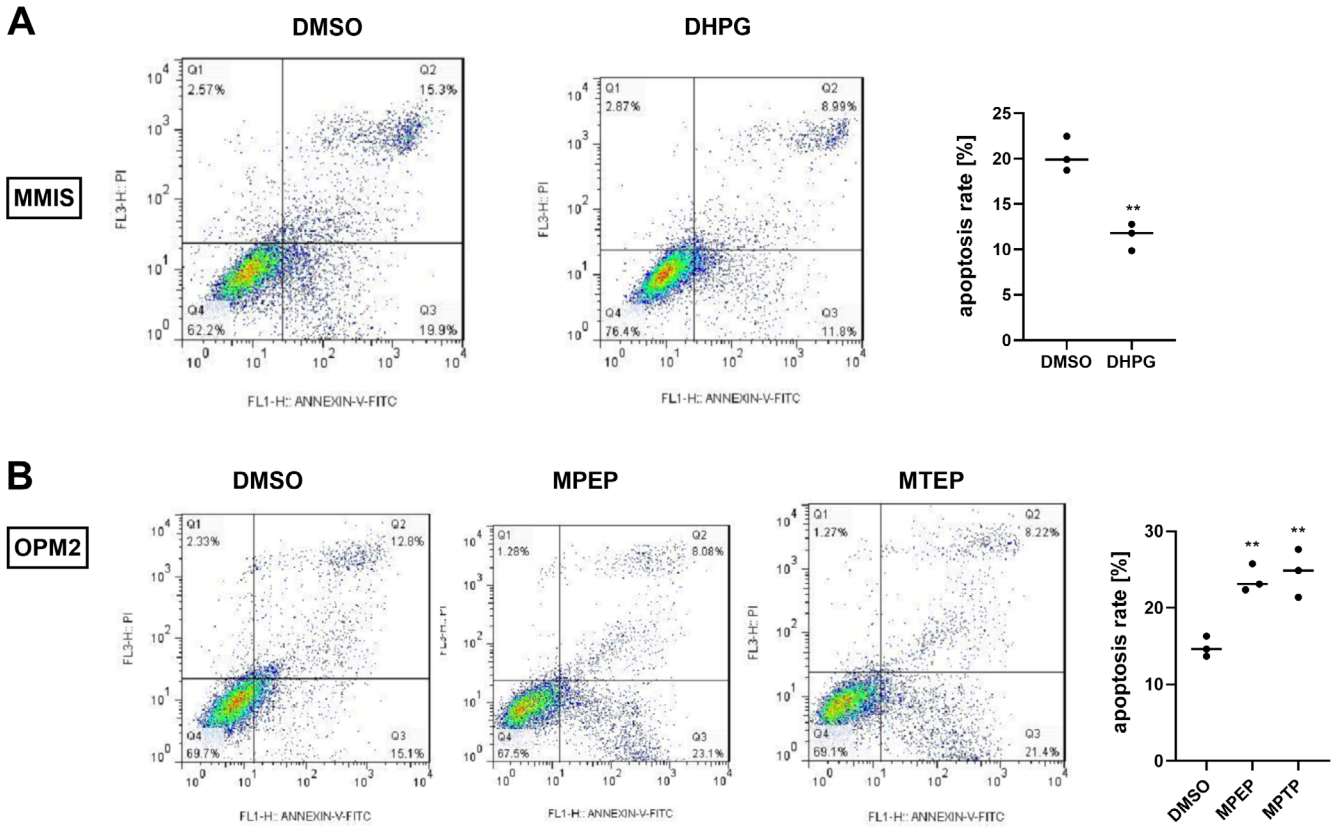

Fig. 3. Agonist-induced *mGluR5* upregulation inhibited apoptosis in multiple myeloma (MM) cells. A,B. Flow cytometry (FCM) apoptosis assays measured the cell apoptosis changes when MM1S cell line was treated with dimethyl sulfoxide (DMSO) as a control group and 3,5-dihydroxyphenylglycine (DHPG) as an agonist group, and OPM2 cell line was treated with DMSO as a control group, with 2-methyl-6-(phenylethynyl)-pyridine (MPEP) or 3-[(2-methyl-1,3-thiazol-4-yl)ethynyl]pyridine (MTEP) as antagonist groups. Each assay was performed thrice independently. Kruskal–Wallis test (K–W) with Dunn's post hoc test and Kolmogorov–Smirnov test (K–S) were applied in the statistical analysis (Fig. 3A. p = 0.0269, K–S; Fig. 3B. DMSO compared to MPEP, p = 0.0274; DMSO compared to MPTP, p = 0.0225; K–W, Dunn's post hoc)

lowered cell viability compared with the control groups (Fig. 4B). On the other hand, FCM apoptosis assays found that the oe-mGluR5 group displayed lower cell apoptosis rate, while the si-mGluR5 group presented higher cell apoptosis in MM cell lines compared to their control groups (Fig. 4C,D). The MTT assay revealed that the oe-mGluR5 induced an increase in cell viability, while si-mGluR5 lowered cell viability compared with the control groups (Fig. 4B).

mGluR5 upregulation activates the Ras signaling pathway in MM cells

Western blot assays revealed that in the oe-mGluR5 group, the protein expression of *mGluR5* was upregulated in comparison to the oe-NC, while in the si-mGluR5 group, the *mGluR5* protein expression was downregulated (Fig. 5A,D). The investigation into the correlation between *mGluR5* and Quantitative real-time polymerase chain reaction (qRT-PCR) signaling was carried out in MM cells. The protein levels of Ras, p-Raf1 and Raf1 were measured using western blot. The inhibition of *mGluR5* in MM1S cells showed decreased protein levels of Ras and phosphorylated Raf1, and the upregulation of *mGluR5* in OPM2 cells had an opposite effect on the protein levels of the signaling pathway (Fig. 5B–D). These data indicated that the upregulation of *mGluR5* in MM cells could activate the Ras–MAPK pathway by promoting Ras and the phosphorylation of Raf1 in MM cells (Fig. 5B–D).

Discussion

Previous studies have demonstrated that *mGluR5* plays different roles in various human diseases. Studies verified that *mGluR5* was upregulated in human tongue cancer and its overexpression promoted cell invasion, migration and adhesion.[13] As displayed by Oncomine online tool, *mGluR5* is aberrantly upregulated in MM compared to normal tissues. Therefore, we presumed that *mGluR5* might serve as an oncogenic gene in MM, similarly to its role in tongue cancer and prostate cancer.[14] Yet, there have been few research studies concerning the impact of *mGluR5* on MM. Therefore, our study was dedicated to explore how *mGluR5* regulated the cell apoptosis and proliferation of the MM and the possible mechanisms beneath. Firstly, through qRT-PCR we confirmed the expression of *mGluR5* in various cell lines of MM and the control cell line HUVEC, and we discovered that *mGluR5* was upregulated in MM

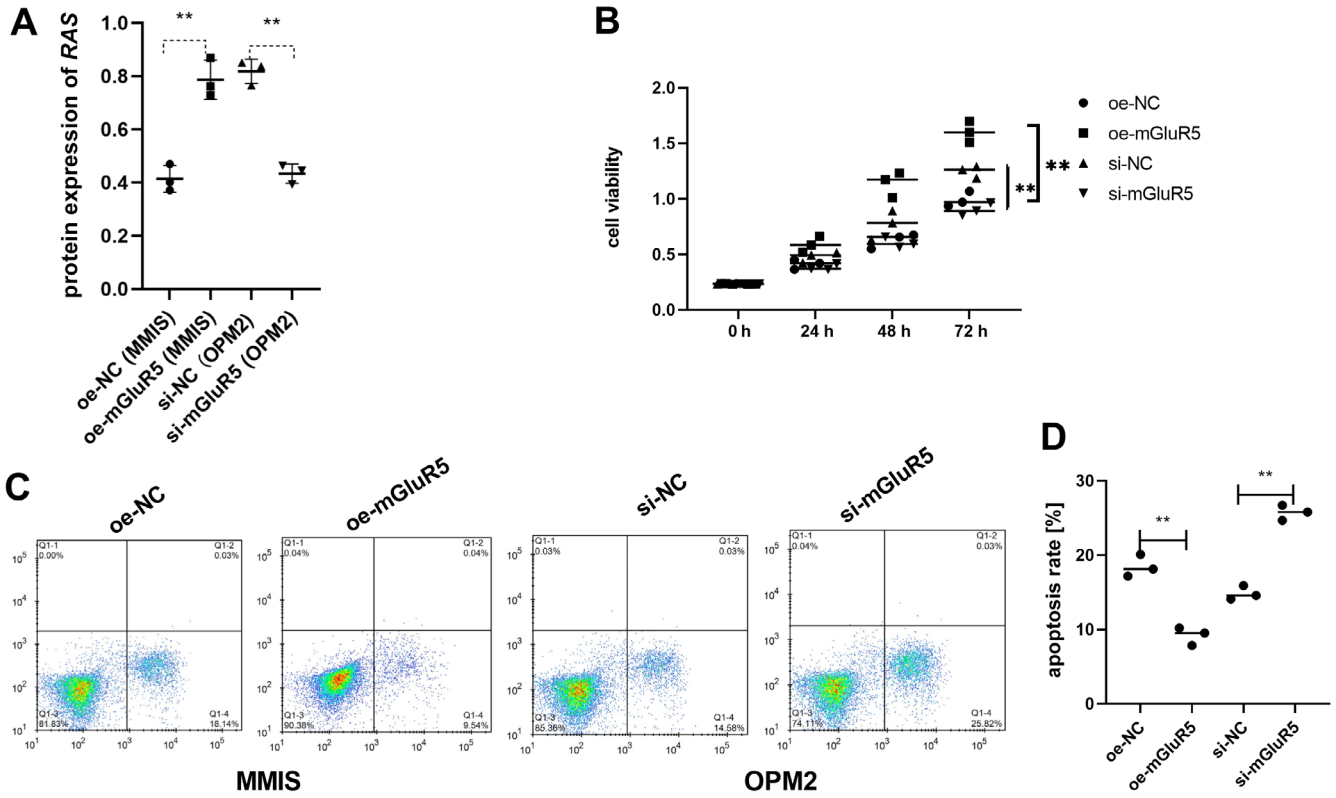

Fig. 4. Transfection-induced upregulation of *mGluR5* promoted cell viability and inhibited cell death in multiple myeloma (MM) cells. MM1S cell line was transfected with si-NC or si-mGluR5 plasmids while OPM2 cell line was selected to knock down by transfection with oe-NC and oe-mGluR5 plasmids. A. Quantitative real-time polymerase chain reaction (qRT-PCR) was performed to evaluate the relative mGluR5 mRNA expression among all the groups in both cell lines; B. Cell viability was measured with MTT method; C,D. Flow cytometry (FCM) apoptosis assay was used to measure cell apoptosis. Each assay was performed thrice independently. Kruskal–Wallis test (K–W) with Dunn's post hoc test and Kolmogorov–Smirnov test (K–S) were applied in the statistical analysis (Fig. 4A. oe-NC (MMIS) compared to oe-mGluR5 (MMIS), p = 0.0163; si-NC (OPM2) compared to si-mGluR5 (OPM2), p = 0.0319; K–W, Dunn's post hoc; Fig. 4B. oe-NC (MMIS) compared to oe-mGluR5 (MMIS), p = 0.0094; si-NC (OPM2) compared to si-mGluR5 (OPM2), p = 0.0308; K–W, Dunn's post hoc. Fig. 4C; oe-NC(MMIS) compared to oe-mGluR5 (MMIS), p = 0.0268; si-NC (OPM2) compared to si-mGluR5 (OPM2), p = 0.0325; K–W, Dunn's post hoc)

cells compared to the HUVECs, which was consistent with Oncomine analysis and also suggested that *mGluR5* might be an oncogene in MM. Among all the MM cell lines, OPM2 presented the highest expression of *mGluR5*, while MM1S the lowest. Furthermore, we decided to differentiate the expression of *mGluR5* in MM cell lines, selecting OPM2 and MM1S cell lines. To inhibit or stimulate the *mGluR5* expression in cells, transfection with si-mGluR5 and oe-mGluR5, and treatments with inhibitors or agonists are available. As for *mGluR5*, DHPG is commonly used to activate *mGluR1/5*[15,16]; MPEP and MTEP are effective inhibitors of *mGluR5*.[17,18] In addition, it has been discovered that *mGluR5* takes part in central nervous system (CNS) disorders that are induced by the hypersecretion of glutamate, such as epilepsy, neurogenic or inflammatory pain, dyskinesia, headaches, drug addiction, and psychosis.[19,20] In this study, we adopted both the transfection and treatments with agonist DHPG and inhibitors MPEP and MTEP in MM cells in order to upregulate *mGluR5* in MM1S and downregulate *mGluR5* in OPM2. The qRT-PCR and western blot confirmed the upregulation of *mGluR5* by agonist DHPG or oe-mGluR5 in MM1S, and inhibition of *mGluR5* by antagonists MPEP and MTEP and si-mGluR5 in OPM2 cells in mRNA and protein levels. After confirmation, we carried out MTT and apoptosis assays to measure the changes in cell viability and apoptosis. The results indicated that the upregulation of *mGluR5* by oe-mGluR5 or DHPG in MM1S promoted the cell viability and inhibited cell apoptosis compared with the DMSO control group, which supported the claim that *mGluR5* behaved as an oncogene in the MM cell lines. Likewise, it was revealed that the downregulation of *mGluR5* by si-mGluR5 and antagonists MPEP and MTEP in OPM2 cells suppressed cell viability and induced cell apoptosis. The results verified that *mGluR5* acted as oncogene in MM, which was in accordance with our hypothesis. To investigate the potential mechanism involved, we did further research with regard to Ras–MAPK signaling.

Ras–mitogen activated protein kinase pathway is an essential intracellular signaling pathway that regulates multiple cellular functions, including cell proliferation and apoptosis.[21] The most frequently mutated gene concerning the MAPK signaling pathway is *Ras*, which is related to the superfamily of small GTPases and is the first intracellular effector of the Ras–MAPK signaling pathway.[22] *Ras* can be stimulated by many extracellular stimuli, which helps to transform *Ras* from a GDP-bound inactivated form to a GTP-bound activated form, and recruit

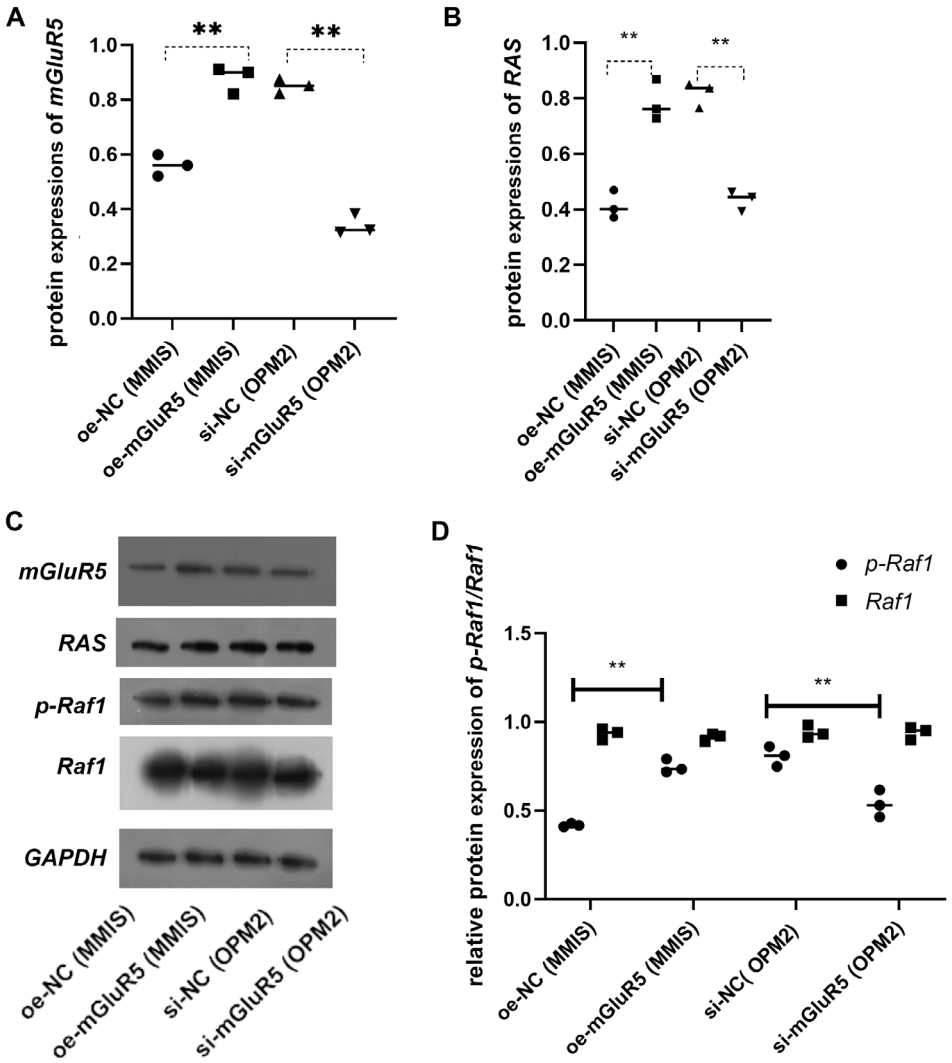

Fig. 5. Transfection-induced upregulation activated the Ras–mitogen activated protein kinase (MAPK) signaling pathway in multiple myeloma (MM) cells. MM1S cell line was transfected with si-NC or si-mGluR5 plasmids, while the OPM2 cell line was selected to be knocked down by transfection with oe-NC and oe-mGluR5 plasmids. Western blot was used to examine the protein levels of *mGluR5* (A) and RAS (B) as well as phosphorylation of Raf1 (C) among all the groups in both cell lines after transfection. Each assay was performed thrice independently. Kruskal–Wallis test (K–W) with Dunn's post hoc test and Kolmogorov–Smirnov test (K–S) were applied in the statistical analysis (Fig. 5A. oe-NC (MMIS) compared to oe-mGluR5 (MMIS), p = 0.0039, si-NC (OPM2) compared to si-mGluR5 (OPM2), p = 0.0254; K–W, Dunn's post hoc; Fig. 5B. oe-NC (MMIS) compared to oe-mGluR5 (MMIS), p = 0.0174, si-NC (OPM2) compared to si-mGluR5 (OPM2), p = 0.0232; K–W, Dunn's post hoc; Fig. 5D. oe-NC (MMIS) compared to oe-mGluR5 (MMIS), p = 0.0094, si-NC (OPM2) compared to si-mGluR5 (OPM2), p = 0.0163; K–W, Dunn's post hoc)

the Raf-1 kinase to the plasma membrane.[23] The activation of the Ras–MAPK pathway is an early event in cancers and a promoter to chemoresistance.[24] In MM, previous studies showed that Ras–MAPK pathway is activated in MM patients and its activation could promote the progression of MM.[25]

In the present study, western blot was used to determine the changes in *Ras* and p-Raf1/Raf1 in response to *mGluR5* regulation in MM1S and OPM2 cells. It was found that the upregulation of *mGluR5* could activate the Ras–MAPK pathway, exerting a promotive impact on MM cell proliferation and a suppressive effect on apoptosis.

Limitations

This study only focused on the in vitro cellular model, which makes the results and findings relatively limited.

Conclusion

The results from the present research indicate that the overexpression of *mGluR5* promoted cell proliferation and inhibited apoptosis by activating Ras–MAPK pathway in MM. The findings suggest that *mGluR5* inhibitors MPEP or MTEP might be used in the future as a tumor suppressor in MM after extensive in vivo research and clinical trials.

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