Activation of hypermethylated P2RY1 mitigates gastric cancer by promoting apoptosis and inhibiting proliferation

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Research Article

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Abstract

P2RY1 receptor is known to cause cancer by activating the ERK signal pathway, its DNA methylation status or even the corresponding regulatory mechanism remains unknown. In this study, DNA methylation chip was used to profile the genome-wide DNA methylation level in gastric cancer tissues. Proliferation and apoptosis of the SGC7901 gastric cancer cell line were determined after treatment with a selective P2RY1 receptor agonist, MRS2365. The promoter region of P2RY1 was found to be highly methylated with 4 hypermethylated sites ($|\Delta\beta| > 0.2$) in diffuse gastric cancer and then were validated by bioinformatic analysis in TCGA database. Analysis of MRS2365-treated cells by annexin-V/PI staining and Caspase-3 activity assays indicated the induction of apoptosis in SGC7901 cells. P2RY1 receptor activation in human SGC7901 gastric cancer cells via the MRS2365 agonist induced apoptosis and reduced cell growth. High DNA methylation in the promoter region of P2RY1 may have contributed to the reduced expression of P2RY1's mRNA, which is likely responsible for the "aggressive" nature of the diffuse type gastric cancer.

1 Introduction

Gastric cancer is a growing concern due to its high prevalence worldwide and the increasing numbers of deaths related to the disease (1). While the etiology of gastric cancer carcinogenesis is thought to be multifactorial, molecular and genome-wide approaches have identified various genetic alterations associated with gastric tumorigenesis and progression (2). Two types of gastric cancers have been identified based on histological differences, epidemiology, etiology, pathogenesis and biological behavior. First is the diffuse type, which is characterized by infiltrating cells, poor differentiation, and noncohesive cancer cells with vast fibrous stroma, and the second is the intestinal type, which mostly features cohesive, and glandular-like cells (3). Among these, the diffuse type is more prevalent among younger individuals and metastasis of this type of gastric cancer is often identified in the peritoneum or lymph nodes, which makes prognosis extremely difficult. Currently, diffuse gastric cancer is treated with chemotherapy and targeted therapy using molecular approaches.

G protein-coupled receptors (GPCRs), which form the largest family of cell surface receptors, have been shown to modulate most physiological functions in the body. However, their roles in cancer development are not clearly understood (4, 5). Recently, P2RY1, a member of GPCRs has emerged as a cancer target because of its critical role in tumor growth and metastasis. P2RY1 activation via the endogenous agonist, ADP, was shown to alter multiple physiological functions (6–8). Interestingly, the P2RY1 receptor was shown to regulate cell growth and death in the following cancer cell lines; 1321N1 astrocytoma cells (9, 10), A375 melanoma cells (11, 12) and PC3 prostate cancer cells (13). Particularly, blocking the P2RY1 receptor using the antagonist MRS2179, reversed cell proliferation, suggesting that the P2RY1 receptor may have an anti-proliferative effect (11, 12). Additionally, other P2RY receptor subtypes, such as the Gq-coupled P2RY2, P2RY6, and P2RY11 receptors and the Gi-coupled P2RY12 and P2RY13 receptors have also been reported (14–17) to regulate cell death and growth (10, 14, 15, 18–20). Studies have shown that in aggressive gastric cancer tissues the levels of P2RY1 mRNA were low when compared to

noncancerous gastric tissues. This indicates that the lack of P2RY1 may contribute to the development of aggressive gastric cancer growth. However, studies on the role of P2RY1 receptor signaling in gastric cancer are lacking. Although most cancers have been linked to specific DNA methylation, the epigenetic factors have not been associated with diffuse gastric cancer.

In this study, we used DNA methylation chip technology and previously published data to analyze the role of the P2RY1 receptor in gastric cancer. Our results showed that gastric cancer tissues have high levels of DNA methylation and low levels of the P2RY1 receptor protein expression when compared to noncancerous gastric tissues. Then, we used gastric cancer cells to analyze the involvement of P2RY1 receptor in cell death and growth. Subsequent activation of P2RY1 receptor using a selective P2RY1 receptor agonist, the ADP analogue MRS2365 (21) revealed that it induced apoptosis and inhibited cell proliferation. These results indicate that the P2RY1 receptor may be a potential target for the treatment of gastric cancer.

2 Materials And Methods

2.1 Cancer Tissues

All experimental procedures were approved by the Zhongshan Affiliated Hospital of Xiamen University. The Human Research Ethics Committee of Zhongshan Hospital also approved this study. Patient participation and sample collection were performed in accordance with the ethical standards of the Zhongshan Affiliated Hospital of Xiamen University committee and the 1964 Helsinki declaration or later amendments. All patients signed the informed consent form. Tissue samples for the study were obtained from 6 gastric cancer patients (2 intestinal and 4 diffuse type cancers with matched normal tissue) at the Department of Surgical Oncology of the Zhongshan Hospital in China. Two samples were obtained from each patient: gastric tumor samples and noncancerous samples from the adjacent areas. After collection, the samples were flash frozen in liquid nitrogen and stored at -80°C until further use. A QIAamp DNA Mini Kit (QIAGEN) was used to extract DNA from individual samples based on the manufacturer's protocol. Additional informed consent was obtained from all patients for which identifying information is included in this article.

2.2 Methylation chip experiment

Microarray hybridization and scanning data were conducted according to standard protocols. The whole genome from each tissue received bisulfite treatment, and was amplified, fragmented with restriction enzymes and hybridized to the Illumina Infinium Human Methylation 450 BeadChip kit, which can analyze $>450,000$ methylation sites that have been reported to cover 99% of the genes in RefSeq. These methylated regions have been also reported to be distributed along the entire gene, including the transcription start site (TSS), 5' untranslated region (UTR), 3' UTR, and the exons. In this study, we filtered the TSS and first exon, which are known to mostly regulate a gene. Following hybridization, allele specific single-base extension and staining were performed. Then, the BeadChips were imaged on the Illumina BeadArray Reader platform. The Illumina's BeadScan software was used to extract image intensities.

Methylated and unmethylated regions in the genome were recognized by the differences in fluorescence intensities, which were marked as data points after subtracting the background fluorescence. The intensities of methylated and unmethylated signals were normalized using the Illumina Genome Studio program. To array data points, the average methylation (β) value was calculated from 30 replicates of methylation data from both methylated (Cy5) and unmethylated (Cy3) alleles. In these analyses, we excluded methylation data from the X chromosome. The filtered methylation sites were then mapped to their potential corresponding gene defined in the UCSC Genome Browser HG19 RefSeq database.

2.3 Cell culture

Noncancerous human gastric cells (GES1) and gastric cancer cells (BGC823, MKN45, SGC7901, HGC27, AGS1 and MGC803) were cultured in RPMI-1640 or DMEM/F-12 media containing the following antibiotics and supplements: 10% FBS, 100 U/ml penicillin, 100 mg/ml streptomycin, and 3 mM/l glutamine. Cells were maintained at 37°C with a constant supply of 5% CO₂ in a humidified incubator.

2.4 Western blotting

To extract total proteins from tissues, the following protocol was used. Tissues collected from patients were lysed 20 min in 1 ml cell lysis buffer on ice after thoroughly washing with PBS. Then, the lysates were centrifuged at 14000 $\times$ g for 30 min to collect the supernatant. On the other hand, total proteins from cell cultures were extracted using the RIPA protein extraction reagent (Solabio, Beijing, China) containing a protease inhibitor cocktail (Roche, Basel, Switzerland) according to the manufacturers' protocol. Then, the total protein concentration in the tissue supernatants and cell lysates was measured by the BCA method. About 30 μ g of total proteins from each tissue sample and 50 μ g of total proteins from each cell culture were each resolved on a 10% SDS-PAGE, transferred to polyvinylidene fluoride (PVDF) membranes (Millipore) and processed for Western blotting. Briefly, anti-P2RY1 (1:1000) was used as the primary antibody (Sungon, China) and goat anti-rabbit IgG (1:5000) was used as the secondary antibody (Santa Cruz Biotechnology, CA). GAPDH was used as the control to verify the loading of equal quantity of proteins. The protein bands were visualized by the ECL-based chemiluminescence signals, which were recorded using an ImageQuant LAS 4000 mini instrument. To detect phosphorylation, SGC7901 cells were first cultured for 4 h in serum-free medium and then incubated in serum supplemented medium to which various pharmacological reagents were previously added. Cells were cultured in these conditions at 37°C for various time points. Then, the media were replaced with 100 ml cold RIPA buffer containing proteinase inhibitors to terminate the reactions. To detect Caspase-3 activity, SGC7901 cells (2×10^5 cells/well) were grown to 70% confluence in 6-well plates. Then, apoptosis inducers (Beyotime, China) were added to the cells 60 min before treatment with the P2RY1 receptor agonist, MRS2365, for 8 h. The control groups did not receive the apoptosis inducers.

2.5 Cell viability assay

To investigate cell viability, 1×10^4 SGC7901 cells were cultured overnight in 100 μ l medium containing 10% serum/well in a 96-well plate at 37°C. Then, MRS2365 (1 mM) was added to the cells and incubated for 24, 48 or 72 h in an incubator maintained with 5% CO₂ at 37°C. The control group was not treated with

MRS2500. The MTT Toxicology Assay Kit (Sigma–Aldrich, St. Louis, MO) was used to assess cell viability according to the manufacturer’s instructions. Cell viability was quantified by measuring the absorbance at 560 nm on MKV reader (Thermofisher, CA). Cell viability was estimated as a percentage of live cells in the treated groups vs. the control.

2.6 Cell cycle distribution

To determine cell cycle arrest by MRS2365, 1×10^5 SGC7901 cells/well in a 6-well plate were treated with MRS2365 for 36 h. Then, the medium was removed, and cells were fixed overnight in 70% ethanol at 4°C. Cell cycle distribution was analyzed after staining with PI (100 µg/ml) (Sigma-Aldrich) using a Guva Easysite flow cytometer (Millipore).

2.7 Cell migration assays

To perform migration assays, a transwell system with an insert (8-µm pore size; Millipore, Bedford, MA, USA) was used. In the upper chamber, 5×10^4 cells were plated in serum-free medium and in the lower chamber medium supplemented with 10% FBS was added. Then, 1 mM MRS2365 was added to the upper chamber, and 24 h later the cells in the lower chamber that had migrated through the insert were detected by staining with 0.5% crystal violet. The cells remaining on the upper part of the insert were removed with cotton wool and analyzed. All cells were then visualized through a ZEISS microscope (New York, USA) to obtain pictures and cells in 6 random fields per well were counted and used to estimate cell migration.

2.8 Apoptosis assay

Apoptosis was measured using the FITC Annexin V Apoptosis Detection kit (BD Pharmingen™, USA) according to the manufacturer’s instructions. SGC7901 cells were cultured in the presence or absence of apoptotic inducers from the Apoptosis Inducers Kit (Beyotime, China), harvested, mixed with 500 µl of Annexin V binding buffer and incubated with 5 µl each of Annexin V-FITC and PI were added for 15 min in dark. Positive staining was analyzed using the GUAVA Easycite flow cytometry (Millipore, USA).

2.9 Statistical analysis

All data represent mean $\pm$ SD. Statistical analysis was performed using R 2.3.0 (22). Statistical significance (*P < 0.05) of the differences between the treated and control groups were analyzed using a 2-tailed t-test.

3 Results

3.1 P2RY1 DNA is hypermethylated in the promotor region that lowers protein expression in gastric cancer tissues

We performed the DNA methylation chip experiment to establish the methylation spectrum of human diffuse gastric cancer (Fig. 1a). Then, we validated/extended our data compared our data with previously published results (23) by constructing a Venn diagram of methylated genes and mRNA that were

significantly different between gastric cancer and noncancerous tissues (Fig. 1b). Among the genes analyzed, P2RY1 had 4 high methylated sites (**Table 1**) within its promoter region while its mRNA level was low in gastric cancer tissues. After compared with TCGA/STAD database, the 4 sites also showed high methylation level status comparing with normal gastric samples in Fig. 1c. In this study, we had 2 samples that were identified histopathologically to be intestinal gastric cancer types and 4 samples that were identified as diffuse cancer types. The expression of P2RY1 differed markedly among the 2 sample types with the diffuse gastric cancer tissues showing significantly lower levels of P2RY1 mRNA when compared to the intestinal gastric cancer tissues (Fig. 1d). Thus, low expression of P2RY1 appears to be a critical factor in establishing diffuse gastric cancer. Moreover, we analyzed the expression levels of P2RY1 in various gastric cancer cell lines by Western blotting to determine how P2RY1 affected the development of gastric cancer. We found that SGC7901 had the higher level and BGC823 had the lower level of P2RY1 proteins among all the lines tested (Fig. 1e). Based on these results, SGC7901 cells were chosen for further experiments for P2RY1 function.

3.2 P2RY1 receptor-mediated activation of ERK1/2 signal pathway

Treatment of SGC7901 cells with the P2RY1 agonist, MRS2365 (1 mM) increased ERK1/2 phosphorylation and ELK1/c-Fos/c-Jun phosphorylation to the maximum in the first 5 min followed by a decrease to the basal level within 15 min (Fig. 2). In contrast, treatment of MGC803 and MKN45 cell lines (high levels of P2RY1) with MRS2365 did not result in an increase in ERK1/2 phosphorylation. These results indicate that the P2RY1 agonist, MRS2365, activates the ERK1/2 signal pathway in gastric cell lines with P2RY1 protein.

3.3 Activation of the P2RY1 receptor inhibits proliferation and gastric cancer cell metastasis

In order to investigate whether activation of the P2RY1 receptor results in an anti-proliferative effect, we used the MTT assay to determine cell proliferation. The results showed that treatment of SGC7901 cells with MRS2365 for 24 h, 48 h or 72 h significantly lowered cell numbers than in the control group (Fig. 3a; $P < 0.05$, paired test) indicating an anti-proliferative effect. To determine the mechanism by which MRS2365 inhibited the growth of SGC7901 cells, we determined the stages of cell cycle arrest in treated cells using flow cytometry. After 24 h of treatment, we found that 34.9% (± 0.81) of the cells were in the S phase when compared to 39.7% (± 1.75) in the control group ($P < 0.05$) (Fig. 3b). This indicated that MRS2365 may inhibit cell growth by arresting the cell cycle in the G2 phase. We then used a transwell system to investigate the effect of P2RY1 on gastric cancer cell migration. We found that activation of P2RY1 decreased SGC7901 cell migration (Fig. 3c). Together, these results suggest that activation of the P2RY1 receptor can in part inhibit cell proliferation/metastasis.

3.4 Effect of P2Y1 receptor activation on apoptosis

P2RY1 receptor activation is known to induce apoptosis in PC3 prostate cancer cells (24). Therefore, we examined the effect of P2RY1 activation via the MRS2365 agonist on the apoptosis of SGC7901 cells by measuring Caspase-3 activity in Fig. 4a/b, as is described (25). As is shown in Fig. 4c, MRS2365 (1 mM)

promoted Caspase-3 activity in SGC7901 cells cultured with apoptotic inducers. In contrast, MRS2365 did not increase Caspase-3 activity in the absence of apoptotic inducers.

4 Discussion

Over the past 50 years, gastric cancer incidence and related mortality have decreased significantly worldwide. However, in Asia, gastric cancers still remain prevalent and result in loss of life in large numbers. This is largely due to poor prognosis, which results in metastasis and subsequent death (26). In this study, we found that the P2RY1 receptor may play a critical role in causing gastric cancer. Our results have shown that 4 higher methylated sites of P2RY1's promoter region could be used as markers for the prognosis of gastric cancers.

In the diverse MAPK signaling pathway, expression of early genes such as c-Fos, c-Jun, and Elk1 is regulated by phosphorylation of the transcription factors (27, 28). Specifically, c-Fos and c-Jun phosphorylation has been shown to increase astrocyte proliferation and GFAP expression. Besides, *in vitro* and *in vivo* studies have shown that ATP can upregulate the expression of the c-Fos protein and the formation of AP-1 transcriptional complexes (29, 30). In the present study, we found that P2RY1 receptor activation in SGC7901 can induce apoptosis. This result is consistent with previous reports after the activation of the P2RY1 receptor (9–13). Previous studies have also shown that the P2RY1 receptor-mediated apoptosis in astrocytoma cells (1321N1) and prostate cancer cells (PC3) correlated with ERK1/2 activation. Our study further examined the potential involvement of MAPK signaling in gastric cancer cells. We showed that the selective activation of P2RY1 via its agonist, MRS2365, can induce ERK1/2 phosphorylation and subsequent ELK1/c-Fos/c-Jun phosphorylation, suggesting a crucial role of ERK1/2 signaling in gastric cancer cells (Fig. 5). The Ras-Raf-MEK-ERK pathway activation is known to be critical for the proliferation of many human tumors (31–33) thus this pathway may serve as an important molecular target for anticancer therapy (34, 35). Specifically, the P2RY1 receptor may serve as a novel anticancer target.

Besides inducing apoptosis, P2RY1 receptors are also known to inhibit cell proliferation. The anti-proliferative effect of P2RY1 receptors was first demonstrated in 1321N1 astrocytoma cells that expressed a recombinant human P2RY1 receptor(36). We also found that selective activation of P2RY1 receptors using the agonist, MRS2365, can inhibit SGC7901 cell proliferation, which is consistent with previous studies (11, 37).

In previous studies (38–41), the expression of the P2RY1 receptor is low in gastric cancer tissues. In this study, we also showed the beneficial effects of the P2RY1 agonist, which has been shown in other cancer models such as melanoma, where P2RY1 receptor was highly expressed (12). The enhanced *in vivo* stability of the di-nucleotide P2RY1 agonists (42) also increases their potential use to treat other diseases such as diabetes or thrombosis (43).

In summary, we have demonstrated that, in gastric cancer tissues, the P2RY1 gene is highly methylated, while P2RY1 mRNA are expressed at relatively low levels when compared to noncancerous tissue. We

also showed that MRS2365, the selective agonist of the P2RY1 receptor, can induce ERK1/2 phosphorylation that may inhibit cell proliferation/migration and induce apoptosis. Together, these findings suggest that P2RY1 plays an important role in the development of gastric cancer. Finally, our results underscore the potential therapeutic application of P2RY1. The pro-apoptotic and anti-proliferative effects induced by P2RY1 receptor activation indicate that the P2RY1 receptor might be an attractive target for the treatment of gastric cancer.

Declarations

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Author contribution

Liangliang Cai participated in the design of the study, performed experiments and participated in the writing of the paper. Yinggang Hua participated in the design of the study and performed experiments. Long Li participated in the design of the study and collected patients' samples. Guoyan Liu designed the study and participated in the writing of the paper.

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Table 1

Table 1. Methylation loci sites within P2RY1’s TSS region.

TargetID	Δbeta value	Gene location	chromosome
cg02841941	0.28431	TSS200	chr3
cg10949611	0.28524	TSS200	chr3
cg21143560	0.20043	TSS200	chr3
cg26125811	0.26865	TSS200	chr3

Figures

Figure 1

Chip data analysis and western blot analysis of P2RY1 receptor expression in human intestinal and diffuse gastric cancers and endogenous P2RY1 expression in gastric cancer cell lines. (A) Hierarchical clustering of differentially methylated sites in gastric cancer tissue compared to the normal tissue; (B) Venn diagram depicting the overlap between genes differentially expressed in chip methylation data and mRNA expression data (published). The cut-off value for significant methylation site is $|\Delta \text{beta value}| > 0.2$. 'a' represents genes with low mRNA expression in reference; 'b' represents genes with high mRNA expression; 'c' indicates genes that are hypomethylated in the chip experiment in this study; 'd' indicates genes that are hypermethylated in the chip experiment in this study; (C) Distribution of beta value was shown according to 4 high methylation sites. Data about beta value are shown as mean $\pm$ SD. * $p < 0.05$. The number of normal, intestinal type, diffuse type and NOS type were 8, 48, 22, 97; (D) Expression of

P2RY1 protein in the intestinal/diffuse gastric cancer tissues and the corresponding noncancerous tissues using western blotting; (E) P2RY1 protein expression in gastric cell lines using western blotting.

Figure 2

MRS2365-induced activation of ERK1/2 in SGC7901 cells promotes ELK1/c-Fos/c-Jun activation. SGC7901 cells were treated with 10 ng/mL MRS2365 and MRS2500 at 37°C for 1 h.

Figure 3

Effects of MRS2365 on the proliferation and migration of SGC7901 gastric cancer cells. (A) MTT assay. SGC7901 cells were cultured with MRS2365 (1 mM) in 96-well plates for 3 days. The controls did not receive MRS2365. Then, MTT was added and MTT activity was measured after 4 h at 37°C. Control cells did not receive MRS2365. All experiments had 3 replicates. Data are presented as mean $\pm$ SD. (B) The effect of the P2RY1 agonist on the cell cycle. SGC7901 cells were grown overnight to ~30% confluence in complete RPMI1640 medium in 6-well plates. Then, they were treated with MRS2365 (100 nM) for 24 or 72 h and cell cycle analysis was carried out with PI staining. MRS2365 caused G1 cell cycle arrest. Data represent the results of 2 independent experiments; (C) Flow cytometry results from 3 independent experiments. Statistical significance of the data was analyzed by Student's t-test. Data represent mean $\pm$ standard deviation (SD). ** $p < 0.01$; * $p < 0.05$.

Figure 4

P2RY1 receptor activation induces apoptosis in SGC7901 cells. (A) Cells were stained with annexin V and propidium iodide (PI) to determine apoptosis by flow cytometry. Viable cells stained negative for annexin V and PI (lower left quadrant), cells in early apoptotic stage stained positive for annexin V but negative for PI (lower right quadrant); and necrotic cells, or those in late apoptotic stage, stained positive for both annexin V and PI (upper right quadrants). (B) Data represent mean $\pm$ standard deviation (SD) of 3 independent experiments. ** $p < 0.01$, control vs. MRS2365 treated group. (C) Western blot analysis of caspase-3 expression. Proteins were extracted from cells treated with MRS2365 (1 mM) in the presence of apoptotic inducer for 8 h. Proteins were separated on SDS-PAGE, blotted onto PVDF membranes and probed with anti-caspase-3 antibodies. GAPDH served as the reference for the loading of equal quantity of proteins. Asterisks represent protein expression that is significantly different when compared to the apoptotic inducer + MRS2365 group.

Figure 5

MRS2365 affects the RAS-RAF-MEK-ERK signaling pathway. In human tumors, the MAPK pathway is activated by P2RY1 agonist (MRS2365) binding to the P2RY1 receptor. The sequential activation of Ras-Raf-MEK-ERK is known to phosphorylate nuclear targets (C-Fos, ELK1, and c-Jun) that cause apoptosis, migration and proliferation of cells. Each tier in the pathway consists of 3 Rafs (1, A and B), 2 MEKs (1 and 2) and 2 ERKs (1 and 2). Phosphorylation of ERK eventually controls the transcription of genes that promote cell cycle progression and tumor survival. This is critical because a number of genes have been reported as targets of ERK.