Original Article
Overexpression of glycosylphosphatidylinositol anchor attachment protein 1 suppresses activation of MAPks in human colorectal cancer COLO205 cell line

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Abstract: Objective: Overexpression GPAA1 is associated with human cancer pathogenesis and poor prognosis of cancer. However, there was no report focusing on the effect of GPAA1 expression on the activation of MAPks in colorectal cancer (CRC). This study aimed to investigate the association of GPAA1 expression with MAPks in CRC. Methods: The expressions of GPAA1 mRNA in several human CRC cell lines were detected to selected appropriate cell lines for transfection. Then the constructed GPAA1 expression plasmids were transfected into cells and expression of GPAA1 as well as MAPKs were detected to investigate the association of GPAA1 with MAPKs signaling pathway. Results: GPAA1 expressed the higher in SW480 and CaCo-2 than in SW116, HT29, HCT116 and COLO205. The constructed GPAA1 expression plasmids were transfected into COLO205 cell line with moderate GPAA1 expression levels. GPAA1 transfection obviously upregulated GPAA1 expression in COLO205 cells. Moreover, the expression of p-JNK, -ERK, and -p38 were significantly reduced by GPAA1 transfection. Conclusion: The transfection of exogenous GPAA1 expression plasmids inhibited activation of MAPKs by downregulating expression of p-JNK, -ERK, and -p38 and upregulating total JNK, ERK, and p38 proteins. We speculated that the GPAA1 GPAA1 overexpression associated cell invasion might relate to other signaling pathways which opposite to MAPKs.

Keywords: GPAA1, colorectal cancer, MAPK, human colorectal cancer, COLO205 cell line

Introduction

Human cancers are generally caused by the abnormal accumulation of genetic or epigenetic alterations [1]. Usually, the abnormal accumulation of two main genes categories, inactivation of tumor suppressor genes and activation of oncogenes which could be caused by chromosomal translocation and mutation of gene, were essential for tumorgenesis [1, 2]. For example, the genetic fusion of ALK and RET are associated with colorectal cancer (CRC) and lung cancer tumorgenesis [3], BRAF (V600E) mutation is related to CRCs [4, 5], and the abnormal accumulations of oncogenic factors such as YAP1 [6], oncogenic KRAS and MAPK signaling and others were associated with CRCs [7].

Glycosylphosphatidylinositol (GPI) anchor attachment protein 1 (GPAA1) is one of the 5 submits of GPI transamidase complexes and is required for the recognition of GPI. GPAA1 is located in human chromosome 8q24.3, near by the site of oncogene c-myc, 8q24.1 [2]. GPI lipid-anchoring of proteins plays great medical implications on post-transfection modification in a variety of conditions associated with various diseases [8]. GPAA1 is evidenced to associate with pathogenesis of human cancer by involving gene amplification [9, 10]. GPAA1 could presents the target proteins without transmembrane domains on the cell surface through transferring GPI anchor to the C-terminus of target protein, and thus is essential for the actin of protein without transmembrane domains [8, 11, 12]. Moreover, GPAA1 has been regarded as an oncogene on account of the fact that increased expression of GPAA1 has been reported to be associated with poor prognosis of cancer including human breast [2], cancer hepatocellular carcinoma [9, 13], human CRCs [14], and so on.

Upregulation of GPAA1 is related to cell proliferation [14, 15]. Paxillin phosphorylation is a crucial factor for the activation of mitogen-activated protein kinases (MAPKs) and cell invasion. MARK/ERK (extracellular signal-regulated kinase) signal-regulated pathway is a frequent event in tumorigenesis [16, 17]. GPAA1 and PIG-T suppressions could reduce paxillin phosphorylation, and the siRNA-paxillin decreased the expression of p-ERK, as well as cell migration, proliferation and invasion ability of LnCAP and PC3 cells [18, 19]. However, there was no direct evidence focusing on the association of GPAA1 expression with MAPKs in CRC.

This study aimed to investigate the association between GPAA1 expression and MAPKs in CRC. Human CRC cell lines were employed, and expression level of GPAA1 in transfected cells or controls were detected using quantitative RT-PCR, Western blotting analysis, as well as immunohistochemistry. Moreover, the expression of MAPKs JNK, ERK, and p38 were detected. This study would provide us with direct information on the association with MAPKs signaling pathway and GPAA1 expression in CRC cell lines.

Materials and methods

Cell line and culture conditions

Six human CRC cell lines cell lines (SW480, SW1116, CaCo-2, HT29, COLO205, HCT116) were primarily obtained from ATCC (Rockville, MD, USA). Cells were cultured in RPMI-1640 (Gibco Laboratories, Grand Island, NY, USA) supplemented with 10% FBS (Gibco Laboratories, Grand Island, NY, USA), 5% glutamine and penicillin-streptomycin (Life Technologies, Gaithersburg, MD, USA) at 37°C with 5% CO_2. Total RNA from these 6 cell lines was extracted and converted into single-strand cDNA for the detection of the mRNA expression levels of *GPAA1*.

GPAA1 expression in CRC cell line

The full length of *GPAA1* cDNA sequence was cloned and inserted into pcDNA3.1 (Clontech, Palo Alto, CA, USA) to construct the overexpression plasmid of *GPAA1*. Naked pcDNA3.1 plasmid was used as a negative control. Cells plated into 24-well plate were transfected with the expression plasmids or naked control using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) following the manufactures' instruction, and were cultured at 37°C 5% CO_2.

RNA isolation and quantitative RT-PCR

For the qRT-PCR analysis of GPAA1 gene, total RNA was extracted from cells. The first-strand cDNA was synthesized. The mRNA expression levels of GPAA1 was determined using an iScript cDNA synthesis Kit (BioRAD, Hercules, CA, USA) and an ABI 7500 system (Applied Biosystems, Carlsbad, Calif., USA). The primers of GPAA1 were synthesized by Shanghai Sangon Biologic Engineering Technology and Services Co., Ltd. (Shanghai, China): Forward primer 5'-TCTCAAGGCTCTGGAACTG-3' and reverse primer 5'-GCCCCACACCCTGTGATG-3'. A total of a final 20 μL volume reaction mix were amplified according the following reaction conditions: initial desaturation at 95°C for 5 min, followed by denaturation at 95°C for 30 s, annealing at 60°C for 40 s and extension at 72°C for 10 s for 40 cycles. GAPDH was used as an internal control and all reactions were run in triplicate. The relative mRNA expression level was calculated by the $2^{-\Delta\Delta Ct}$ method.

Western blot analysis

Transfected human CRC cell lines were seeded into 6-well plates at a density of 1.0×10^6 in RPMI-1640 and harvested at 48 h after transfection. Cells were then lysed in the RIPA buffer (Shanghai Solarbio Bioscience & Technology Company, Beijing, China) and a BCA protein assay kit (Thermo Scientific, Rockford, IL, USA) was used to determine protein concentration. A total of 15 μl cell lysates was separated by 10% SDS-PAGE gels and was transferred to PVDF membranes (Invitrogen Corp., Carlsbad, CA, USA) which were then blocked with 5% skimmed milk (BD BYL40422, Biosciences, Franklin Lakes, NJ, USA) and incubated with primary antibody against GPAA1 (dilution 1:1000), ERK (dilution 1:1000), phosphorylated (p)-ERK (dilution 1:1000), JNK (dilution 1:1000), p-JNK (dilution 1:2000), p-p38 (dilution 1:1000), p38 (dilution 1:1000) and GAPDH (dilution 1:2000) at 4°C overnight and HRP-conjucted secondary antibodies for 1 h. All the antibodies were purchased from Cell Signaling Technology (CST, Denvers, MA, USA), The polypeptide bands were captured by a Tanon-5200 C hemiluminescent Imaging System (Tanon Science & Technology Co., Ltd. Shanghai, China).
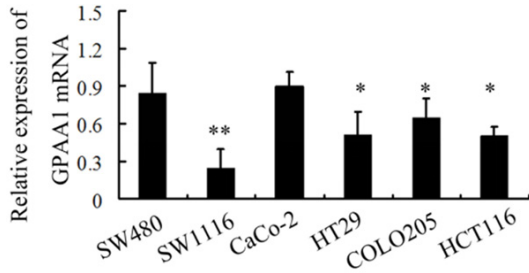
Figure 1. Relative expression level of GPAA1 mRNA in human CRC cell lines. The expression of GPAA1 mRNA was detected using qRT-PCR. * and **indicates significant levels at P < 0.05 and P < 0.01 level, respectively, vs. SW480.

Immunohistochemical analysis

For immunohistochemistry, cells slides were performed. Slides were fixed with cold acetone, blocked using goat serum and cultured with primary antibody against GPAA1 at 4°C overnight. After washing with PBS for three times, slides were then incubated with biotin-conjuct-ed secondary antibody. Signal visualization was performed with diaminobenzidine (DAB) staining. Histological scoring was evaluated by a double blind scoring method. Cells with brown granules were considered as GPAA1 positive cells. Positive GPAA1 cell percentages in cell slides were calculated as considered as (positive GPAA1 cell number)*100%/total cell number. All experiments were performed in triplicate and the average percentages were used.

Statistical analysis

Data were expressed as mean ± standard deviation (SD) of 3 triplicates. Statistical analysis was performed using Student's t test or a one-way analysis of variance (ANOVA) test. A *P* value of less than 0.05 was considered to be statistically significant.

Results

GPAA1 differentially expresses in CRC cell lines

To detect the expression pattern of *GPAA1* mRNA in the 6 human CRC cell lines (SW480, SW1116, CaCo-2, HT29, COLO205, HCT116), the total RNA in these cells were extracted and expression of *GPAA1* mRNA were detected using qRT-PCR. Data from qRT-PCR show-
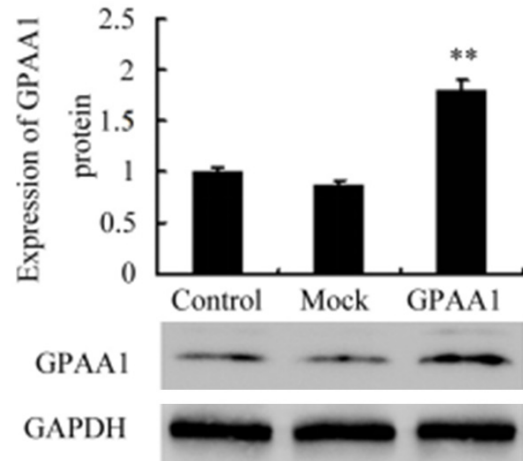

Figure 2. GPAA1 protein level were upregulated in transfected cells. The human CRC COLO205 cell line were transfected by GPAA1 expression plasmids and the expression of GPAA1 protein were detected using Western blotting analysis. **indicates significant levels at P < 0.01 level, vs. Control.

ed that the expression level of *GPAA1* mRNA in cell lines was different, with the highest expression level in CaCo-2 cell line or SW480 cell line (P < 0.05, **Figure 1**), and the lowest expression level in SW1116 (P < 0.05, **Figure 1**). No differences were found in the *GPAA1* mRNA expression among the other 3 cell lines HT29, COLO205, and HCT116 (**Figure 1**). COLO205 cell line with the moderate expression level of *GPAA1* was selected to be transfected with the *GPAA1* expression plasmids.

GPAA1 was upregulated by overexpression plasmid transfection

The expression level of *GPAA1* in COLO205 cell line was modulated using *GPAA1* expression plasmids. The expression of GPAA1 protein in transfected cells were detected using Western blotting analysis as well as immunohistochemistry. Results showed that the expression of GPAA1 protein were significantly upregulated by GPAA1 transfection (P < 0.01, **Figures 2** and 3). Western blotting analysis showed the GPAA1 protein in transfected cells was almost 1.8 fold higher than that in the control or mock transfected cells (P < 0.01, **Figure 2**). The percentage of GPAA1 positive cells by immunohistochemistry showed it was increased to 82% in transfected cells, which was obviously higher than the < 20% positive cells in the control or mock transfected cells (P < 0.01, **Figure 3**).
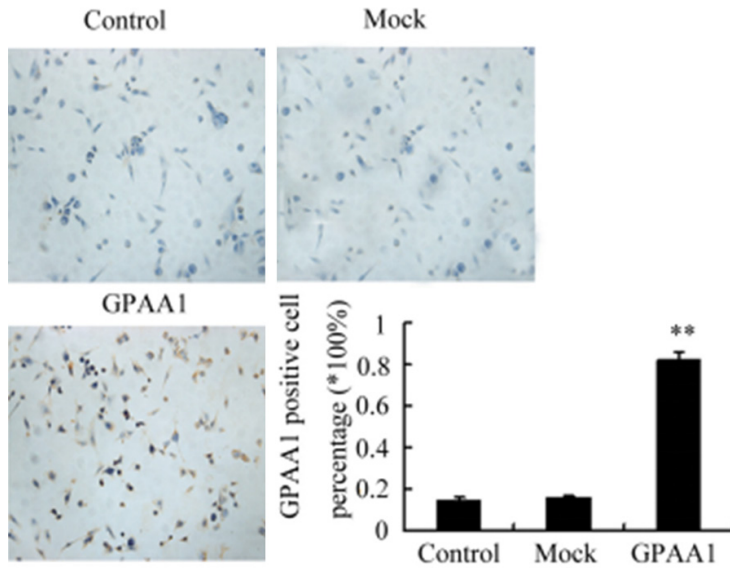
Figure 3. GPAA1 positive cells were increased in transfected cells. The expression of GPAA1 protein was detected using immunohistochemistry using antibody anti GPAA1. Positive cell numbers in transfected CRC COLO205 cell line were significantly increased by GPAA1 expression plasmids. **indicates significant levels at P < 0.01 level, vs. Control.
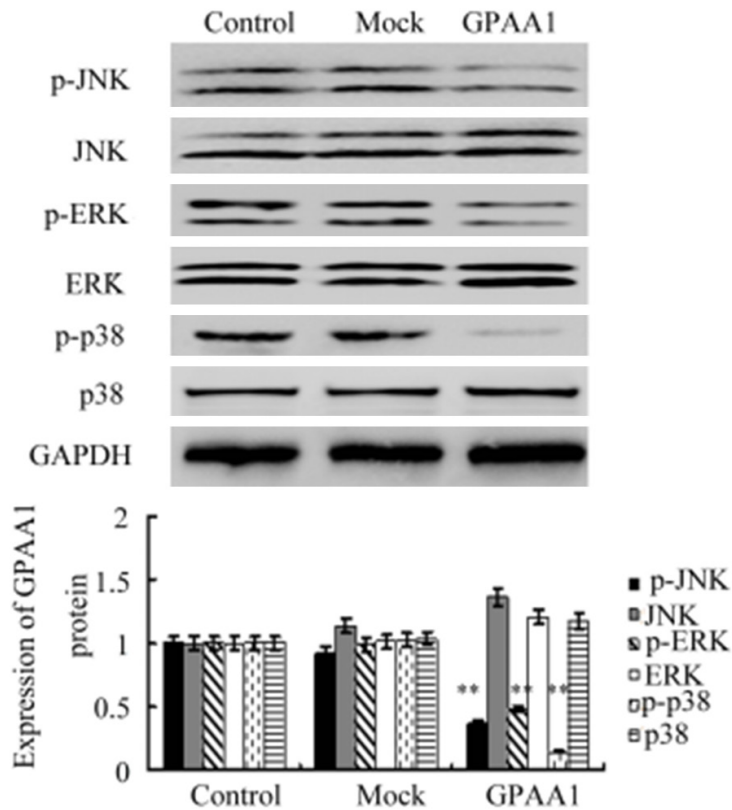

Figure 4. MAPks protein levels were modulated by GPAA1 transfection. The expression of MAPks protein levels in transfected cells were detected using Western blotting analysis. The results showed the phosphorylated (p)-JNK, -ERK, and -p38 were inhibited by GPAA1 overexpression. **indicates significant levels at P < 0.01 level, vs. Control.

These results showed transfection of exogenous GPAA1 expression plasmids successfully resulted in the overexpression of GPAA1 in COLO205 cells.

GPAA1 transfection modulates the expression of MAPks signaling pathways

To investigate the effect of GPAA1 on the expression of MAPks signaling pathway, we detected the expression of MAPks JNK, ERK, and p38 using Western blotting analysis. Data from the transfected cells and the controls showed that expression of these 3 MAPks were modulated by GPAA1 overexpression. The expression of p-JNK, p-ERK, and p-p38 were obviously downregulated by GPAA1 transfection in the transfected COLO205 cells in comparison with the control and mock cells (P < 0.01, **Figure 4**). On the contrary, the expression of JNK, ERK, and p38 protein were upregulated to some extent. However, there were no differences in the downregulation of JNK, ERK, and p38 protein expression compared with the controls (**Figure 4**). These showed GPAA1 expression inhibited the activity of MAPks signaling pathways.

Discussion

GPAA1 is associated with human cancer pathogenesis by involving gene amplification, and the increased expression of GPAA1 has been reported to be associated with poor prognosis of cancer including human breast [2], cancer hepatocellular carcinoma [9, 13], human CRCs [14], and so on. However, there was no report focusing on the effect of GPAA1 expression on the activation of MAPks. In this study, we successfully overexpressed GPAA1 in transfected COLO205 cells which showed the moderate

endogenous GPAA1 expression. Furthermore, we confirmed that the expression of phosphorylated JNK, ERK, and p38 were significantly downregulated by GPAA1 transfection. While the expression of total JNK, ERK and p38 were faintly upregulated to some extent.

GPAA1 is one submit of GPI transamidase complex and required for GPI recognition. GPAA1 is essential for the properly exercise of functions of protein without transmembrane domains by presenting the proteins on the cell surface through transferring GPI anchor to the C-terminus of target protein [8, 11, 12]. GPI lipid-anchoring of proteins plays great medical implications on post-transfection modification in a variety of conditions associated with various diseases [8]. As reported, the transcript level of GPAA1 was frequently up-regulated on gene expression profile in human tumor [9, 13, 14]. Evidence had proved that GPAA1 was tightly involved into the cancer cell invasion, metastasis, as well as the pathogenesis of human cancers [14]. This showed that GPAA1 might be an oncogene and played an important part in the metastasis of human cancers.

In this study, we detected there were relative higher expression of *GPAA1* mRNA in SW480, and CaCo-2, COLO205, HCT116 and HT29 cell lines that that in SW1116. This was basically in accordance with the fact that SW480 and CaCo-2 cell lines were high or moderate differentiated and invasive cell lines, comparing with the low or poorly differentiated CRC cell lines SW1116 and COLO205 [20-22]. However, the fact that there was a relative higher expression of GPAA1 mRNA in poorly differentiated, non-metastatic CRC cell line COLO205 lead to the suggestion of there might be a positive relationship between cell metastasis and GPAA1 expression insufficient. Thus the association of GPAA1 expression with tumor progression, metastasis and invasion needed to be deeply explored.

The implications of MAPKs in cell migration, regulation of apoptosis and other cell functions had been well explained [16]. As reported in tumorigenesis, the activation of the MAPK/ERK pathway is a frequent event [16], and activation status of MAPKs of p-JNK, p-ERK, and p-p38 are positively associated with cell invasion, metastasis and migration [23-25]. As had

been shown that GPAA1 might be an oncogene in CRC and other cancer [26] and upregulation of GPAA1 is related to cell proliferation and adhesion ability promotion [14, 15]. Accordingly, we speculated that there were positive associations between GPAA1 and activation of MAPKs.

To investigate the association, we detected the expression of MAPKs p-JNK, p-ERK, and p-p38 in the GPAA1 expression plasmid transfected cells. However, we confirmed in this study that the upregulation of GPAA1 in transfected cells significantly reduced the activation of MAPKs downregulating the expression of activation status of MAPKs of p-JNK, p-ERK, and p-p38. This was contrary to the results of GPAA1 and PIG-T suppressions reduced paxillin phosphorylation, which is crucial for the activation of MAPKs, as well as cell invasion and proliferation [19, 27]. Additionally, Sen *et al* confirmed that the siRNA-paxillin decreased both expression of paxillin and p-ERK, as well as cell migration, proliferation and invasion ability of LnCAP and PC3 cells [18]. Then we speculated that there might be a negative relationship between GPAA1 expression and MAPKs p-JNK, p-ERK, and p-p38. The GPAA1 overexpression associated cell invasion might relate to other signaling pathways which opposite to MAPKs.

Conclusion

In this study, we successfully overexpressed GPAA1 in human CRC COLO205 cell line. Contrary to the other studies, we confirmed that the overexpression of exogenous GPAA1 in COLO205 cells reduced the activation status of MAPKs p-JNK, p-ERK, and p-p38. We speculated that the GPAA1 overexpression associated cell invasion might relate to other signaling pathways which opposite to MAPKs, and there might be a negative relationship between GPAA1 expression and MAPKs p-JNK, p-ERK, and p-p38. However, this hypothesis would be testified by experiments focusing on cell invasion, migration, proliferation, expression of more signaling pathways as well as GPAA1 expression.

Acknowledgements

best experimental environment. The study is financial supported by all authors personally.

Disclosure of conflict of interest

None.

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