Abstract. p120-catenin (p120), an E-cadherin regulator, has been implicated as central to a series of genetic and epigenetic changes that ultimately lead to tumor progression and metastasis. Ras-related C3 botulinum toxin substrate 1 (Rac1) and p21-activated kinases (PAKs) are effectors of p120. In the present study, we examined the expression of p120, Rac1 and Pak1 using immunohistochemistry in human gastric cancer tissues. Then, we used the gastric cancer SGC7901 and AGS cell lines to explore the possible mechanism of p120, Rac1 and Pak1 in the progress of gastric cancer. Western blotting was used to detect the expression of p120, Rac1 and Pak1 in the two cell lines. Next, p120 was silenced using p120 siRNA or overexpression of p120 by transfection of the plasmid p120 1A into the two cell types, western blotting was used to investigate the expression changes of Rac1 and Pak1. Furthermore, the effects of p120 siRNA-mediated knockdown or overexpression on the proliferation and invasive ability of gastric cancer cells were investigated using wound healing test and Matrigel invasion assays. The results showed that p120 was downregulated in both poorly differentiated group and well differentiated human gastric cancer. However, Rac1 and Pak1 were upregulated in poorly differentiated tissues and remain low in well differentiated gastric cancer tissues. In the two gastric cancer cell lines, although the expression of Rac1 and Pak1 remained unchanged after the p120 knockdown, the expressions of Rac1 and Pak1 protein were decreased after p120 overexpression in both SGC7901 and AGS cells. Furthermore, knockdown of p120 promoted gastric cancer cell proliferation and invasion; overexpression of p120 reduced the proliferation and invasion of gastric cancer cells. In conclusion, based on our results, we speculate that p120 participates in the progress of gastric cancer through regulating Rac1 and Pak1, which provides a potential prevention and a promising therapeutical approach for the patients with gastric cancer.

Introduction

Gastric cancer is one of the most common malignancies worldwide (1,2). Recent advances in early diagnosis and treatment have resulted in significant improvement in long-term survival for gastric cancer patients. However, the prognosis for advanced gastric cancer remains poor. A majority of patients with advanced gastric cancer die due to complications caused by metastases. Therefore, invasion and metastasis are critical determinants of gastric cancer morbidity.

Ras-related C3 botulinum toxin substrate 1 (Rac1) is an important member of the small molecule G-protein Rho family (Ras homologue) and is an important class of intracellular signaling molecules. It affects tumor growth, invasion and metastasis, and tumor angiogenesis (3,4). p21-activated kinase 1 (Pak1) is a conserved serine/threonine protein kinase that is an important downstream target protein of Rho-GTPase Cdc42 and Rac1, which are involved in numerous cellular activities and play an important role in cytoskeletal reorganization, cell migration, apoptosis and survival, cell cycle, gene transcription regulation and cell transformation (5,6). Activation of Pak1 increases cell motility in non-metastatic MCF-7 breast carcinoma cells (7), and overexpression of Pak1 was recently found in NSCLC (6) and gastric cancer (8). Many research groups have shown that Rac1 and Pak1 may be important biomarkers of gastric carcinoma invasion and metastasis (8,9).

p120-catenin (p120) belongs to the armadillo protein superfamily and is originally identified as a substrate for oncogenic Src family tyrosine kinase (10). It is best known for binding directly to the cytoplasmic domain of cadherin or VE-cadherin and contributing to regulation of cell-cell adhesion (11-13). Due to its stabilizing function in the AJ, p120 has caught much attention in the context of tumor development and progression. The absence of membrane p120 or nuclear translocation of p120 in colon, breast, bladder, lung, pancreas, prostate and stomach tumors is well recognized, which has been associated with tumor malignancy (14).
Research has focused on the relationship between p120 and the expression of Rac1 and Pak1 in gastric carcinoma. On one hand, several results indicated that p120-catenin also controls the activity of small GTPases. For instance, overexpression of p120-catenin represses RhoA activity (15,16) and activates Rac1 (16,17). In contrast, there is a study revealing that Pak5 and p120 co-localized in neuroblastoma cells (18), Pak4, Pak5 and Pak6 were the founding members of group B Paks, and Pak1, Pak2 and Pak3 compose the group A Paks (19,20). For this reason, we hypothesize that p120 participates in the development of gastric cancer through regulating Rac1 and Pak1.

Materials and methods

Immunohistochemistry in gastric carcinoma tissues. Gastric carcinoma tissue specimens both poorly differentiated and well differentiated, were obtained from the Institute of Pathology, Tongji Hospital, Tongji Medical College, Wuhan, China. The specimens were fixed, dehydrated and embedded in paraffin, then cut into 3-µm thin slices. After dewaxing and rehydration, they were autoclaved for 2 min and were then incubated with 3% hydrogen peroxide for 10 min at room temperature to remove endogenous peroxidase activity. The slices were added with 5% BSA for 30 min, followed by incubating with anti-p120, anti-Pak1, anti-Rac1 (p120; Santa Cruz Biotechnology; Pak1 and Rac1; CST Co.) antibodies at 4˚C overnight, then washed in phosphate-buffered saline (PBS) for 2 min three times and incubated with secondary antibodies at 37˚C for 1 h, then stained with DAB substrate chromogen solution for 5 min at room temperature.

AGS and SGC7901 cell culture. Human gastric cancer SGC7901 and AGS cell lines were cultured in RPMI-1640 with 10% fetal bovine serum (FBS), 200 µg/ml streptomycin, 200 IU/ml penicillin at 37˚C under 5% carbon dioxide.

Western blotting in AGS and SGC7901 cells. To investigate the level of the protein expression, AGS and SGC7901 cells were cultured in 6-well inserts board, cells were rinsed twice with ice-cold PBS, lysed in RARP buffer with 1% protease inhibitor cocktail. Lysates were then cleared by centrifugation, and protein concentration was determined by a BCA kit. Equal amounts of proteins were fractionated by SDS-PAGE and transferred to a nitrocellulose (NC) membrane or polyvinylidene fluoride (PVDF) membrane. The membranes were blocked with 5% fat-free milk in TBS and incubated with anti-p120 (1:300), anti-Rac1 (1:500), anti-Pak1(1:500) and anti-β-actin (1:2,500) overnight at 4˚C. The signal was detected using a horseradish peroxidase-conjugated secondary antibody and ECL and was then exposed to X-ray film (Fuji, Japan).

Plasmids and transient transfection. Plasmid of RcCMV mp120-1A was generously provided by Professor Enhua Wang (21). Transient gene transfection was performed on cells in the exponential phase of growth using Lipofectamine 2000 according to the manufacturer's recommendations and the method described by Tucker et al (22) with minor modification. Forty-eight hours after transfection cells were treated for further analysis.

RNA interference. The small interfering RNA (siRNA) oligonucleotides of human p120 were purchased from Shanghai GenePharma Co. Ltd. Cells were grown on a 6-well plate to 50% confluency with complete medium and transfected with the siRNA using Lipofectamine 2000 according to the manufacturer's recommended procedure. Efficiency of knockdown by siRNA was assessed by western blot analysis. The non-silencing siRNA (scramble) was used as control. Forty-eight hours after transfection cells were treated for further analysis.

Wound healing assay. A wound healing assay was performed to examine the capacity of cancer cell migration as previously described (23). Twenty-four hours after transfection with p120 siRNA, the AGS and 7901 cells were resuspended with serum-free RPMI-1640 in 6-well plates, when cancer cells were 90-95% confluent, a single scratch wound was generated with a 200 µl disposable pipette tip. The migration of the cells at the edge of the scratch was analyzed at 0, 24 and 48 h. The images were captured with a fluorescence microscope.

Transwell assay. Twenty-four hours after transfection with p120 siRNA, the AGS and 7901 cells were resuspended in serum-free RPMI-1640 to adjust the density to 10^5/ml. Twenty-four-well 8.0 µM Transwell inserts (3422; Corning) were used for the experiments. We added 400 µl RPMI-1640 containing 10% FBS to the lower chamber and 100 µl medium that containing the cells to the upper chamber. After incubation for 24 h, the cells that did not migrate to the upper chamber were removed with a cotton swab. Then the migrated cells were fixed with 4% paraformaldehyde for 20 min, stained with crystal violet for 15 min, and were counted and photographed with a fluorescence microscope at a magnification of x200.

Statistical analysis. All data are expressed as the means ± standard deviation (SD) of experiments repeated at least three times. The statistical software SigmaStat was used to analyze the data. t-test and one-way ANOVA were used for statistical analysis, and statistical significance was assumed at p<0.05.

Results

Expression of p120, Pak1 and Rac1 in different types of gastric cancer tissues and the AGS and SGC7901 cells. In order to determine the protein expression of different stages of gastric cancer patients, immunohistochemistry was used to detect the expression level of p120, Pak1 and Rac1. As shown in Fig. 1, immunohistochemistry revealed that expression level of Rac1 and Pak1 proteins were low in well differentiated gastric cancer tissues, yet were high in poorly differentiated tissues. Differently, the expression level of p120 proteins were low both in poorly differentiated group and well differentiated ones. Nevertheless, there was a significant nuclear location of p120 in poorly differentiated tissues.

Next, we explored the expression of p120, Rac1 and Pak1 in two types of GC cells. Western blotting showed that expression of Pak1 and Rac1 in SGC7901 cells were both higher than that in the AGS cells. p120 isoform 1 and 3 was
detected in SGC7901 cells, while only p120 isoform 1 was detected in AGS cells, which was identical with a previous study (24) (Fig. 2).

Overexpression of p120 1A inhibits the expression of Pak1 and Rac1 in GC cells. To investigate the relationship between downregulated p120 and upregulated Rac1 and Pak1, we transfected plasmid of p120 1A into the two cell types to detect the expression of Rac1 and Pak1. The transfection efficiency was detected by western blotting (Fig. 3). Compared with MT groups, the expression of Rac1 and Pak1 were both downregulated when p120 1A was overexpressed in GC cells (Fig. 3).

In contrast, we used p120 siRNA to silence p120, then detected the changes of Rac1 and Pak1. Compared with the scrambled groups, the silencing efficiency of p120 was detected by western blotting (Fig. 4). Notably, the expression of Rac1 and Pak1 remained unchanged (Fig. 4).

Overexpression of p120 1A decreases the proliferation and invasion of AGS and SGC7901 cells. Cell migration and invasion were considered to have important value in progress of cancer (25), were one of the crucial events in metastasis of cancer cells. Therefore, in order to find whether p120 impacts the progress of gastric cancer, we used wound healing and Transwell assays to explore the biological behavior changes of SGC7901 and AGS cells in overexpression of p120 1A.

Overexpression of p120 inhibited the migration capacity of the two GC cell types at 24 and 48 h, while the GC cells covered the wound at 48 h in MT groups (Fig. 5). Moreover, the serum-stimulated Matrigel invasion assay demonstrated that overexpression of p120 1A significantly decreased the
Figure 3. Overexpression of p120 1A inhibits the expression of Pak1 and Rac1 in GC cells. The expression of p120, Pak1 and Rac1 were detected by western blotting in SGC7901 and AGS cells which were transfected with the p120 1A plasmid. The ratio of mean of the band which were measured are shown in the right and under the panel. Each bar represents the mean ± SD in one representative experiment repeated at least three times. Representative images of western blotting are shown. Statistical analysis by t-test, *p<0.05. Pak1, p21-activated kinase 1; Rac1, Ras-related C3 botulinum toxin substrate 1; GC, gastric cancer.

Figure 4. Expression of Rac1 and Pak1 remains unchanged when p120 is silenced. The expression of p120, Pak1 and Rac1 were detected by western blotting in SGC7901 and AGS cells which were transfected with p120 siRNA. The ratio of mean of the band were measured and shown in the right and under the panel. Each bar represents the mean ± SD in one representative experiment repeated at least three times. Representative images of western blotting are shown. Statistical analysis by t-test, *p<0.05. Rac1, Ras-related C3 botulinum toxin substrate 1; Pak1, p21-activated kinase 1.
invasiveness of AGS and SGC7901 cells at 24 and 48 h, compared to the MT groups (P<0.05, Fig. 6).

Silencing of p120 increases the proliferation and invasion of the GC7901 and AGS cells. To further confirm the changes
of p120 effects in the biological behavior of SGC7901 and AGS cells, we silenced p120 to observe the proliferation and invasion of the cells. The wound assay showed that when p120 was silenced, the migration of SGC7901 and AGS cells were increased.

Figure 7. Silencing of p120 increases the proliferation and invasion of the two GC cell types. SGC7901 and AGS cells were transfected with p120 siRNA, and migration was measured by wound healing assay. Representative images of wound healing assay are shown. Similar results were obtained in at least three experiments.

Figure 8. Silencing of p120 increases the proliferation and invasion of two GC cells. SGC7901 and AGS cells were transfected with p120 siRNA, and the potential ability of invasion were detected with Transwell assay, the number of cell/field are shown in the right panel. Each bar represents the mean ± SD in one representative experiment repeated at least three times. Representative images of Transwell assay are shown. Statistical analysis by t-test, *p<0.05. GC, gastric cancer.
increased in 24 h and 48 h, particularly AGS cells were more prominent (Fig. 7). Moreover, knockdown of p120 significantly reduced the invasiveness of the two GC cell types at 48 h when compared to the scrambled group (P<0.05, Fig. 8).

**Discussion**

In the present study, p120 was downregulated and Rac1 and Pak1 were upregulated in the different tissues of human gastric cancer by immunohistochemistry. Then western blotting showed that expression of Pak1 and Rac1 in SGC7901 cells were higher than in the AGS cells. p120 isoform 1 and 3 was detected in SGC7901 cells, while only p120 isoform 1 was detected in AGS cells. Next, overexpression of p120 1A downregulates the expression of Pak1 and Rac1 in SGC7901 and AGS cells. Notably, the expression of Rac1 and Pak1 remained unchanged when silencing the p120 by p120 siRNA. Furthermore, overexpression of p120 1A decreased the migration and invasion of the GC cells, while silencing of p120 increased the migration and invasion of the two GC cell types. In conclusion, we speculated that in addition to Rac1 and Pak1, p120 also participates in the progress of gastric cancer and this may be through regulating Rac1 and Pak1, which provides a potential potential and a promising therapeutical approach for patients with gastric cancer.

In previous studies, Pak1 and Rac1 signaling pathway was shown to play a crucial role in malignant tumors (26,27). Positive rates of Rac1 and Pak1 expression in normal tissue, dysplasia and gastric carcinoma showed an increasing trend and were correlated with tumor lymph node metastasis and TNM stage (9). We found that the expression of Rac1 and Pak1 was higher in poorly differentiated than well differentiated gastric cancer tissues. p120 has been shown to be crucial in contributing to the cell-cell adhesion and strengthen the stability of cadherin-catenin complex (28). A number of studies have shown that an absence of p120 expression is common in colon, bladder, stomach, breast and prostate cancer (15), and in many cases the absence of p120 expression is associated with poor prognosis, indicating that reduced expression of p120 correlates closely with the progression of cancer. For the first time, we found that p120 was also absent in gastric cancer. Four different subtypes of p120 exist as a result of differential splicing. Our results indicated that p120 isoform 1 and 3 were mainly expressed in SGC7901 cells and p120 isoform 1 was expressed in AGS cells. The present study further confirmed that p120 isoform 1 was involved in promoting cell invasiveness (29).

It has been found that Rac1 and Pak1 were downstream factors of p120 (30). Thus, we overexpressed or silenced p120 to explore the relationship among p120, Rac1 and Pak1 at the cellular level. Notably, overexpression of p120 1A decreased the expression of Rac1 and Pak1 in both GC cell types. Silencing p120 did not change the expression of Rac1 and Pak1, but possibly the activity of Rac1 and Pak1 changed. Rac1 and Pak1 may be important biomarkers of gastric carcinoma invasion and metastasis (10). p120 can partially regulate the migration and invasiveness of GC cells via Rac1 and Pak1. Overexpression of p120 1A decreases the migration and invasion of the GC cells, while silencing p120 increases the migration and invasion of two GC cell types. These results indicated that p120 may be an important biomarker of gastric carcinoma invasion and metastasis.

In conclusion, our studies demonstrated that not only Rac1 and Pak1, but also p120 participates in the progress of gastric cancer and this may be through regulating Rac1 and Pak1. Most importantly, p120 as an upstream protein of Rac1 and Pak1 may be a new target for the treatment of gastric cancer, which provides a potential prevention and a promising therapeutical approach for patients with gastric cancer.

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**References**


