Expression and significance of Rac1, Pak1 and Rock1 in gastric carcinoma

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Abstract

Aims: Rac1, Pak1 and Rock1 are indicators related to gastric cancer invasion and metastasis, but few reports discuss all three kinds of protein in research on gastric cancer invasion and metastasis. The aim of this study was to investigate the expression and clinical significance of Rac1, Pak1 and Rock1 in gastric carcinoma.

Methods: Rac1, Pak1 and Rock1 expression in 158 cases of gastric carcinoma were investigated via immunohistochemical staining and clinical analysis.

Results: The positive expression rates of Rac1, Pak1 and Rock1 in normal tissue, intraepithelial neoplastic tissues and gastric carcinoma showed an increasing trend \( (P < 0.05) \). Their expression in lymph node metastasis was significantly higher than in patients with lymph-node metastasis than in those without lymph nodes metastasis \( (P < 0.05) \). Their expression in tumor (TNM stages III and IV) were significantly higher than that in stages I and II \( (P < 0.05) \). Rac1, Pak1 and Rock1 expression did not differ significantly with patients’ sex \( (P > 0.05) \).

Conclusion: Positive rates of Rac1, Pak1 and Rock1 expression in normal tissue, dysplasia and gastric carcinoma show an increasing trend and are correlated with tumor lymph node metastasis and TNM stage. Rac1, Pak1 and Rock1 may be important biomarkers of gastric carcinoma invasion and metastasis.

Key words: gastric neoplasm, immunohistochemistry, Pak1, Rac1, Rock1, tissue microarray.

INTRODUCTION

Gastric cancer has one of the highest incidences of cancer worldwide. Invasion is a major cause of recurrence and patient death in gastric cancer. 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. 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 many important cellular activities and play an important role in cytoskeletal reorganization, cell migration, apoptosis and survival, cell cycle, gene transcription regulation and cell transformation. The Rock1 gene is highly expressed abnormally in a variety of tumor cells and plays a role in tumor cell invasion and metastasis. At present, little research has focused on the relationship between the expression of Rac1, Pak1 and Rock1 in gastric carcinoma and clinical pathology. In the present study, immunohistochemistry and a tissue microarray were used in the detection of Rac1, Pak1 and Rock1 protein expression levels in gastric cancer cells, intraepithelial neoplasia and normal tissues. The correlation between lymph node metastasis and TNM stages was analyzed.
METHODS

Clinical data
The specimens (resection specimens of 158 cases of gastric cancer) were recruited from the Department of Pathology, Xiangtan Affiliated Clinical College of Nanhua University from 2004 to 2010. All specimens were sorted according to the World Health Organization classification of digestive system cancer in 2010, and confirmed using hematoxylin–eosin (HE) slice biopsy. The patients had not received radiotherapy and chemotherapy before surgery.

Tissue microarray
The primary tumor tissue was taken. Based on the slice determined using H–E staining, the representative lesion distribution was determined to construct the tissue microarray.

Immunohistochemistry
Substance P (SP) immunohistochemistry was carried out according to the manufacturer's instructions. Phosphate buffered saline instead of primary antibodies were used as the negative control. A known cancer-positive biopsy was used as the positive control.

Results determination
The results were determined according to the method described by Wang et al. The positive rate was determined by three pathology experts. We checked 10 high power fields from each slice randomly, and made a positive cell count score and staining intensity score. Rac1, Pak1 and Rock1 protein positive expression consisted of tan or brown granules located in the cytoplasm and/or cell membrane. Based on the degree of positive staining and the percentage of stained cells, the specimens were scored as follows: 0 corresponds to unstained, 1 point corresponds to brown and 2 points correspond to dark brown; 0 for stained cells <5%, 1 for 5–25%, 2 for 26–50% and 3 for above 50%. Two kinds of scoring were employed: ≥2 points was considered positive, <2 points was considered negative.

Statistical analysis
The results were analyzed via a χ² test using SPSS 17.0 (SPSS, Chicago, IL, USA) statistical software, and differences of P < 0.05 were considered statistically significant.

RESULTS

Clinical data
The subjects included 112 men and 46 women with a mean age of 56.25 years (28–83-years old). Gastric cancer with lymph node metastasis was found in 109 cases, and 49 cases did not exhibit lymph node metastasis. A total of 67 cases were classified into TNM stages I to II and 91 were diagnosed with stages III–IV. Intraepithelial neoplastic tissue was collected in 54 cases and normal gastric mucosa (gastric resection specimens from the foci of the cancer more than 10 cm of normal gastric mucosa as the control group) was collected from 64 cases.

Expression of Rac1, Pak1 and Rock1 protein
The expression of Rac1, Pak1 and Rock1 in normal epithelium and intraepithelial neoplastic epithelium was weakly positive or positive; however, a large number of positively stained cells were heterogeneously distributed in the gastric carcinoma tissues. Positive staining was tan-yellow, with bulky granules that were heterogeneously distributed in the cell membrane and cytoplasm. The Rac1 expression rates in the normal gastric tissue, intraepithelial neoplastic tissue and gastric carcinoma were 27, 43 and 68 percent, respectively. The Pak1 expression rates in the three groups were 20, 35 and 60%, respectively, whereas those of Rock1 in the three groups were 16, 28, and 58%, respectively. The differences among the groups were statistically significant (P < 0.05) (Fig. 1).

Relationship between Rac1, Pak1 and Rock1 protein expression and clinicopathological indicators
As shown in Table 1, Rac1, Pak1 and Rock1 expression levels and patients’ sex were not statistically significant (P > 0.05). Rac1, Pak1 and Rock1 expression in the lymph node metastasis group (75, 71 and 66%) were significantly higher than in the group without metastasis (51, 43 and 41%). Rac1 expression in stages I and II was 48 percent, which was significantly lower than that in stages III and IV (82%). Pak1 expression in stages I and II was 46 percent, which was significantly lower than that in stages III and IV (74%). Rock1 expression in stages I and II was 42 percent, which was significantly lower than that of stages III and IV (70%) (P < 0.05).
The relationship between Rac1, Pak1, and Rock1 expression and clinical pathology

The correlation among the expression of Rac1, Pak1 and Rock1 in gastric cancer was analyzed using Spearman’s rank correlation. Rac1, Pak1 and Rock1 expression in gastric cancer was positively correlated ($r = 0.555$, $P < 0.05$).

Relationship between Rac1, Pak1 and Rock1 expression and survival of gastric cancer patients

Kaplan–Meier survival curves showed that the median survival time of the 158 patients with follow up was 26 months. The 3-year and 5-year overall survival rates of the whole group were 47 and 41 percent, respectively. Median survival was significantly shorter in Rac1, Pak1 and Rock-positive patients than in Rac1, Pak1 and Rock-negative patients (19 months vs 78 months, $\chi^2 = 6.857$, $P = 0.009$) (Fig. 2). Univariate survival analysis showed that Rac1, Pak1 and Rock1 expression was a risk factor affecting the survival of the patients.

The results of multivariate analysis by Cox regression for all patients among the four prognostic factors (tumor differentiation, lymph node metastasis, TNM stage and expression of the markers) showed that the expression of the markers may be recognized as the significant independent factor related to disease-free survival ($\chi^2 = 17.594$, $P < 0.001$).

DISCUSSION

The characteristics of the cytoskeletal structure result in different exercise capacities, which are related to the genetic diversity of tumor cells and normal cells and different metastatic potentials of the tumor cells.8 The cytoskeleton is the intracellular structure mainly composed of protein fiber. It plays an important role in maintaining cell morphology and the internal structure of cells, as well as cell movement, material transport, energy conversion, information transfer and cell differentiation.9

The Rac1 gene, located on the short arm of human chromosome 7 (7p22), encodes a small G-protein that is an important member of the Rho (Ras homologue) family. The Rac1 protein has two states, Rac1-GDP (inactive) and Rac1-GTP (activated).2 The biological functions of Rac1 depend on its conversion between the two states. When Rac1 is activated it participates in the formation of actin stress fibers and adhesion plaques, promotes cytoskeleton reorganization, regulates sheet pseudopodia and filopodia extension, affects the structure and polarization of the cell, promotes cell motility and migration and inhibits apoptosis.10 Studies in recent years have shown that Rac1 expression in colon, breast

Figure 1 (a) Positive Rac1 in gastric tube adenocarcinoma (substance P (SP) × 400); (b) positive Pak1 in mixed gastric adenocarcinoma (SP × 400); (c) positive Rock1 in stomach cancer of poor adhesion (SP 400).
and lung cancer, among others, was significantly increased. Moreover, Rac1 expression is closely related to invasion and metastasis.\textsuperscript{11,12} Rac1 cell motility signaling, which promotes cancer cell invasion and metastasis, is quite complex. This process may be achieved through the following ways: (i) activated Rac1 promotes the assembly of cell surface integrin protein molecules in the surface of the head of the cells and passes regulative signals to the actin cytoskeleton, thus inducing actin filaments to aggregate in the plasma membrane and form sheet pseudopodia, leading to cell membrane multi-polarization, which eventually affects the movement of cell migration\textsuperscript{12} (ii) Rac1, through the activation of type IV collagenase type 2 matrix metalloproteinase to increase collagenase type I expression, promotes extracellular matrix degradation and enhances the penetration ability of tumor cells.\textsuperscript{13} (iii) Rac1 regulates nuclear factor kappa-light-chain-enhancer of activated B cells activity and increases intracellular superoxide anion concentration to suppress apoptosis.\textsuperscript{14}

Pak1 is a class of evolutionarily conserved serine/threonine protein kinase that is widely expressed in many tissues as downstream target proteins of the small molecule G-protein Rho family Cdc42 and Rac 1. Pak1 can be activated by growth factors and other extracellular signals, either through the GTPase-dependent signaling pathway or not. It has a variety of biological effects. PAK, as an important biological regulator, plays an important role in a series of cell functions of mammals, such as cell motility, cell survival, cell cycle, angiogenesis and the regulation of gene transcription. Recent research indicates that Pak1 activation of lysophosphatidic acid and toxins from the body induces cell motility in melanoma cells.\textsuperscript{15} Head and neck cancer was found to have higher Pak1 activity than normal tissue.\textsuperscript{16} Combined analysis using gene hybridization array and tissue microarray confirmed that Pak1 is an upregulated

| Clinical pathological features | Rac1 Positive rate (%) | P value | Pak1 Positive rate (%) | P value | Rock1 Positive rate (%) | P value |
|-------------------------------|------------------------|---------|------------------------|---------|------------------------|---------|
| Sex                           |                        |         |                        |         |                        |         |
| Male                          | 112                    | 73 (65) | 0.350                  |         | 67 (60)                | 0.586   |
| Female                        | 46                     | 34 (74) | 31 (68)                |         | 29 (63)                |         |
| Group                         |                        |         |                        |         |                        |         |
| Normal epithelial             | 64                     | 17 (27) | 0.000                  |         | 13 (20)                | 0.000   |
| Epithelial neoplasia          | 54                     | 23 (43) | 19 (35)                |         | 15 (28)                |         |
| Gastric cancer                | 158                    | 107 (68)| 98 (62)                |         | 92 (58)                |         |
| Lymph node metastasis         |                        |         |                        |         |                        |         |
| No                            | 49                     | 25 (51) | 0.003                  |         | 21 (44)                | 0.001   |
| Yes                           | 109                    | 82 (75) | 77 (71)                |         | 72 (66)                |         |
| TNM classification            |                        |         |                        |         |                        |         |
| I, II stage                   | 67                     | 32 (48) | 0.000                  |         | 31 (46)                | 0.001   |
| III, IV stage                 | 91                     | 75 (82) | 67 (74)                |         | 64 (70)                |         |

[Correction added on 19 March 2013, after first online publication: Positive rate (%) of Rac1 for Male was amended to be 65.]
key cancer target gene and it is positively correlated with cyclin D1 expression. \(^{37}\) Studies have shown that with increasing Pak1 expression, the malignant evolution of colorectal cancer is increased. \(^{18}\) Moreover, 55 percent of breast cancer had a high expression of Pak1. \(^{19}\)

Rock (Rho-associated kinases) are direct downstream target proteins of RhoA, which are involved in a variety of cell functions, such as smooth muscle contraction, cytoskeleton construction, cell adhesion and movement and gene expression. The Rock gene has two subtypes, Rock1 and Rock2. Rock1 is located on chromosome 18 and encodes a 1354 amino acid protein. Rock2 is located on chromosome 12 and encodes a 1388 amino acid protein. Rocks include an amino acid kinase domain, a carboxyl terminal cysteine-rich region, are coiled coil domains in the middle, which includes the Rho-binding domain. Rock1 and Rock2 have 65 percent amino acid homology and the kinase domain has 92 percent homology. Rock1 is a GTP-dependent serine/threonine protein kinase that interacts with the Rho G-protein through its Rho-binding domain, thereby mediating Rho signaling. Rock1 overexpression or activation stimulates Rho activity. In addition, Rock1 can independently stimulate Rho and directly regulate cell biological behavior. Activated Rock1 induces a variety of proteins that can regulate cytoskeleton phosphorylation, thus producing corresponding biological effects, such as the reliance of Rock1 on MLC kinase or direct phosphorylation of serine 19 of MLC. Through the phosphorylation of LIMK-1 of Section 508 threonine and LIMK-2 of Section 505 threonine and the ezrin-radixin-moesin family proteins and adducin to promote cell cortex actin network formation and actin filament contact with the cell membrane. \(^{17,20}\)

Some studies have shown that GTP enzymes, such as Rho, Rac and Cdc42, through the downstream effectors Pak1, Pak4 and Rock activate LIMK1. \(^{17,21}\) Previous studies have shown that LIMK1 is closely related to the differentiation of gastric cancer, lymph node metastasis and TNM stage, and plays an important role in invasion and metastasis in gastric cancer. Rac1, Pak1 and Rock1, through the phosphorylation activation of the threonine residue within the LIMK1 ring, regulates the activity of LIMK1 and plays a role in cancer invasion and metastasis. \(^{22}\) LIMK is regulated by a variety of upstream signals, where the main upstream signal involved in the migration and invasion is the Rho GTP enzyme family. The Rho GTP enzyme family, including Rho, Rac and Cdc42, are activated by different transmembrane receptors and transmit signals to downstream effector proteins Rock1 and Pak1. Rock1, a Rho-associated protein kinase 1, can activate the protein function, and Rac can indirectly activate LIMK1 by Pak1 (P21-activated protein kinase 1). Conformational changes of Rock1 and Pak1 caused by connecting to the active GTP enzyme leads to the first 508 threonine phosphorylation of LIMK1, thereby causing the third serine phosphorylation of coflin1, ultimately causing actin dynamics, \(^{23-24}\) caused the formation of a signaling pathway regulation of cell migration and invasion of Rho-Rac1-ROCK1/PAK1-LIMK1-Cofilins. (actin-depolymerizing factor)/cofilins belongs to actin depolymerization factor, is a key factor regulating the actin cytoskeleton. It includes three members: destrin (ADF), coflin1 and coflin2, low concentration monomers G-actin, maintaining the actin monomers pool; high concentration by nucleation effect, promoting the formation of pseudopodia, drive tumor cell migration. The results indicate that Rac1 expression in normal gastric tissue, intraepithelial neoplastic tissues and gastric carcinoma were 27, 43 and 68 percent, respectively.

Pak1 expression levels in normal gastric tissue, intraepithelial neoplastic tissues and gastric carcinoma were 20, 35 and 60 percent, respectively, whereas Rock1 expression was 16, 28 and 58 percent, respectively. The difference between the groups was significant (\(P < 0.05\)). The Rac1 expression rate in lymph node metastasis was 75 percent, which is significantly higher than in the group without metastasis (51%). The Pak1 expression rate in lymph node metastasis was 71 percent, which is significantly higher than in the group without metastasis (43%). The Rock1 expression rate in stage II was 47.8 percent, which was significantly lower than that in stage III (82%). The Pak1 expression rate in stage II was 46 percent, which is significantly lower than that in stage III (74%). The Rock1 expression rate in stage II was 42 percent, which is significantly lower than that in stage III (72%) (\(P < 0.05\)). The correlation of Rac1, Pak1 and Rock1 expression in gastric cancer was analyzed using Spearman’s rank correlation, and the expression of the three groups in gastric cancer was positively correlated (\(r = 0.555, P < 0.05\)). Using a Kaplan–Meier curve to analyze the survival of 138 patients with follow up, it was found that Rac1, Pak1 and Rock expression was closely associated with survival and was a risk factor for survival.

We found that there were significant differences of Rac1, Pak1 and Rock expression in gastric cancer, epithelial neoplasia and normal epithelial tissue, which was...
an early molecular event in gastric cancer; Rac1, Pak1 and Rock expression, closely related to lymph node metastasis, depth of invasion and degree of differentiation, were valuable indicators in evaluating the degree of malignancy in gastric cancer and thus could contribute to the predication of invasion and metastasis of gastric cancer. Rac1, Pak1 and Rock expression was closely related to the survival of patients and could help in predicting the prognosis of patients. The possible mechanism of interaction between Rac1, Pak1 and Rock and other genes in gastric cancer deserves further study.

Rac1, Pak1 and Rock1 expression in gastric cancer is closely related with the degree of gastric cancer lymph node metastasis and TNM stage and they play an important role in the invasion and metastasis of gastric cancer and might be key biological markers for invasion and metastasis. The expression of these genes might be valuable indicators for evaluating the degree of malignancy of gastric cancer, perhaps as new markers of the biological behavior of gastric cancer. They could contribute to predicting gastric cancer invasion, metastasis and prognosis of patients. The actin cytoskeleton dynamics mechanism in tumor biology behavior, as the regulation of the actin cytoskeleton in cancer prevention and control in the sense Rac1, Pak1 and Rock1 is worth studying. Drugs including paclitaxel and cytochalasin B in making the cytoskeleton stable have been used for the treatment of cancer, but the wide range of their toxic effects make people worry. The development of drugs regulating the expression of Rac1, Rock1 and Pak1 may more accurately regulate actin activity which may be more beneficial in the prevention and treatment of diseases such as cancer.

ACKNOWLEDGMENTS

This study was supported by the Scientific and Technological Project of Hunan Province (2008SK3010); and the Science and Technology Planning Project of Xiangtan Science and Technology Bureau (SF20081003).

REFERENCES

1 Gómez del Pulgar T, Bandrés E, Espina C et al. Differential expression of Rac1 identifies its target genes and its contribution to progression of colorectal cancer. Int J Biochem Cell Biol 2007; 39: 2289–302.
2 Rathinam R, Berrier A, Alahari SK. Role of Rho GTPases and their regulators in cancer progression. Front Biosci 2011; 17: 2561–71.
3 Kumar R, Vadlamudi RK. Emerging functions of p21-activated kinases in human cancer cells. J Cell Physiol 2002; 193: 133–44.
4 Ong CC, Jubb AM, Haverty PM et al. Targeting p21-activated kinase 1 (PAK1) to induce apoptosis of tumor cells. Proc Natl Acad Sci U S A 2011; 108: 7177–82.
5 Liu X, Choy E, Hornicke F et al. ROCK1 as a potential therapeutic target in osteosarcoma. J Orthop Res 2011; 29: 1259–66.
6 Bosman FT, Carneiro F, Hruba RH, Theise ND. World Health Organization Classification of Tumours of the Digestive System. International Agency for Research on Cancer, Lyons 2010; 1–155.
7 Wang JX, Zhou YN, Zou SJ, Ren TW, Zhang ZY. Correlations of P21-activated kinase 1 expression to clinicopathological features of gastric carcinoma and patients prognosis. Chin J Cancer 2010; 29: 649–54.
8 Stengel K, Zheng Y. Cdc42 in oncogenic transformation, invasion, and tumorigenesis. Cell Signal 2011; 23: 1415–23.
9 Prasain N, Stevens T. The actin cytoskeleton in endothelial cell phenotypes. Microvasc Res 2009; 77: 53–63.
10 Baranwal S, Alahari SK. Rho GTPase effector functions in tumor cell invasion and metastasis. Carr Drug Targets 2011; 12: 1194–201.
11 Arias-Romero LE, Chernoff J. P21-activated kinases in Erbb2-positive breast cancer: a new therapeutic target. Small Gtpases 2010; 1: 124–8.
12 Schnelzer A, Prechtel D, Knaus U et al. Rac1 in human breast cancer: overexpression, mutation analysis, and characterization of a new isoform, Rac1b. Oncogene 2000; 19: 3013–20.
13 Binker MG, Binker-Cosen AA, Richards D, Gaisano HY, de Cosen RH, Cosen-Binker LI. Hypoxia-reoxygenation increase invasiveness of PANC-1 cells through Rac1/MMP-2. Biochem Biophys Res Commun 2010; 393: 371–6.
14 Spindler V, Waschke J. Role of Rho GTPases in desmosomal adhesion and pemphigus pathogenesis. Ann Anat 2011; 193: 177–80.
15 Pavey S, Zuidervaart W, van Nieuwpoort F et al. Increased p21-activated kinase-1 expression is associated with invasive potential in uveal melanoma. Melanoma Res 2006; 16: 285–96.
16 Yang Z, Bagheri-Yarmand R, Wang RA et al. The epidermal growth factor receptor tyrosine kinase inhibitor ZD 1839 (Iressa) suppresses c-Src and Pak1 pathways and invasiveness of human cancer cells. Clin Cancer Res 2004; 10: 658–67.
17 Arber S, Barbayannis FA, Hanser H et al. Regulation of actin dynamics through phosphorylation of cofilin1 by LIM-kinase. Nature 1998; 393: 805–9.
18 Okada T, Lopez-Lago M, Giancotti FG. Merlin/NF-2 mediates contact inhibition of growth by suppressing recruitment of Rac to the plasma membrane. J Cell Biol 2005; 171: 361–71.
19 Carter JH, Douglass LE, Deddens JA et al. Pak-1 expression increases with progression of colorectal carcinomas to metastasis. *Clin Cancer Res* 2004; 10: 3448–56.

20 Lou Z, Billadeau DD, Savoy DN, Schoon RA, Leibson PJ. A role for a RhoA/ROCK1/LIM-kinase pathway in the regulation of cytotoxic lymphocytes. *J Immunol* 2001; 167: 5749–57.

21 Ozawa T, Araki N, Yunoue S et al. The neurofibromatosis type 1 gene product neurofibromin enhances cell motility by regulating actin filament dynamics via the Rho-ROCK-LIMK2-cofilin pathway. *J Biol Chem* 2005; 280: 39524–33.

22 Wu YJ, Tang Y, Li Z et al. Clinicopathological significance of cofilin1 in gastric cancer. *Chin J Clin Exp Pathol* 2011; 27: 658–60.

23 Schmitz AA, Govek EE, Böttner B et al. Rho GTPases: signaling, migration, and invasion. *Exp Cell Res* 2000; 261: 1–1.

24 Delorme V, Machacek M, DerMardirossian C et al. Cofilin1 activity downstream of Pak1 regulates cell protrusion efficiency by organizing lamellipodium and lamella actin networks. *Dev Cell* 2007; 13: 646–62.

25 Hall A, Rho GTPases and the actin cytoskeleton. *Science* 1998; 279: 509–14.

26 Edwards DC, Sanders LC, Bokoch GM et al. Activation of LIM-kinase by Pak1 couples Rac/Cdc42 GTPase signalling to actin cytoskeletal dynamics. *Nat Cell Biol* 1999; 1: 253–9.

27 Kamai T, Tsujii T, Arai K et al. Significant association of Rho/ROCK1 pathway with invasion and metastasis of bladder cancer. *Clin Cancer Res* 2003; 9: 2632–41.

28 Ohashi K, Nagata K, Maekawa M et al. Rho-associated kinase ROCK1 activates LIM-kinase 1 by phosphorylation at threonine 508 within the activation loop. *J Biol Chem* 2000; 275: 3577–82.