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Original Article

Expression and clinical significance of the RAS/RAF/MAPK cell signaling pathway in gastric cancer

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Abstract: Objective: To explore the correlation between the Ras/Raf/MAPK signaling pathway and the development of gastric cancer. Methods: 65 tissue samples of primary gastric cancer were collected from patients who had undergone radical gastrectomy for gastric cancer as the study group, and 70 normal gastric mucosal tissue samples were collected as the control group. The expression of Ras, p-Raf-1, MAPK, and CyclinD1 proteins in the two groups of tissues were detected by immunohistochemistry. The expression of MAPK and CyclinD1 mRNAs in the two groups was detected by nucleic acid in situ hybridization, and the average light density of each test index was calculated by the CCD imaging system. Results: The expression levels of Ras and p-Raf-1 proteins in the study group were higher than that in the control group and the difference was statistically significant ($P<0.05$); the expression levels of MAPK protein and mRNA in the study group were significantly higher than that in the control group ($P<0.01$) and the expression intensities of CyclinD1 protein and mRNA in the study group were higher than that in the control group ($P<0.05$). In the study group, there was a positive correlation between Ras and p-Raf-1 protein expressions, p-Raf-1 and MAPK protein expressions, and CyclinD1 and MAPK protein expressions. Conclusion: Ras, p-Raf-1, MAPK, and CyclinD1 proteins and MAPK and CyclinD1 mRNAs, were highly expressed in the RAS/RAF/MAPK cell signal conduction pathway. The excessive expression of the RAS/RAF/MAPK pathway proteins in gastric cancer may be one of the important mechanisms of gastric cancer and may be exploited as a potential target for its treatment.

Keywords: Gastric cancer, Ras/Raf/MAPK, signal conduction pathway, targeted therapy

Introduction

Gastric cancer is one of the most common malignant tumors in China. The occurrence of gastric cancer is related to environmental factors and biological genetics [2]. At present, the clinical therapy of gastric cancer consists mainly of combination treatment of surgery and chemical drugs. However, as the early stage of gastric cancer lacks specific clinical symptoms, diagnosis is mostly made when patients are in the middle or advanced stages of gastric cancer, which leads to a low first diagnosis rate [1, 2]. Gastric cancer progresses rapidly, with radical gastrectomy offering little benefit. In addition, the specificity of chemotherapeutic drugs is poor, with many side effects, resulting in both decreased survival time and quality of life of the patients [3]. In recent years, research on molecular targeted therapy, based on cellular signal conduction, has shown characteristics of high efficiency, accuracy, and low toxicity, and has opened a new avenue for the treatment of cancer [4]. Cellular signal conduction systems are often abnormally activated in tumor cells, and the signaling pathways play an important role in regulating the proliferation and apoptosis of tumor cells [5].

Ras/Raf/MAPK signal conduction pathway is an important pathway for the transmission of extracellular signals into cells. This pathway is considered to be one of the most active pathways that regulates cell growth, and is closely related to the proliferation and differentiation of cells [6]. The phosphorylation of proteins in the body is a mechanism of signal conduction in various cells. Ras is phosphorylated by the stimulation of extracellular signals and in turn, transmits these signals into cells [8]. After activation of Ras, its downstream molecule Raf, appears to undergo conformational changes;

Raf-1 protein is the target point of Ras. After activation of Raf-1, MAPK (also called ERK) is phosphorylated and activated. This series of reactions constitute a protein kinase cascade. The Ras/Raf/MAPK signaling pathway ultimately induces the expression of the cyclin D1 (CyclinD1), which is an important regulatory factor involved in cell cycle transition from the G1 to S phase. The abnormal expression of CyclinD1 may cause an alteration in the expression of genes or proteins related to cell growth, differentiation, and other functions [7-9]. An abnormal activation of Ras/Raf/MAPK signal conduction pathway may cause abnormal proliferation of cells, and the natural apoptosis process of the cells is blocked, which further leads to the invasion and metastasis of tumor cells [10]. Studies have shown that the obvious enhancement of the Ras/Raf/MAPK signal conduction pathway is closely related to the occurrence and development of many malignant tumors, which has been confirmed in studies of breast cancer, lung cancer, colon cancer etc. [10-12].

There are few studies on the role of Ras/Raf/MAPK signaling pathway in the generation and development of gastric cancer. Therefore, in this study, we have investigated the expression of related proteins and genes of the Ras/Raf/MAPK signal conduction pathway in gastric cancer tissues and analyzed the correlation between the pathway and the occurrence and development of gastric cancer, so as to provide a reference for the targeted treatment of gastric cancer.

Materials and methods

Experimental reagents

Mouse anti human Ras polyclonal antibody, mouse anti human Raf polyclonal antibody, mouse anti human CyclinD1 polyclonal antibody and mouse anti human MAPK polyclonal antibody were purchased from Santa Cruz company (Santa Cruz, CA, USA); in situ hybridization kit of human MAPK mRNA oligonucleotide probe, in situ hybridization kit of human CyclinD1 mRNA oligonucleotide probe, 1% Polyformaldehyde and DEPC water were purchased from Wuhan BOSHIDE Biological engineering Co. Ltd.; DAB kit, AEC kit and PBS buffer solution were purchased from Fuzhou Maxim Biotechnology Co., Ltd.; the CCD microimaging system was purchased from the Pixera company (United States).

Experimental objects

Sixty-five tissue samples of primary gastric cancer were collected from patients who had undergone radical gastrectomy for gastric cancer, in our hospital as the study group, and 70 normal gastric mucosal tissue samples, confirmed by gastroscopy, were collected as the control group. None of the gastric cancer patients were treated by chemoradiotherapy before operation, and gastric cancer was diagnosed by histopathological examination before operation. The tissue samples were fixed with 10% neutral buffered formalin, embedded in conventional paraffin, 4 μm serial sections were cut and hematoxylin and eosin (HE) stained conventionally.

Protein expression detection in tissues by immunohistochemistry

The paraffin-cut sections of gastric cancer tissues and normal gastric mucosal tissues were placed on glass slides, and conventional dewaxing was carried out at 60°C for 30 min, incubated with 3% H_2O_2 at warm temperature for 10 min, and rinsed with PBS, 3 times, for 3 minutes each. Thereafter, sections were treated with 0.5% TritonX-100 for 30 min and rinsed with PBS 3 times, for 3 min each. Sections were then incubated with 10% goat sera for 10 min. After removing the serum, primary antibody was added (50 L), put in a wet box and incubated overnight (4°C). Following incubation, sections were rinsed with PBS 3 times, for 3 min each. Secondary antibody was added and incubated at room temperature for 10 min. Thereafter, slides were rinsed with PBS 3 times, for 3 min each. Sections were then incubated with horseradish peroxidase (37°C for 30 min), followed by rinsing with PBS 3 times, for 3 min each. Finally, DAB reagent was added and hematoxylin was applied to re-stain for 2 min. PBS and deionized water were used for rinsing and sealing, and a light microscope was used for observation. The optical density value was detected by CCD microscope imaging system.

Detection of mRNA in tissue by in situ hybridization

The paraffin-cut sections of gastric cancer tissues and normal gastric mucosal tissues were

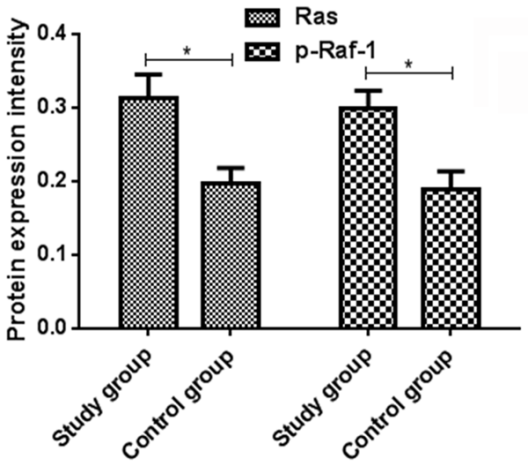

Figure 1. Comparison of expression intensity of Ras and Raf proteins.The immunohistochemistry results show that the expression intensities of Ras and p-Raf-1 proteins in the study group are higher than that in the control group, and the difference is statistically significant (P<0.05). *P<0.05.

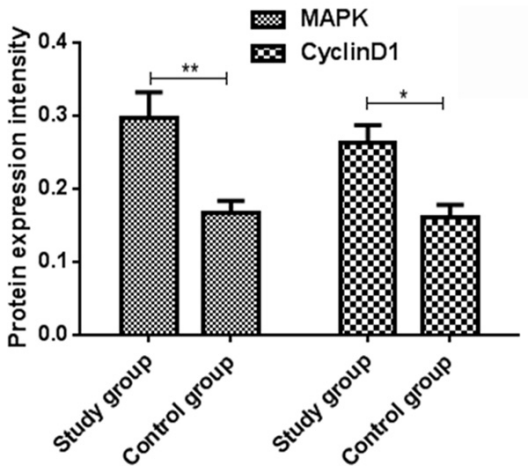

Figure 2. Comparison of expression intensity of MAPK and CyclinD1 proteins. The immunohistochemistry results show that the expression intensity of MAPK protein in the study group is higher than that in the control group and the difference is statistically significant (P<0.01); the expression intensity of CyclinD1 protein in the study group is higher than that in the control group and the difference is statistically significant (P<0.05). *P<0.05, **P<0.01.

placed on glass slides, and conventional dewaxing was carried out. Thereafter, they were incubated for 10 min with 3% H_2O_2 and rinsed with PBS, 3 times for 3 min each. This was followed by addition of 3% citric acid pepsin, allowed to stand at room temperature for 10 min, and rinsed with PBS, 3 times for 5 min each. Thereafter, 1% polyoxymethylene was added and allowed to stand at room temperature for 10 min. Slides were then washed with DEPC-treated water, 3 times, for 3 min each. Twenty microliters of prehybridization solution was added and incubated for 2 h at 37°C. Twenty μL of probe hybridization solution was then dripped onto the slides and allowed to stand at a constant temperature of 37°C. Stringency washes were done as follows: 37°C, 2 x SSC; 37°C, 0.5 x SSC and 37°C, 0.2 x SSC. Sealing solution was dripped onto the slide (37°C for 30 min), rat digoxin was added, and incubated at room temperature for 2 h. Thereafter, SABC-POD was added and placed at room temperature for 30 min. Biotinylated peroxidase was added and incubated for 30 min, followed by washing with PBS. The AEC developer was then added and incubated at 37°C for 5 min. Hematoxylin was added for 2 min for re-staining, distilled water was used for rinsing and sealing. Slides were observed under a light microscope.

Observation of experimental results

The cells whose cytoplasm/nuclei showed yellow particles during the immune-histochemical test, and the cells whose cytoplasm/nuclei showed red particles during in situ hybridization, were considered positive. The CCD imaging system was used to observe 5 fields at random, for each slide under the high-power lens. The expression intensity of positive staining was detected by microscopic image analysis system (mean value of light density, the greater the value is, the higher the positive expression intensity will be).

Statistical analysis method

The SPSS13.0 software (IBM, Armonk, NY, USA) was adopted for statistical analysis, and the GraphPad Prism 5 software for mapping. The quantitative data were analyzed by t test, and the results were expressed by $\bar{x} \pm s$. The correlation was analyzed by Pearson's correlation analysis. All *P* values indicate bilateral probability and significance was set at 0.05.

Results

Basic clinical data of two groups of patients

Of the 65 cases of gastric cancer, 36 were males and 29 were females with an average age of 64±2.58 years; 40 cases were diagnosed with moderately and well differentiated

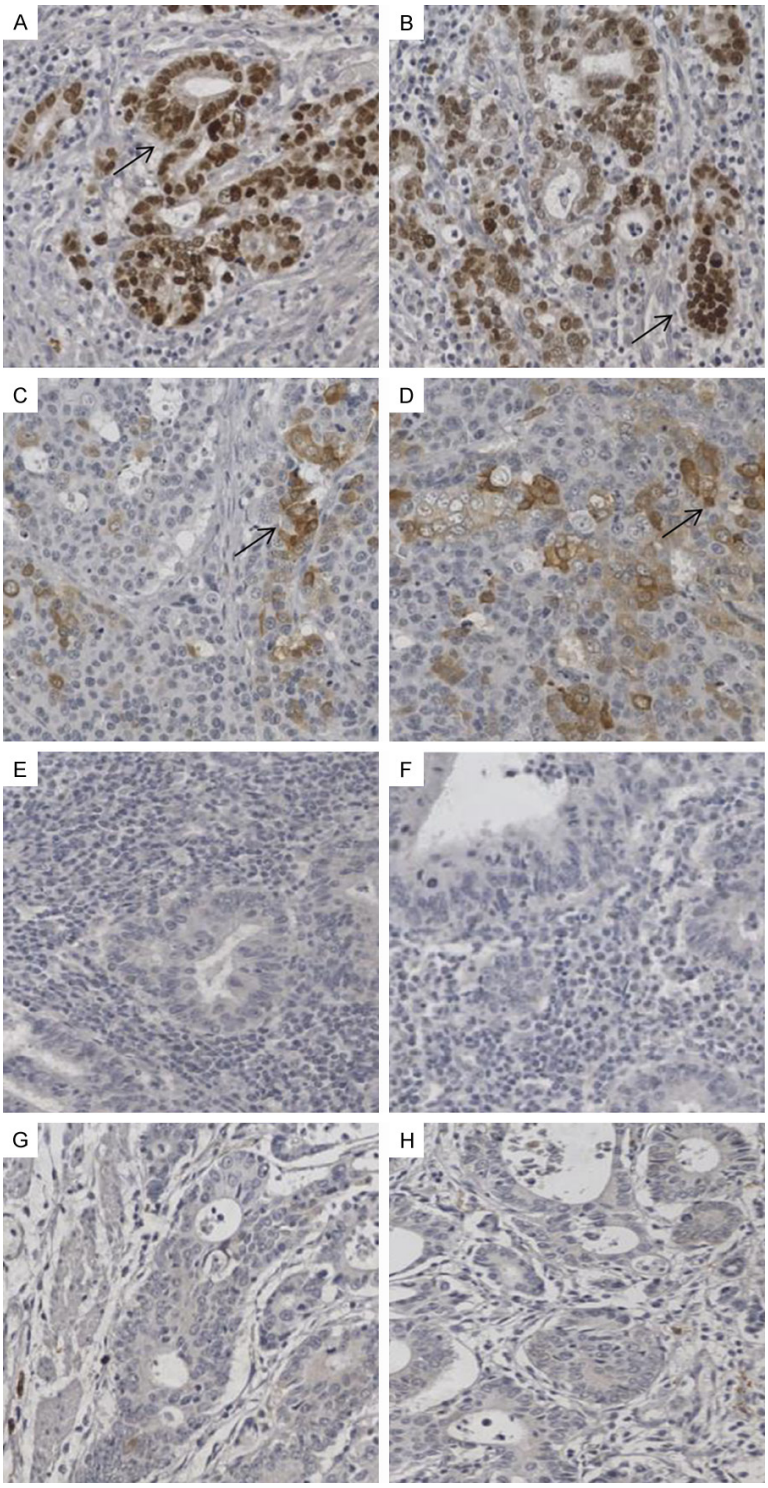

Figure 3. Immunohistochemical expression of gastric cancer in case group and control group. A: Expression of Ras protein in the case group. The cells were brown and yellow. B: The expression of p-Raf-1 protein was brown. C: The expression of MAPK protein was located in cytoplasm or nucleus and was brown. D: In the case group, the expression of CyclinD1 protein was located in cytoplasm or nucleus, and was brown. E: The expression of Ras protein in control group. F: The expression of CyclinD1 protein in control group. G: The expression of MAPK protein in control group. H: Control group Cyclin Expression of D1 protein.

gastric cancer while 25 cases with poorly differentiated gastric cancer; 18 cases had tumors not infiltrating into the serous layer and 47 cases had tumors infiltrating the serous layer; 43 cases were detected with lymph node metastasis and 22 cases were detected without lymph node metastasis; 6 cases were in TNM stage I, 21 cases in stage II, 33 cases in stage III, and 5 cases in stage IV. Of the 70 cases undergoing gastroscopy examination, 42 were males and 38 were females, with an average age of 65±3.02 years. There was no significant difference in gender and age between the two groups of patients (P>0.05).

The expression intensity

The results of immunohistochemical staining showed that the expression intensity of Ras protein (0.312±0.033) and p-Raf-1 protein (0.298± 0.025) in the study group was higher than that in the control group (0.197±0.021, 0.188 + 0.026), and the differences were statistically significant (t=8.040, P<0.05; t=7.251, P<0.05) (**Figure 1**).

Expression intensity of MAPK and CyclinD1 proteins in the two groups of patients

The MAPK protein and CyclinD1 protein appear of brownish yellow color, mainly in the cytoplasm or nuclei. The results of the immunohistochemistry analysis showed that the expression intensity of MAPK protein (0.296± 0.036) in the study group was higher than that in the control group (0.166±0.018) (t=9.853, P<0.01); the expression intensity of CyclinD1 protein (0.263±0.024) in the

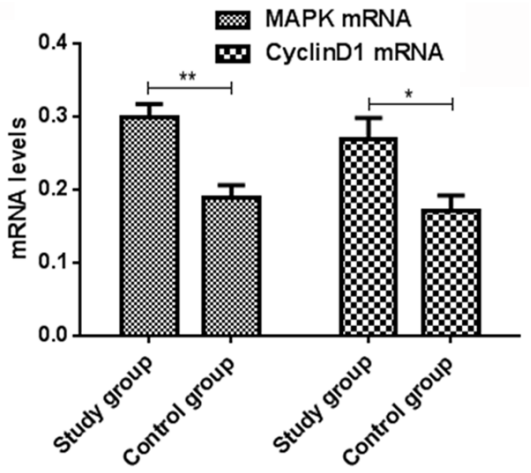

Figure 4. Comparison of MAPK and CyclinD1 mRNA levels. The results of in situ hybridization demonstrate that MAPK mRNA in the study group is higher than that in the control group and the difference is statistically significant (t=11.201, P<0.01); the expression intensity of CyclinD1 mRNA in the study group is higher than that in the control group (0.171±0.021), and the difference is statistically significant (t=8.710, P<0.05). *P<0.05, **P<0.01.

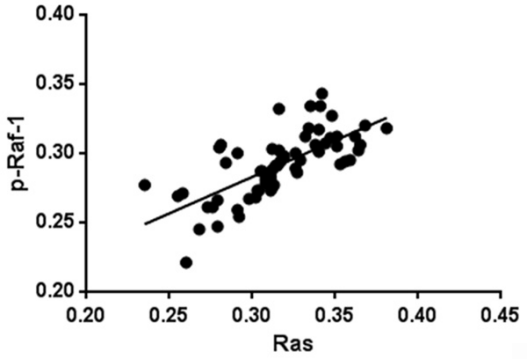

Figure 5. Correlation between the expressions of Ras and Raf proteins. The Pearson correlation analysis result demonstrates that Ras and p-Raf-1 proteins in gastric tissues (r=0.700, P<0.001) are positively related.

study group was higher than that in the control group (0.161±0.017) (t=7.798, P<0.05) (**Figure 2**).

The immunohistochemistry results and *in situ* hybridization results of Ras, p-Raf-1, MAPK and CyclinD1 are shown in **Figure 3**.

MAPK and CyclinD1 mRNA levels in the two groups

MAPK and CyclinD1 mRNAs appear red under the microscope and are mainly located in the

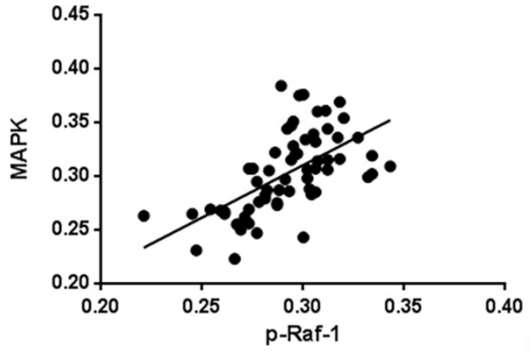

Figure 6. Correlation between the expressions of p-Raf-1 and MAPK proteins. The Pearson correlation analysis result demonstrates that p-Raf-1 and MAPK proteins in gastric tissues are positively related.

nucleus. The results of *in situ* hybridization showed that the MAPK mRNA (0.298±0.019) in the study group was higher than that in the control group (0.188±0.018), and the difference was statistically significant (t=11.201, P<0.01). The expression intensity of CyclinD1 mRNA (0.268±0.030) in the study group was higher than that in the control group (0.171±0.021), and the difference was statistically significant (t=8.710, P<0.05) (**Figure 4**).

Correlation analysis of the expression of Ras, Raf, MAPK, and CyclinD1 proteins and MAPK and CyclinD1 mRNAs in gastric cancer tissue

The results of Pearson's correlation analysis demonstrated that there was a positive correlation between Ras and p-Raf-1 proteins (r=0.700, P<0.001), p-Raf-1 and MAPK proteins (r=0.601, P<0.001), MAPK and CyclinD1 proteins, and MAPK and CyclinD1 mRNAs (**Figures 5-8**).

Discussions

The clinical treatment of advanced gastric cancer is mainly based on surgical treatment, supplemented by chemotherapy. The early detection of gastric cancer and the adoption of targeted measures to improve the effectiveness of treatment are the keys to improving the patient's survival time and quality of life [13]. The Ras/Raf/MAPK signaling pathway plays an important role in cell growth and differentiation [8]. When the Ras/Raf/MAPK signaling pathway is activated by the stimulation of extracellular signals, it causes a cascade reaction, which eventually leads to the increase of the

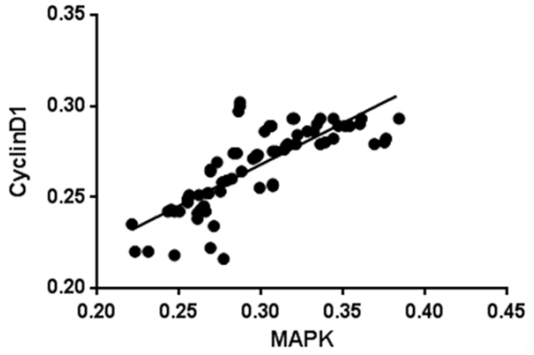

Figure 7. Correlation between the expressions of MAPK and CyclinD1 proteins. The Pearson correlation analysis result demonstrates that MAPK and CyclinD1 proteins in gastric tissues (r=0.780, P<0.001) are positively related.

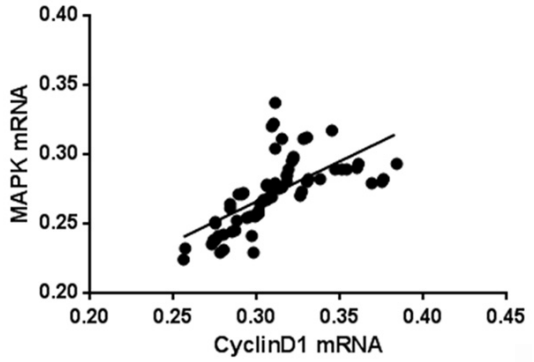

Figure 8. Correlation between the expressions of MAPK and CyclinD1 mRNAs. The Pearson correlation analysis result demonstrates that MAPK mRNA and CyclinD1 mRNA in gastric tissues (r=0.662, P<0.001) are positively related.

expression of cyclin, CyclinD1, induces the differentiating cells to progress from the G0/G1 phase to the S phase, and further promotes the proliferation of cells [14]. Studies have shown that the sustained activation of Ras/Raf/MAPK signaling pathway is closely related to the occurrence and development of multiple malignancies [10, 15]. Therefore, controlling the signaling pathway can be an effective tool for targeted treatment of malignant tumors and help improve the treatment of cancer patients [5, 8].

In order to explore the relationship between the Ras/Raf/MAPK signaling pathway and the occurrence of gastric cancer, we used immunohistochemistry and nucleic acid in situ hybridization to detect the related proteins and genes in the Ras/Raf/MAPK signaling pathway in gastric cancer and normal gastric mucosal tissues. Thereafter, we compared the differences in the expression levels between the two groups, and analyzed the correlations between the cytokines, in order to explore the clinical value of this signaling pathway in targeted therapy of gastric cancer. This study only analyzed the Ras/Raf/MAPK signaling pathway and hence, the expression of CyclinD1, and the relationship between other related molecules and the tumor, and the relationship between the pathway and other signal conduction pathways, need to be further verified.

The results of the immunohistochemical method showed that the expression intensity of Ras, p-Raf-1, and MAPK proteins in the study group was higher than that in the control group, which indicates that the Ras/Raf/MAPK signal transduction pathway is abnormally activated in gastric cancer tissues. An earlier study found that there were Ras mutations in about 30% of tumors and the mutation of the Ras gene enables the malignant transformation of tumor cells [16, 17]. It is generally believed that there is a significant association between the RAS gene and the occurrence of tumors [18-22]. In recent years, it has been found that MAPK is overexpressed in many kinds of tumors, such as breast, colon, and lung cancers, and is considered to be associated with the occurrence of tumors [10-12]. The activation of KRAS is related to the occurrence and development of thyroid cancer; the RAS pathway is activated, and the expression of RAS protein is increased in patients with thyroid cancer [23]. Studies by Diego, et al. [19] found that the inactivation of RAS inhibitors enabled the activation of the Ras/Raf/MAPK signaling pathway. There is a downregulation or loss of Ras inhibitors in liver cancer tissue, which causes the continuous activation of the Ras/Raf/MAPK signal transduction pathway and the upregulation of the expression of Raf and MAPK proteins.

In this study, we found that the expression intensity of CyclinD1 protein and mRNA in the study group was higher than that in the control group. The correlation analysis showed that there was a positive correlation between Ras and p-Raf-1 proteins, p-Raf-1 and MAPK proteins, MAPK and CyclinD1 proteins, and MAPK and CyclinD1 mRNAs. All the signals of the Ras/Raf/MAPK signaling pathway eventually induce

the activation of CyclinD1. The activated CyclinD1 regulates the cell proliferation cycle by binding to proteins related to the cell cycle, and induces differentiating cells to transition from the G1 to S phase, which causes the malignant proliferation of the cells, and results in the occurrence or malignant transformation of tumors [24, 25]. Earlier studies have shown that the increased expression of Ras and CyclinD1, as well as other genes or proteins in the Ras/Raf/MAPK signal transduction pathway, can be detected in early tumor tissues. Therefore, the abnormalities of the pathway may be the trigger point for the malignant transformation of the tumor, which is closely related to the degree of cell carcinogenesis. Indeed, timely discovery of the abnormalities will enable action to be taken early, which will be of great significance for early detection, diagnosis, and treatment of malignant tumors [26-28]. In studies conducted on breast cancer, lung cancer and thyroid cancer, it was found that the Ras/Raf/MAPK signaling pathway is involved in cell growth, proliferation, and differentiation, which is associated with the occurrence and development of tumors. Therefore, this signaling pathway may be identified as a potential target of antitumor therapy in the tumors above [29].

In this study, the expressions of related proteins and genes in the Ras/Raf/MAPK signal conduction pathway in gastric cancer tissues were detected by immunohistochemistry and in situ hybridization. The results showed that there was an abnormal activation of the Ras/Raf/MAPK signal conduction pathway in gastric cancer tissues, and the expressions of related proteins and genes in the signaling pathway were significantly correlated, indicating that genes or proteins related to this pathway will be promising targets for gastric cancer treatment.

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Disclosure of conflict of interest

None.

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