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Research on the Regulatory Mechanism of Twist on Epithelial-Mesenchymal Transition and Vasculogenic Mimicry in Human Gastric Cancer Tissues

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Abstract: Objective: To explore the promoting effect of Twist on the formation of vasculogenic mimicry (VM), so as to enrich and improve the theory of vasculogenic mimicry. Methods: For gastric cancer tissues and normal gastric tissues, observe the expression status of Twist, epithelial-mesenchymal transition (EMT) marker proteins (including E-cadherin and Vimentin), and VM marker CD34 - PAS. Analyze the correlations between Twist, EMT marker proteins and the clinicopathological features of gastric cancer. Conduct in-depth research on the associations between VM and EMT marker proteins as well as between Twist and EMT marker proteins, and then verify the situation that Twist promotes the formation of VM by promoting EMT in gastric cancer. Results: There are statistical differences between EMT marker proteins and VM, and Twist has a certain correlation with EMT marker proteins. Conclusion: Twist may induce the occurrence of epithelial-mesenchymal transition (EMT) in gastric cancer cells and promote the formation of vasculogenic mimicry (VM) in gastric cancer.

Key words: Epithelial-Mesenchymal Transition (EMT); Twist; Invasion; Correlation

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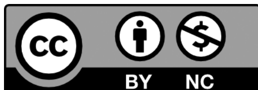

Twist is a major regulator in human embryonic development. Among them, the Twist1 gene has been proven to be conducive to the metastatic spread of cancer cells through its ability to induce epithelial-mesenchymal transition (EMT). Previous studies have shown that a large proportion of human cancers overexpress the Twist1 or Twist2 gene [1]. Gastric carcinoma (GC) is a malignant tumor originating from the abnormal proliferation of gastric mucosal epithelial cells. The epidemiological manifestations of gastric cancer show significant differences in different regions. In particular, the incidence rates in the northwest and eastern coastal areas are significantly higher than those in the southern regions. Gastric cancer usually tends to occur in people over 50 years old. However, with the changes in modern dietary structures, the increase in work pressure, and the influence of various factors such as Helicobacter pylori infection, gastric cancer is showing a trend of getting younger. Although the prevalence and mortality rates of gastric cancer in developed countries (such as Europe and the United States) are on a downward trend, which is related to social and economic development and the improvement of living standards, gastric cancer remains a high-incidence disease in Asia and Latin America. In recent years, the early diagnosis rate, comprehensive treatment effect, surgical resection rate, and postoperative survival rate of gastric cancer have all improved [2].

1. Tumor Angiogenesis and Vasculogenic Mimicry

The traditional angiogenesis theory holds that the growth of tumors depends on the formation of new blood vessels.

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Once the diameter of a solid tumor exceeds 2 mm, it is urgent to induce angiogenesis to obtain sufficient blood supply to maintain its continuous growth; otherwise, tumor cells may undergo necrosis due to a lack of sufficient blood oxygen supply [3]. Anti-angiogenic therapy has emerged as the times require. However, with the wide application of anti-angiogenic drugs, some scholars have found that the expected results have not been achieved. In 1999, Maniotis et al. in the United States [4] discovered and named a new blood supply pattern - VM (vasculogenic mimicry) when studying the microcirculation of highly invasive human uveal melanoma. The discovery of VM broke the traditional theory that the only way for tumors to obtain blood supply was through endothelial blood vessels and explained the biological behaviors of malignant tumors with high invasiveness and high metastasis. VM is an irregular grid-like vascular-like channel formed by tumor cells instead of endothelial cells (endothelial cell marker CD34 negative) through self-shaping and extracellular matrix remodeling under a specific microenvironment, with a layer of basement membrane-like substance inside (PAS positive, which is a marker for the composition of normal blood vessel walls and VM walls) [5]. Three-dimensional culture is currently a rather important evaluation method for studying VM in vitro [6]. The role of Matrigel is similar to that of the basement membrane of mammalian cells and is one of the ideal scaffold sources for studying VM in vitro.

2. EMT and Vasculogenic Mimicry

Tumor cells participating in the formation of VM mimic the functions and phenotypes of endothelial cells, and endothelial cells themselves are a kind of mesenchymal cells. Therefore, the transformation of tumor cells from the epithelial type to the mesenchymal type of endothelial cells may occur, and this phenomenon is EMT [7]. EMT is a dynamic pathological process in which closely connected epithelial cells transform into loosely connected spindle-like mesenchymal cells under certain conditions, and corresponding changes occur in cell morphology at the same time. Through EMT, tumor cells acquire higher metastatic and invasive abilities and play an important role in the occurrence and development of tumors.

3. Materials and Methods

3.1. Research Subjects

Collect paraffin specimens of primary gastric cancer that have been surgically resected and confirmed by pathology in clinical practice. The patients did not receive tumor-specific treatments such as chemotherapy, radiotherapy, and immunotherapy before surgery. Collect data on patients' ages, tumor diameters, clinical stages, histological grades, presence or absence of lymph node metastasis, and formation of vascular tumor thrombi. In addition, specimens of normal gastric tissue after pathological confirmation of benign gastric ulcer surgery or gastric weight loss surgery were collected as controls for comparative studies. Patients signed the informed consent forms and complied with the relevant regulations of the institutional medical ethics committee.

3.2. Methods

Immunohistochemical Staining of Proteins: The S-P immunohistochemical method was used to detect the protein expressions of Twist, E-cadherin, and N-cadherin. The specific operations were as follows:

(1) Paraffin sections with a thickness of 3 μm were baked at 60 - 65°C for 4 hours.

(2) Rinse with PBS buffer twice, 5 minutes each time.

(3) Tissue antigen retrieval was carried out.

(4) Wash with PBS buffer for 3 minutes.

(5) Add 50 μL of peroxidase solution to each slide to block it, and incubate at room temperature for 10 minutes.

(6) Rinse with PBS for 33 minutes.

(7) Remove PBS, add 50 μL of non-immunological animal serum to each slide, and incubate at room temperature for 10 minutes.

(8) Rinse with PBS for 35 minutes.

(9) Remove PBS, add the solution to each slide, and incubate at room temperature.

(10) Rinse with PBS for 33 minutes. Remove PBS, add 1 drop to each slide, incubate at room temperature for 10 minutes, and then rinse with PBS three times, 3 minutes each time.

(11) Remove PBS, add fresh DAB to each slide 1 - 2 times, and develop the color for 3 - 10 minutes. Positive staining appears brown or yellow.

(12) Rinse with tap water, counterstain with hematoxylin, and then rinse with tap water for 10 - 15 minutes.

(13) Prepare the DAB chromogenic reagent. Dehydrate and dry the sections with gradient alcohol, use xylene for clearing, and perform the procedure in reverse according to the "section dewaxing and hydration" process. Finally, mount with neutral balsam.

CD34-PAS Double Staining:

(1) Cut the embedded specimens into sections with a thickness of 4 μm using a paraffin microtome.

(2) The sections were subjected to baking treatment, dewaxed with xylene, and then treated with gradient alcohol until hydrated.

(3) Place the sections into 0.1 mol/L citric acid antigen retrieval solution with a pH of 6.0, and conduct boiling water bath treatment for 20 minutes. After stopping heating, let it cool naturally for 20 - 30 minutes.

(4) Wash with 0.01 mol/L PBS three times, 3 minutes each time. Circle the tissue with a water-blocking pen, add 3% hydrogen peroxide, and block for 10 minutes to remove endogenous peroxidase.

(5) Wash with 0.01 mol/L PBS three times, 3 minutes each time to wash away the hydrogen peroxide. Spin dry the PBS, add 5% goat serum, and block at room temperature for 1 hour.

(6) Absorb the excess blocking solution with filter paper. According to the results of the preliminary experiment or the recommended concentration in the antibody instruction manual, add the appropriate concentration of the primary antibody, place it in a humid box, and incubate at 4°C overnight.

(7) On the second day, wash with 0.01 mol/L PBS for 3 minutes, a total of 5 times to remove the primary antibody. After shaking off the excess PBS, add the secondary antibody, and place the sections in an incubation box at room temperature for 30 minutes of incubation.

(8) Wash with 0.01 mol/L PBS five times, 3 minutes each time to wash away the excess secondary antibody. Shake off the excess PBS. While keeping the sections moist, add DAB chromogenic solution. Observe under a microscope for positive results and record the optimal chromogenic time. The remaining sections are developed for the same time.

(9) Wash away the excess DAB with water (DAB is a class of carcinogens. The waste liquid after washing with water needs to be neutralized with sulfuric acid - potassium permanganate aqueous solution and can be safely discharged).

(10) Gently wash the glass slides with deionized water to replace the tap water. Spin dry the water on the glass slides, add periodic acid solution, and treat at room temperature for 10 minutes. Wash with deionized water 2 - 3 times, with a standing time of 1 - 2 minutes each time.

(11) Shake off the water on the glass slides. Take out the PAS reagent at 4°C and directly drip it for staining. Incubate at room temperature for 10 minutes. Note that this reaction needs to be protected from light in time. Just cover it with light-proof paper and do not move it to prevent the staining solution from flowing.

(12) After the PAS staining time is up, wash the glass slides with sulfurous acid rinse solution 3 - 5 times, with a standing time of 1 - 2 minutes each time.

(13) Counterstain by adding hematoxylin staining solution, incubate at room temperature for 3 minutes. Wash away the excess staining solution with 0.01 mol/L PBS, three times for 3 minutes each time.

(14) Conduct gradient alcohol rehydration treatment: soak in 80% alcohol for 5 seconds first, then soak in 95% alcohol for 10 seconds, then soak in 100% alcohol for 3 minutes, and then soak in 100% alcohol for 5 minutes. Next, use xylene II for clearing for 5 minutes, and then use xylene I for clearing for 5 minutes (if it is not completely clear, the time can be appropriately extended).

(15) Mount with neutral balsam. After the balsam is completely dry, conduct microscopic examination.

Result Judgment and Scoring: The judgment of immunohistochemical results adopted the semi-quantitative integral method of immunoreaction. The total score was equal to the product of the staining intensity integral and the positive cell proportion integral. The staining intensity integral was scored as follows: 0 points for no color development, 1 point for light yellow, 2 points for brownish-yellow, and 3 points for brownish-black. The positive cell proportion integral was calculated by randomly counting 10 fields under a low-power microscope to ensure that the total number of tumor cells

was not less than 1,000, and calculating the proportion of positive cells, which were scored as 0 points (0%), 1 point (1% - 10%), 2 points (11% - 50%), 3 points (51% - 80%), and 4 points (> 80%). The total score ranged from 0 to 12 points, and 0 - 3 points were defined as negative, and 3 points or more were defined as positive. CD34-PAS Double Staining and VM Judgment: CD34 positive granules were expressed in the cytoplasm/membrane of vascular endothelial cells, and PAS positive was expressed in the basement membrane of the vascular lumen wall, showing purplish-red or cherry-red. The specific judgment criteria were as follows: The PAS positive basement membrane-like substance with an irregular vascular structure inside surrounded by CD34 negative tumor cells (confirmed under a HE light microscope), sometimes with red blood cells visible inside, without bleeding, necrosis, or obvious inflammatory cells around the pipeline, was judged as VM. Among them, the regular annular substances with both CD34 and PAS positive expressions were excluded as endothelial blood vessels.

3.3. Statistical Methods

This study used SPSS 22.0 software for statistical analysis. The measurement data were expressed as $X \pm S$. The count data were analyzed by χ^2 test and adopted. The test standard was set as $\alpha = 0.05$. When the p-value was less than 0.05, the difference was considered statistically significant.

4. Results

4.1. Analysis of VM Expression in Human Gastric Cancer Tissues

In this study, tissue detection of VM expression in paraffin blocks was carried out on 50 colon cancer patients after surgery. The research results showed that VM phenomenon did exist in gastric cancer patients. It was found that among the 50 gastric cancer patients, there were patients with VM expression (+). In addition, the positive expression of VM was related to poorly differentiated tumor tissues, higher clinical stages, recurrence and metastasis, etc., which were statistically significant ($p < 0.05$).

4.2. Correlation Analysis of VM Protein and EMT Protein Expressions in Human Gastric Cancer Tissues

This study found that among 11 VM-positive colon cancer tissues, the high expression rate of Vimentin was 63.7%, while the low expression rate of E-cadherin was 18.2%. In contrast, in the VM-negative group, the low expression rate of Vimentin was 15.4% and that of E-cadherin was 74.4% (see Table 1 for details). Through statistical analysis, it was found that there was a significant difference in the expression patterns of E-cadherin in colon cancer cells between the VM-positive group and the VM-negative group ($p < 0.05$), which was statistically significant. This indicated that in colon cancer tissues with VM, epithelial-mesenchymal transition (EMT) was more likely to occur in tumor cells, and the expressed Vimentin and E-cadherin were correlated.

Table 1. Relationship between VM and EMT-tagged protein expression [n(%)]

VM expression rate	Vimentin expression rate		E-cadherin expression rate		p
	+	-	+	-	
+(n=11)	7(63.7)	4(36.3)	2(18.2)	9(81.8)	0.001
-(n= 39)	6(15.4)	33(84.6)	29(74.4)	10(25.6)	

4.3. Expressions of Twist and EMT Proteins in Gastric Cancer Tissues

Among 50 gastric cancer tissue specimens, the number of cases with positive Twist protein expression was 35(70.00%), the number of cases with positive E-cadherin protein expression was 19(38.00%), and the number of cases with positive Vimentin protein expression was [missing data here]. Through statistical analysis, it was found that the expression of Twist protein had a significant difference in gastric cancer tissues ($p = 0.000$) (Table 2).

Table 2. Relationship between Twist and EMT-tagged protein expression [n(%)]

Group	Number of cases	Twist		Vimentin expression rate		E-cadherin expression rate		p
		+(%)	p	+(%)	p	+(%)	p	
gastric cancer	50	35(70.00)	0.000	24(48.00)	0.000	19(38.00)	0.000	0.001

Among the 50 gastric cancer tissue specimens studied, 35 showed positive Twist protein expression, among which 19 cases of laryngeal cancer tissues showed positive E-cadherin protein expression. In addition, among the 15 gastric cancer tissues with negative Twist protein expression, 11 patients showed negative E-cadherin protein expression. Spearman's correlation analysis of these two proteins showed that the positive correlation coefficient between Twist protein and E-cadherin protein expression was 0.544, and the correlation was statistically significant ($p = 0.010$). Furthermore, among the 35 gastric cancer tissues with positive Twist protein expression, 24 showed positive Vimentin protein expression; however, among the 15 gastric cancer tissues with negative Twist protein expression, 12 patients showed negative Vimentin protein expression. Spearman's correlation analysis of these two proteins showed that the positive correlation coefficient between Twist protein and Vimentin protein expression was 0.362, and the correlation was statistically significant ($p = 0.010$).

5. Discussion

The TWIST gene is an important transcription factor and has been proven to play a key role in various cancers, especially having a significant impact on the development of gastric cancer [8]. Moreover, this study showed that TWIST guides gastric cancer cells to undergo epithelial-mesenchymal transition by regulating genes related to cell adhesion, migration, and invasion. Epithelial-mesenchymal transition makes cancer cells lose the unique structures of epithelial cells and acquire the characteristics of mesenchymal cells, thereby enhancing the migration and invasion abilities of the cells. TWIST also participates in facilitating vasculogenic mimicry in gastric cancer tissues, prompting vascular endothelial cells to undergo mimicry changes and exhibit tumor-related angiogenic features [9]. This further supports the growth and nutrient supply of tumors. At the molecular level, TWIST regulates the expression of a series of genes related to the biological characteristics of cancer cells by interacting with other transcription factors and signaling pathways, including key processes such as promoting changes in the cell cycle, inhibiting apoptosis, and enhancing cell migration and invasion [10, 11]. The overexpression of TWIST is closely related to the clinicopathological features, prognosis, and treatment response of gastric cancer. Therefore, the TWIST gene may become an important marker for the prognosis assessment and treatment target of gastric cancer [12]. Future studies can focus on the following directions: deeply explore the cross-regulation mechanism of TWIST in signaling pathways and clarify its interaction with other key factors; explore the potential value of TWIST in the early diagnosis and prognosis prediction of gastric cancer; conduct drug screening studies to find new treatment targets that can interfere with the role of TWIST. As a key regulatory factor in the development of gastric cancer, TWIST has broad application prospects in diagnosis, treatment, and prognosis assessment. In-depth research on the molecular mechanism of TWIST is expected to provide a theoretical basis for the development of targeted TWIST therapeutic drugs and lay a foundation for individualized treatment and precision medicine. The mechanism of action of TWIST in human gastric cancer tissues is complex and diverse, involving multiple aspects such as epithelial-mesenchymal transition and facilitating vasculogenic mimicry. Future research and applications are expected to deepen the understanding of TWIST in gastric cancer and provide strong support for the formulation of relevant treatment strategies.

In conclusion, the TWIST gene participates in the development and progression of tumors in human gastric cancer tissues through multiple mechanisms such as promoting epithelial-mesenchymal transition and facilitating vasculogenic mimicry. A deep understanding of the role of TWIST in gastric cancer is expected to provide new targets and strategies for the treatment of gastric cancer.

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