

# Expression of Twist protein in colorectal carcinoma and its effect on proliferation and invasion of colorectal cancer cells

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**Abstract:** This paper aims to study Twist protein expression in colorectal carcinoma and its effect on the proliferation and invasion of colorectal cancer cells, and to explore its specific mechanism of promoting cancer. Tissues of 20 patients and specimens of 20 normal people were examined by colonoscopy biopsy; the Twist mRNA expression level was detected by real-time quantitative PCR; the RNA interference in colorectal carcinoma cell line CT-26 cells was performed by lipid transfection method; the effects of Twist interference on proliferation and invasion of colorectal cancer cells were observed by CCK8 assay and tran swell assay; the mRNA and protein levels of mRNA in CT-26 cells were detected by RT-PCR and Western blot. The Twist mRNA level in colorectal cancer tissues was significantly higher than that in corresponding para-carcinoma tissues and control group ( $P<0.05$ ); Twist mRNA expression level in the cancer tissues combined with lymph node metastasis was significantly higher than that without lymph node metastasis ( $P<0.05$ ); the proliferation and migration ability of Twist siRNA transfected CT-26 cells were significantly lower than those of the control group ( $P<0.05$ ); CT-26 cells with Twist interference could significantly decrease the mRNA and protein levels of Matrix Metalloproteinase-9 (MMP-9). Twist protein can increase the proliferation and migration ability of the colorectal cancer cells, and its mechanism may be related to MMP - 9.

**Keywords:** Twist, colorectal carcinoma, MMP – 9.

## INTRODUCTION

Colorectal carcinoma is a common malignant tumor of digestive tract. At present, the morbidity of colorectal carcinoma in China ranks third, and its case fatality rate ranks fourth, and the incidence population of this disease gradually tends to be younger (Zhao and Ding, 2014). Therefore, it is of great significance to explore the molecular mechanism of colorectal carcinoma morbidity and metastasis and find out a new therapeutic target.

Twist protein is one of the transcription factors of the basic helix-loop-helix family. A large number of studies have shown that the Twist gene plays an important role in the promotion of epithelial mesenchymal transition (EMT), the involvement of distant metastasis of solid tumors and the formation of adjacent nodes (Qin *et al.*, 2012; Yang *et al.*, 2009; Zhang *et al.*, 2007; Sasaki *et al.*, 2009). Foreign scholar F Valdes-Mor *et al* have had found that the over-expression of Twist is closely related to the lymph node invasion in primary colorectal carcinoma in male patients (Valdés-Mora *et al.*, 2009). However, there is no report on the expression of Twist in patients with colorectal carcinoma and its influence on the proliferation and migration of colorectal cancer cells. Therefore, this study aims to investigate the expression of Twist in colorectal carcinoma tissues and to investigate the molecular mechanism influencing the colorectal carcinoma migration through the low expression of Twist in colorectal carcinoma cell lines *in vitro*.

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## MATERIALS AND METHODS

### General information

All colorectal carcinoma tissues were derived from surgical resection of patients admitted to our hospital from August 2013 to January 2014. Among them, 20 cases were included in the canceration group, with 11 male cases and 9 female cases, aged between 57 years to 73 years old, and average aged ( $62.2\pm 6.6$ ) years old. All the specimens were confirmed by postoperative pathology as colorectal cancer. The colorectal carcinoma tissue and the corresponding para-carcinoma tissue about 2cm to 6 cm to the tumor edge were taken for detection and analysis, respectively. Meanwhile, colorectal mucosa tissues taken from 20 cases at the corresponding period were collected and included as control group, with 12 male cases and 8 female cases, aged between 60-72 years old, average aged ( $63.52\pm 7.53$ ) years old.

The study had received written approval from the Ethics Committee of Huaihe Hospital of Henan University for Clinical Research. All of the patients participated in the study signed the informed consent form.

### The extraction of the total RNA and the reverse transcriptional reaction in the colorectal carcinoma tissue and plasma specimens

About 100mg colorectal carcinoma tissues and corresponding para-carcinoma tissues were taken out and added 1ml Trizol (Invitrogen Corporation, USA) to be fully homogenized, and the extracted total RNA were dissolved in the 30  $\mu$ l DEPC water solution. The extracted

**RT-PCR sequence**

| Names | Forward              | Reverse                 |
|-------|----------------------|-------------------------|
| MMP-9 | CGCAGACATCGTCATCCAGT | GGATTGGCCTTGGGAAGATGA   |
| Twist | CACGCAGTCGCTGAACGA   | GACCTGGTACAGGAAGTCGATGT |
| GAPDH | AACTGGGACGACATGGAGAA | ATACCCCTCGTAGATGGGCA    |

total RNA were quantitatively analyzed by ultraviolet spectrophotometer, and reverse transcribed using the Femantes reverse transcription kit. The production of cDNA was stored at -20°C. Fluorescence quantitative PCR kits was purchased from Roche Company (Switzerland). Real-Time PCR instrument (7300 Type, Applied Biosystems Inc., USA) was used to amplify. And the PCR reaction condition were: denaturation for 20 s at 95°C, then turned to 20 s at 60°C and 1 s at 70°C, with 40 circulations. The relative quantitative analysis results were analyzed with 2<sup>-ΔΔCt</sup> method.

**Cell transfection**

The CT-26 cells of mouse colorectal cancer cells were cultured in complete DMEM medium until the convergence degree reached about 50%-60%. Micro RNA interference was performed on Twist in mouse colorectal cancer cell line CT-26 using lipofectamine™2000 (Invitrogen Corporation, USA). The targeting sequence of the Twist siRNA was 5'-GGUACAUCGACUCCUGU ATT, and the control sequence was 5'-UCAUAAGUGA UGCUGGAGCTT (Yu *et al.*, 2008). After 48h interference, the transfection efficiency was verified by RT-PCR technique.

**Cell migration**

The colorectal carcinoma cells at logarithmic growth phrase were taken out with its density adjusted to 2\*10<sup>5</sup> /ml to 3\*10<sup>5</sup> /ml, and added to the upper layer of transwell chamber according to 0.1ml/well, with the lower chamber added 1 ml 10% serum culture medium. After 24 h culture, the amount of cells that which had already migrated to the lower chamber were counted (×200 times).

**Cell proliferation detection by CCK-8 reagent**

CCK-8 reagent (Shanghai Biological Technology Co. Ltd., China) was used for analysis according to its specification. CT-26 cells in untreated group and transfection group at 24h, 48 h and 72 h were collected, respectively, and added 10% CCK-8 reagent to incubate for 3 h at 37°C incubator. And then the OD value of 450 nm was detected.

**The detection of protein level by immunoblotting**

The collected cells were added 1×SDS cell lysis buffer to proceed with SDS-PAGE electrophoresis and 110v Transmembrane for 100 min, and then sealed at 37°C. After that, the Rabbit Anti-Human MMP-9 (Biolegend) were added and incubated over night at 4°C, and then incubated at HRP-labelled Mouse-Anti-Rabbit second antibody (NanJing Sun Shine Biotechnology Co., LTD,

China) (1:1000) for 50 min at 37°C. The anti-human β-actin (sigma company, USA) (1:5000) was used as the reference protein. After incubation, PBS-T was used to wash the membrane for 3 times and to detect the electrochemiluminescence (ECL) emission.

**STATISTICAL ANALYSIS**

SPSS19.0 statistical software was used to analyze all the data. The measurement data were expressed as mean ±s standard deviation, which are tested by normality test. The comparison between the two groups were tested by two kinds of independent samples t test, and the multi-comparison between groups were tested by the single-factor analysis variance + two multiple-comparisons (LSD-t test). The comparison of multi temporal data is a single factor analysis of variance. P<0.05 indicated that the difference was statistically significant.

**RESULTS**

**Twist mRNA expression level in the colorectal carcinoma tissue and corresponding para-carcinoma tissue**

The overall comparison of Twist mRNA expression level among the three groups were significantly different (P<0.05). Then the comparison were compared between each two groups, with the results: Twist mRNA expression level in the corresponding para-carcinoma tissues of colorectal cancer was significantly higher than that in the normal mucosa, and the difference was statistically significant (P<0.05); Twist mRNA expression level in the colorectal carcinoma tissues was much higher than that of corresponding para-carcinoma tissues, with statistical significance (P<0.05), which indicating that Twist might be involved in the formation of the colorectal carcinoma tumor. As shown in table 1.

**The relationship among twist mRNA expression and the lymph node metastasis and the duckes stages**

Among all the patients, there was no lymph node metastasis in 8 patients (Dukes A/B stage), and lymph node metastasis was found in 12 patients (Dukes C/D stage). The expression level of Twist mRNA in Dukes C/D stage was significantly higher than that of Dukes A/B stage, and the difference was statistically significant (P<0.05), suggesting that Twist expression in tissues with lymph node metastasis was significantly higher than that without lymph node metastasis, further indicating that Twist played a positive role in promoting the development of colorectal carcinoma. As seen in table 2.

**Table 1:** Twist mRNA expression level in the colorectal carcinoma tissue and para-carcinoma tissue

| Groups                               | n        | Twist mRNA     |
|--------------------------------------|----------|----------------|
| A: The normal tissue                 | 20       | 1.24±0.41      |
| B: Para-carcinoma tissue group       | 20       | 2.41±0.57      |
| C: Colorectal carcinoma tissue group | 20       | 5.25±1.22      |
| Overall analysis                     | F, P     | 128.590, 0.000 |
| B vs A                               | LSD-t, P | 4.547, 0.000   |
| C vs A                               | LSD-t, P | 15.592, 0.000  |
| C vs B                               | LSD-t, P | 11.045, 0.000  |

**Table 2:** The relationship between Twist mRNA expression level relationship and the lymph node metastasis

| Lymphatic metastasis | n  | Twist mRNA | Comparison t, P |
|----------------------|----|------------|-----------------|
| Yes (Dukes A/B)      | 8  | 1.29±0.31  |                 |
| No (Dukes C/D)       | 12 | 3.01±0.51  | 8.505, 0.000    |

**Note:** the pairwise comparison used independent samples t-test

**Table 3:** The effect of lower expression Twist on colorectal carcinoma cells proliferation and invasion (n = 6)

| Groups             | n        | Twist mRNA    | OD450 nm (fig.1B) |              |              |               | Number of cells |
|--------------------|----------|---------------|-------------------|--------------|--------------|---------------|-----------------|
|                    |          | (fig.1A)      | T0:0h             | T1:24h       | T2:48h       | T3:72h        | (fig.1C)        |
| A: Blank           | 6        | 1.03±0.18     | 1.12±0.06         | 1.41±0.11    | 2.27±0.16    | 5.92±0.18     | 34.0±2.6        |
| B: Control         | 6        | 0.98±0.19     | 1.14±0.06         | 1.52±0.17    | 2.52±0.51    | 6.18±0.61     | 32.6±4.6        |
| C: siRNA           | 6        | 0.35±0.06     | 1.09±0.05         | 1.20±0.16    | 1.83±0.40    | 3.25±0.41     | 18.9±1.8        |
| Overall comparison | F, P     | 35.556, 0.000 | 1.213, 0.325      | 7.136, 0.007 | 4.848, 0.024 | 82.439, 0.000 | 35.556, 0.000   |
| B vs A             | LSD-t, P | 0.588, 0.565  | 0.474, 0.642      | 1.269, 0.224 | 1.148, 0.269 | 1.013, 0.327  | 0.588, 0.565    |
| C vs A             | LSD-t, P | 7.579, 0.000  | 1.048, 0.311      | 2.448, 0.027 | 1.932, 0.072 | 10.579, 0.000 | 7.579, 0.000    |
| C vs B             | LSD-t, P | 6.991, 0.000  | 1.522, 0.149      | 3.716, 0.002 | 3.081, 0.008 | 11.592, 0.000 | 6.991, 0.000    |

**Note:** the overall analysis used the single- factor variance analysis, and the pairwise comparison used LSD test.

**Table 4:** The influence of the lower expression of Twist on MMP-9 mRNA and protein levels in the CT-26 cells (n=6)

| Groups             | n        | MMP-9 mRNA    |
|--------------------|----------|---------------|
| A: Blank           | 6        | 1.14±0.22     |
| B: Control         | 6        | 1.02±0.20     |
| C: siRNA           | 6        | 0.31±0.04     |
| Overall comparison | F, P     | 39.381, 0.000 |
| B vs A             | LSD-t, P | 0.607, 0.553  |
| C vs A             | LSD-t, P | 7.971, 0.000  |
| C vs B             | LSD-t, P | 7.365, 0.000  |

**Note:** the overall comparison was analyzed with one-way variance, and the pairwise comparison with LSD test

**The effect of Twist siRNA on CT-26 cells proliferation and invasion**

The effects of siRNA interference on the proliferation and invasion of CT-26 cells were shown in the table 3.

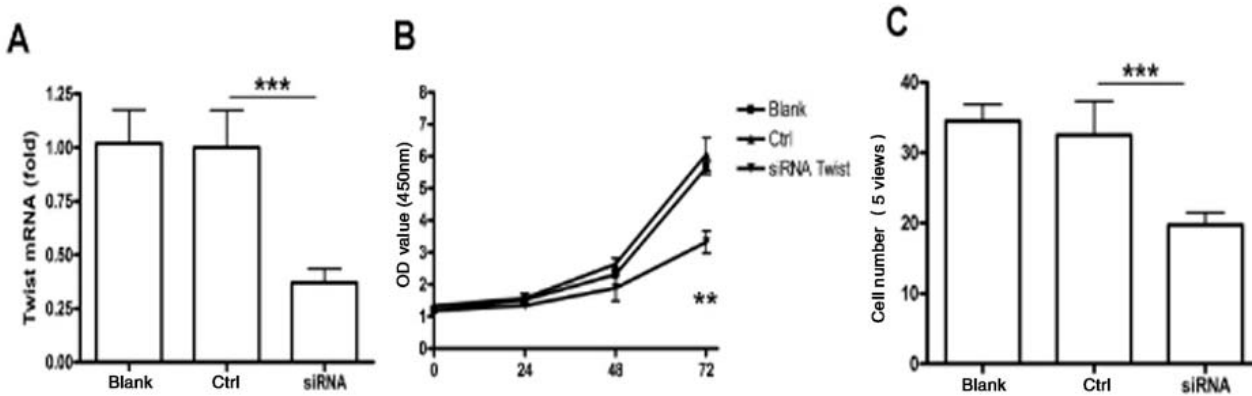
**The overall analysis**

After the transfection of CT-26 cells, the difference of Twist siRNA levels were significant different between the 3 groups (P<0.05); CCK8 assay showed that there was significant difference of OD value of 450nm after 24 h between the three groups (P<0.05); the differences of the

mean value of the cell count of 5 visual field were also statistically significant (P<0.05). Then the detailed comparison were conducted as follows.

**Pairwise comparison**

Twist mRNA level was significantly reduced after CT-26 cells transfected with Twist mRNA (fig.1A), indicating the success of the interference. The findings of CCK8 method showed that: compared with the control group, the proliferation ability of CT-26 cells interfered with Twist gene after 72 h was significantly lower (fig. 1B),



**Fig. 1:** The affect of Twist low expression on proliferation and invasion of colorectal cancer cells (n = 6), compared with the control group, P<0.000

and the difference was statistically significant (P<0.05), suggesting Twist might play a positive role in the proliferation of colorectal carcinoma cells. The further experiment showed that after CT-26 cells transfected with Twist siRNA, the number of cells in the lower layer of tran swell chamber was significantly reduced (fig.1C), and the difference was statistically significant (P<0.05), suggesting Twist could promote the invasion of colorectal carcinoma cells.

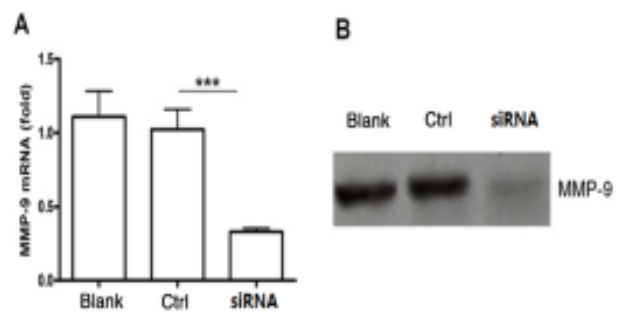
In addition, the results of CCK8 assay about the CT-26 cells transfected with Twist siRNA were analyzed by multiple times point repeatedly, and there were significant differences among different time points (P<0.05). According to the data, although the OD value increased significantly with time, it was still much lower than that of control group, only about half of it, which further verified that Twist played a positive role in the proliferation of colorectal carcinoma cells.

**The effect of twist interference CT-26 cells on protein expression of mRNA and MMP-9**

In order to investigate the mechanism of Twist gene on migration ability of CT-26 cells, the MMP-9 gene and protein levels in CT-26 cells after interfering with Twist were further detected. The findings showed compared with control group, the levels of MMP-9 mRNA (fig. 2A) and protein (fig. 2B) in CT-26 cells interfered with Twist were significantly decreased, which indicated that Twist might affect the migration of colorectal carcinoma cells by promoting the MMP-9 expression. As shown in the table 4.

**DISCUSSION**

Colorectal carcinoma is one of the most common malignant tumors in the digestive tract. In recent years, the incidence and mortality of colorectal cancer are increasing year by year in the world (Brenner *et al.*, 2014). Therefore, it is of great clinical significance to study the cytological and molecular mechanism of pathogenesis of colorectal carcinoma for its prevention and treatment.



**Fig. 2:** The influence of the lower expression of Twist on MMP-9 mRNA and protein levels in the CT-26 cells (n = 6)

Twist is the first gene to be found in fertilized eggs of Drosophila. Due to the fact that the development of Drosophila embryos without Twist gene shows a “distorted” phenomenon, presenting as the formation of gastrula is blocked, and the development of mesoderm disordered, leading to the final death of embryonic development. Thus, this gene is named as “Twist” (Simpson, 1983). In recent years, more and more studies have suggested that Twist gene is involved in the regulation of the epithelial - mesenchymal transition (EMT), which plays an important role in the development and invasion and metastasis of ovarian cancer, pancreatic cancer and other tumor cells (Roesch, 2014; Wang *et al.*, 2014; Nuti *et al.*, 2014). However, the expression of Twist in colorectal carcinoma and its effect on the proliferation and invasion of colorectal cancer cells are not fully understood

This study had found that Twist mRNA level in colorectal carcinoma tissues and para-carcinoma tissues were significantly higher than that in normal mucosa tissues, and the expression of Twist was higher in cancer tissues. What’s more, the expression of Twist in carcinoma tissues with lymph node metastasis was higher than that without lymph node metastasis, which suggested that Twist had a potential role in promoting the development of colon

cancer and colon cancer metastasis. And the findings were consistent with the high expression of Twist in human esophageal squamous cell carcinoma and involved in cancer metastasis, which was studied by Sasaki, K *et al* (Sasaki *et al.*, 2009). In order to further observe the influence of Twist on the proliferation and migration of colorectal carcinoma cells, we attenuated the expression of Twist in colorectal cancer cell line CT-26 by siRNA interference, and the findings showed that the proliferation and migration ability of colorectal carcinoma cells were reduced correspondingly after the low expression of colorectal cancer cells, indicating that Twist played an important role in the proliferation and metastasis of colon cancer cells. One of the important steps in the invasion and metastasis of malignant tumor was the degradation of tumor cells to matrix and MMP-9 had been showed to be involved in the invasion and metastasis of various tumors (Xu *et al.*, 2013; Li *et al.*, 2013; Hsin *et al.*, 2013). In order to further explore the mechanism of Twist affecting the migration of colorectal cancer cells, we detected the expression of MMP-9 after siRNA interference with Twist, and the results showed that low-expression of Twist could significantly inhibit the expression of MMP-9, indicating that Twist could promote the migration of cancer cells by promoting the expression of MMP-9, which was consistent with the study of Cuixiang L *et al* that high-expression of Twist in human peritoneal mesothelial cells could increase cell migration (LiC *et al.*, 2012).

## CONCLUSION

In conclusion, Twist may increase the migration of cancer by promoting the expression of MMP-9, which plays an important role in the occurrence and development of colorectal carcinoma. The detection of Twist in clinical diagnosis of colorectal cancer may have some guiding significance.

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