

# Upregulation of Twist in Gastric Carcinoma Associated with Tumor Invasion and Poor Prognosis

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**Abstract** Tumor invasion and metastasis are the most common causes of death in gastric carcinoma. Twist, a transcription factor of the basic helix-loop-helix class, reportedly regulates cancer metastasis and induces epithelial-mesenchymal transition (EMT). We evaluated the expression of Twist and its effect on cell migration in gastric carcinoma (GC). Twist expression was detected by real-time quantitative RT-PCR in gastric tumor tissue, metastatic lymph nodes and normal gastric tissue from 47 gastric carcinoma patients who had undergone gastrectomies with radical lymph node dissections without preoperative treatment. Twist expression was also analyzed immunohistochemically in 436 gastric cancer cases. GC tumor tissue and metastatic lymph nodes was upregulated compared with normal gastric mucosa ( $P < 0.05$ ). Twist protein expression differed significantly among gastric tumor tissue, matched normal gastric mucosa, and lymph nodes ( $P < 0.05$ ). In stages I, II, and III, 5-year survival rates of patients with high Twist expression were significantly lower than in patients with low expression ( $P < 0.05$ ). In stage IV, Twist expression did not correlate with 5-year survival rates ( $P = 0.07$ ). Further multivariate analysis suggested that depth of invasion,

lymph-node and distant metastases, TNM stage, and up-regulation of TWIST were independent prognostic indicators for GC. Twist expression in gastric cancer is associated significantly with lymph-node and distant metastases, and poor prognosis. Twist may be a useful marker for the development, progression and metastasis of GC.

**Keywords** Gastric carcinoma · Metastasis · RT-PCR · Twist expression

## Introduction

Although the global incidence of gastric cancer has decreased in recent years, its mortality rate in China is the highest among all tumors and represents 25% of gastric cancer mortality worldwide. Despite advances in treatment, the overall 5-year survival rate in China is only 40%. Most gastric cancer is diagnosed at stage III or IV, and the lymph node metastasis rate is higher (50–75%) [1].

Metastasis is a complex process [2]. Numerous genes and proteins involved in essential roles during embryonic development are reportedly mutated or aberrantly expressed in different cancers [3–5]. Epithelial-mesenchymal transition (EMT) is a characteristic of the most aggressive metastatic cancer cells [6–8]. Cells that undergo EMT morphogenesis switch from an apical-basolateral, polarized epithelial phenotype to a spindle-shaped, fibroblast-like mesenchymal phenotype. In their natural state, epithelial cells exist as tight cell clusters that maintain cell–cell or cell–matrix contacts, while mesenchymal cells are loosely organized, unpolarized cells with reduced adhesion and enhanced migratory tendencies. A key feature in the initiation and execution of EMT is downregulated E-cadherin expression [6, 9]. Several mechanisms that

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downregulate E-cadherin expression were recently reported [10–12].

Twist, a transcriptional factor involved in E-cadherin downregulation, plays a crucial role in metastasis. Microarray analysis shows Twist to be predominantly expressed in metastatic cancers [13–15]. Twist-enhanced cancer metastasis occurs in breast cancer, gastric cancer, and hepatocellular carcinoma [14, 16, 17].

Therefore, we conducted this study to determine the prognostic value of Twist expression. We used tissue microarrays to ascertain whether Twist expression was associated with degrees of tumor invasiveness and metastasis.

## Materials and Methods

### Real-Time Quantitative Reverse-Transcription Polymerase Chain Reaction Analysis of TWIST Gene Expression

Total RNA was isolated from frozen gastric cancer tissues ( $n=47$ ) and matched normal tissues and lymph node metastasis ( $n=47$ ) (obtained from the Zhejiang Provincial People's Hospital, China) using Trizol reagent according to the manufacturer's protocol and cDNAs were reverse-transcribed by RevertAid reverse transcriptase. Real-time polymerase chain reaction was carried out using the ABI PRISM 7700 Sequence Detection System (Applied Biosystems) at 50°C for 2 min, 95°C for 10 min, followed by 50 cycles at 95°C for 15 s and at 60°C for 1 min. The primers for GAPDH (224 bp) were 5'-TGAAGTTCG GAGTCAACGG-3' (sense) and 5'-CTGGAAGATGGT GATGGGATT-3' (antisense). The primers for Twist (153 bp, Invitrogen Biotechnology Co.Ltd) were 5'-GGGACAGCAGTGACATCGG-3' (sense) and 5'-CCCAAACATAAGACCCAGAAGAA-3' (antisense). The expression of GAPDH was used to normalize that of the target genes. Each assay was done in triplicate, the average was calculated, and the expression level of Twist was expressed as,  $2^{-\Delta\Delta Ct}$ ,  $\Delta Ct = Ct(TWIST) - Ct(GAPDH)$ .

### Gastric Cancer Tissue Microarray Construction

Gastric cancer tissues were collected from gastrectomy specimens from 436 patients (311 male, 125 female; median, age 60.0 years; age range, 30–91 years) from the Department of Surgery, Zhejiang Provincial People's Hospital, from January 1998 to January 2004. Tissues had been formalin-fixed, paraffin-embedded, and diagnosed clinically and histopathologically at the Departments of Gastrointestinal Surgery and Pathology. All patients had

follow-up records for more than 5 years. The follow-up deadline was December 2008. The survival time was calculated from the date of surgery to the follow-up deadline or date of death. Death was caused mainly by carcinoma recurrence or metastasis. Based on the Lauren classification, 223 cases were intestinal and 213 cases were diffuse gastric cancer. There were 55, 163, and 218 cases from the cardia, body, and antrum, respectively. According to the 2002 WHO histological classification of gastric carcinoma, there were 326 tubular, 16 papillary, 29 mucinous, 65 signet-ring cell; 13 highly differentiated, 128 well- or moderately differentiated, 293 poorly differentiated, and 2 undifferentiated adenocarcinomas. There were 61 cases with distant metastases. Ninety cases were categorized as stage I, 104 as stage II, 173 as stage III and 69 as stage IV. Ninety-two noncancerous human gastric tissues were obtained from gastrectomy of adjacent gastric cancer margins greater than 5 cm. Routine chemotherapy was given to patients with advanced-stage disease following surgery, but no radiation treatment was administered to any of the patients included in our study. Array blocks containing a total of 528 cases were prepared as described previously [18, 19]. Core tissue biopsies (2 mm in diameter) were taken from individual paraffin-embedded gastric tumors (donor blocks) using a trephine and arranged in recipient paraffin blocks (tissue array blocks). Staining results from different intratumoral areas in various tumors show reliable consistency [20], therefore, a single core was sampled in each case. An adequate sample was defined as tumor occupying 10% of the core area [21]. Each block contained more than seven non-neoplastic gastric mucosa internal controls. Four-micrometer-thick sections were cut from each tissue array block, deparaffinized and dehydrated.

### Immunohistochemistry

Immunohistochemical analysis was performed to study altered protein expression in 92 non-cancerous human gastric tissue samples and 436 human GC tissues [22, 23]. Briefly, slides were baked at 60°C for 2 h, followed by deparaffinization with xylene, and then rehydrated. The sections were submerged in EDTA antigenic retrieval buffer, microwaved for antigen retrieval, treated with 3% hydrogen peroxide in methanol to quench endogenous peroxidase activity, followed by incubation with 1% bovine serum albumin to block non-specific binding. Sections were incubated with mouse anti-Twist(Dako USA) overnight at 4°C. Normal goat serum was used as a negative control. After washing, tissue sections were treated with secondary antibody. Tissue sections were then

counterstained with hematoxylin, dehydrated, and mounted.

### Evaluation of Results

Stained Twist was buff-colored in the cytoplasm and nucleus. The degree of immunostaining was reviewed and scored independently by two observers, based on the intensity of staining [24–26]. Staining intensity was graded by the following criteria: 0 (no staining), 1 (weak staining = light yellow), 2 (moderate staining = yellow brown), and 3 (strong staining = brown). Moderate and strong staining indicated tumors with high Twist expression; no and weak staining indicated low Twist expression.

### Statistical Analysis

All statistical analyses were performed using SPSS 12.0 software. Data were analyzed using the  $\chi^2$  or Fisher's exact test and the Wilcoxon test. Survival curves were estimated using the Kaplan–Meier method, and the log-rank test was used to calculate differences between the curves. Multivariate analysis using the Cox proportional hazards regression model was performed to assess prognostic values of protein expression. Correlation coefficients between protein expression and clinicopathological findings were estimated using the Pearson correlation method. Statistical significance was set at  $P < 0.05$ .

## Results

### Upregulation of Twist mRNA in Gastric Tumor Tissue and Lymph Nodes

Twist was over-expressed in 47 gastric tumor tissue samples, and matched lymph nodes. In 47 gastric tumor tissue samples, matched normal gastric mucosa and lymph node metastases, the average expressions were  $4.269 \pm 3.245$ ,  $1.925 \pm 0.865$ , and  $21.338 \pm 36.443$ , respectively; there were significant differences among these three groups ( $P < 0.05$ , Wilcoxon test).

### Expression of Twist in Archived Gastric Tissue Samples and Non-Tumor Mucosa

Twist protein was not detected in human non-tumor mucosa samples, but was detected in 322 of 436 (73.9%) cases of human gastric cancer; high expression of Twist protein was detected in 187 (42.9%) tumors. Twist was localized mainly in the cytoplasm or nucleus of primary cancer and metastatic cells (Fig. 1).

### Correlation Between Twist Up-Regulation and Clinical Features of Gastric Cancer

High expression of Twist correlated with age, tumor location, size, depth of invasion, lymph node and distant metastases, Lauren's classification, regional lymph node stage and TNM stage ( $P < 0.05$ ) (Table 1). Twist expression did not correlate with sex, differentiation, or histological classification ( $P > 0.05$ ) (Table 1).

### Correlation Between Twist Expression and Patient Prognosis

In stage I, II and III tumors, the 5-year survival rate in patients with high expression of Twist was significantly lower than that in patients with low expression ( $P < 0.05$ ). In stage IV tumors, Twist expression did not correlate with the 5-year survival rate ( $P > 0.05$ ) (Fig. 2).

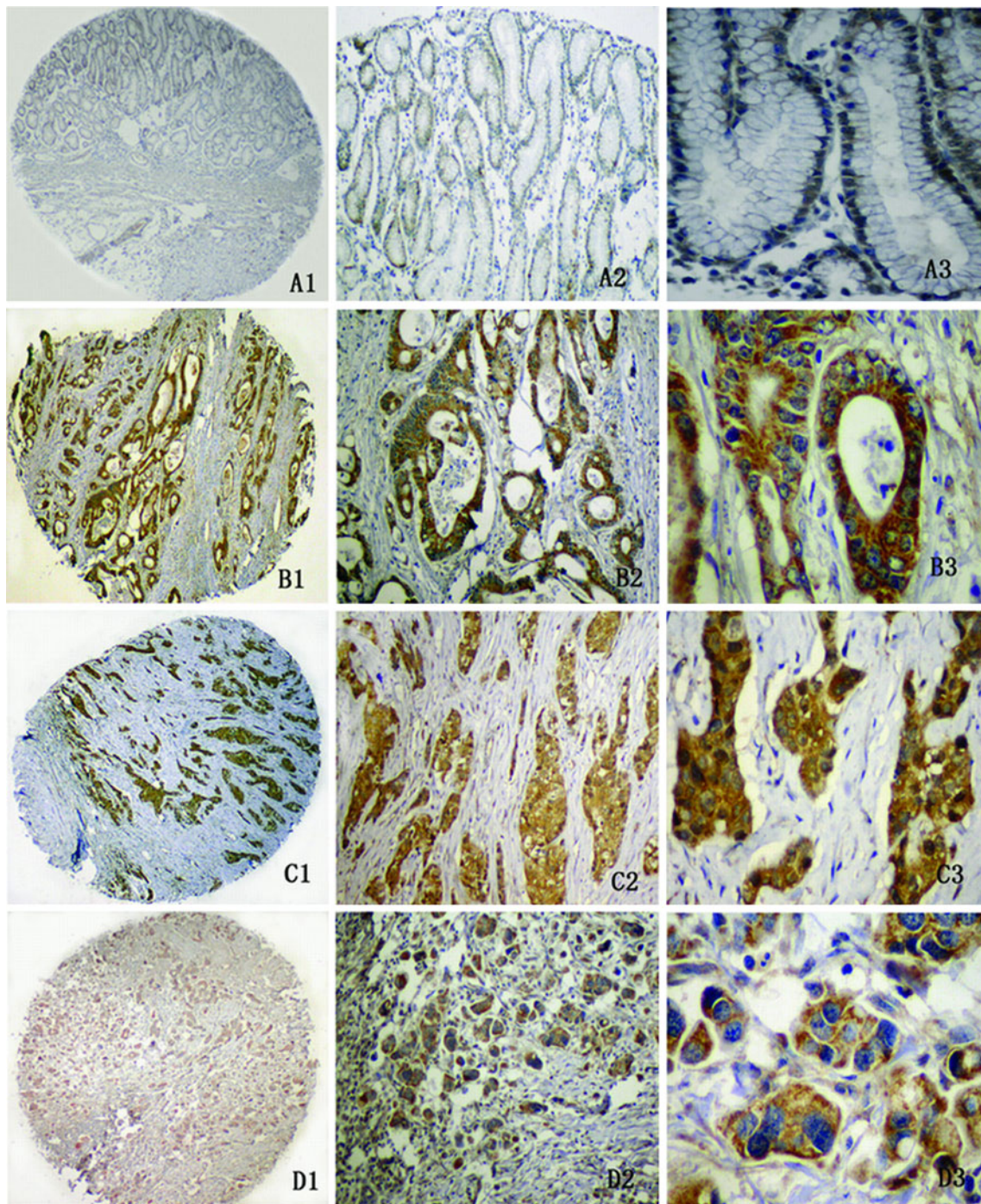
### Multivariate Analysis of Clinicopathological Parameters and Prognosis

Factors with possible prognostic effects in gastric carcinoma were analyzed by Cox regression analysis. The study revealed that lymph node and distant metastases ( $P = 0.005$ ), TNM stage ( $P = 0.005$ ), and Twist expression ( $P = 0.000$ ) are independent prognostic factors in patients with gastric carcinoma. However, age, sex, tumor location and size, histological classification, tumor differentiation, and Lauren's classification, had no prognostic value.

## Discussion

Twist is a transcription factor involved in the regulation of cell movement and tissue reorganization during early embryogenesis [27, 28]. In keeping with the hypothesis that carcinogenesis reflects an embryonic phenotype, Twist has been described as an oncogene and is involved in different oncogenic pathways such as inhibition of cell safeguard programs [29, 30], alteration of cell–cell adhesion, and deregulation of differentiation [31], leading both to cell survival and invasion capability.

The expression pattern of Twist in human tumors and its association with clinical parameters has been extensively investigated. Statistical analysis shows that Twist overexpression has important clinical implications as a prognostic and predictive factor in different tumor types. Increased Twist expression is usually associated with poor prognosis, and specifically, with metastasis and shorter patient survival.



**Fig. 1** Immunohistochemical staining for TWIST in gastric cancer lesions and noncancerous tissues. A1 to A3, TWIST negative in noncancerous tissues; magnifications were  $\times 40$ ,  $\times 100$ , and  $\times 400$ , respectively. B1 to B3, TWIST was highly expressed in tubular adenocarcinoma; magnifications were  $\times 40$ ,  $\times 100$ , and  $\times 400$ , respectively. C1 to C3,

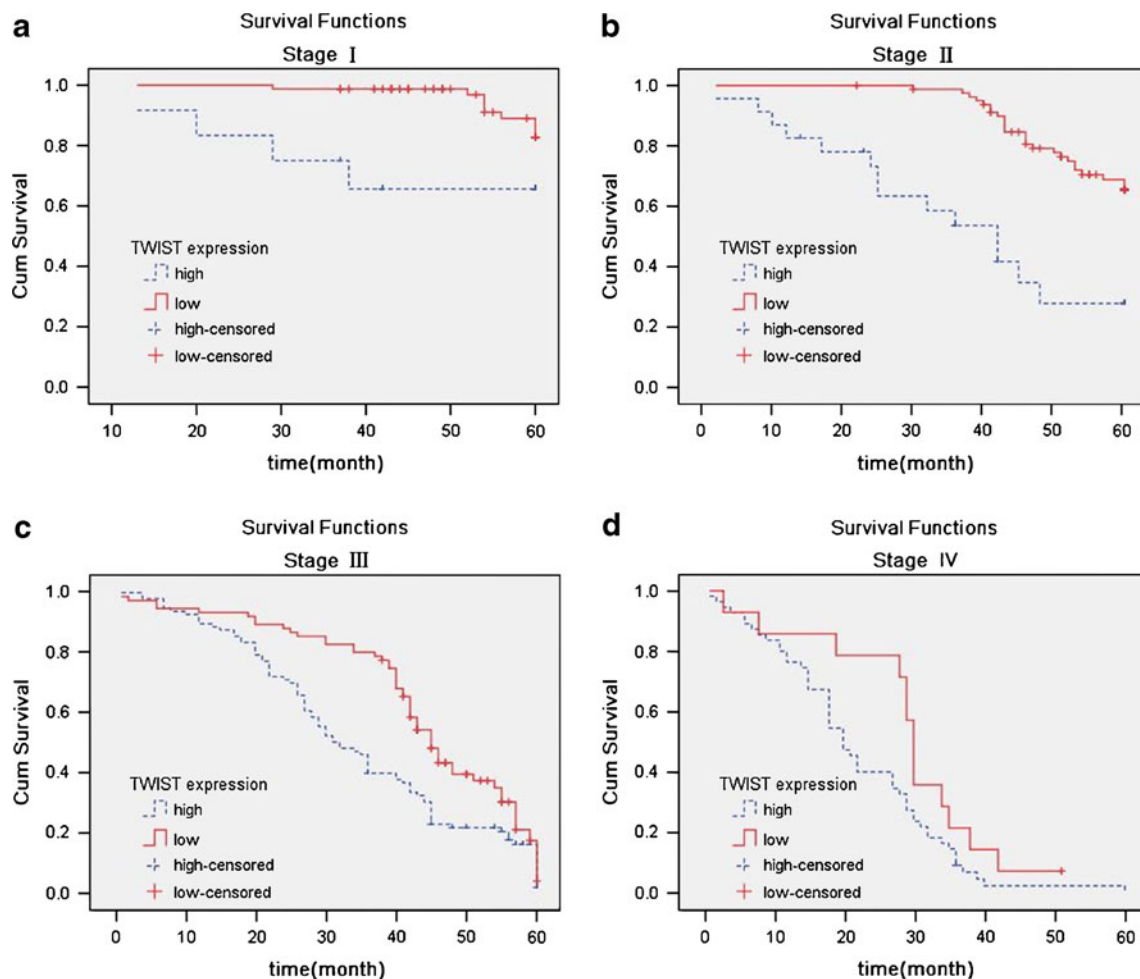
TWIST was highly expressed in poorly differentiated adenocarcinoma; magnifications were  $\times 40$ ,  $\times 100$ , and  $\times 400$ , respectively. D1 to D3, TWIST was highly expressed in Signet-ring cell carcinoma; magnifications were  $\times 40$ ,  $\times 100$ , and  $\times 400$ , respectively

We demonstrated that Twist mRNA and protein levels in gastric carcinoma specimens were both increased compared with non-neoplastic gastric epithelium. Another study demonstrated up-regulation of Twist in a diffuse-type gastric carcinoma in contrast to a well-differentiated

intestinal carcinoma [32]. Our results indicated that positive expression of Twist was correlated with tumor location and size, differentiation, depth of invasion, lymph node and distant metastases, Lauren's classification and TNM stage. We further analyzed the association between Twist expres-

**Table 1** Relationship of TWIST expression with pathological parameters of tumor

Clinical parameters	TWIST		t/ $\chi^2$	P
	Low	High		
Age(yrs)	57.50±10.92	62.13±11.95	4.193	0.000
Gender			0.915	0.491
Male	177(56.9%)	134(43.1%)		
Female	72(57.6%)	53(42.4%)		
Location			13.187	0.001
Proximal	20(36.4%)	35(63.6%)		
Middle	91(55.8%)	72(44.2%)		
Distal	138(63.3%)	80(36.7%)		
Size			25.709	0.000
<5 cm	172(67.2%)	84(32.8%)		
≥5 cm	77(42.8%)	103(57.2%)		
Lauren classification			116.032	0.000
Intestinal	183(82.1%)	40(17.9%)		
Diffuse	66(31.0%)	147(69.0%)		
Histology classification			2.242	0.155
Papillary adenocarcinoma	12(75.0%)	4(25.0%)		
Tubular adenocarcinoma	186(54.9%)	140(45.1%)		
Mucinous adenocarcinoma	12(41.4%)	17(58.6%)		
Signet-ring cell carcinoma	39(60.0%)	26(40.0%)		
Histologic differentiation			2.668	0.102
Well	12(92.3%)	1(7.7%)		
Moderately	74(61.7%)	54(38.3%)		
Poorly	161(54.4%)	132(45.6%)		
Others	2(100%)	0(0.0%)		
Invasion depth			48.285	0.000
T1	48(84.2%)	9(15.8%)		
T2	76(69.7%)	33(30.3%)		
T3	121(49.6%)	123(50.4%)		
T4	4(15.4%)	22(84.6%)		
TNM Stages			100.881	0.000
I	78(86.7%)	12(13.3%)		
II	81(77.9%)	23(22.1%)		
III	76(43.9%)	97(56.1%)		
IV	14(20.3%)	55(79.7%)		
Lymphatic metastasis			32.590	0.000
No	113(76.4%)	35(23.6%)		
Yes	84(45.4%)	101(54.6%)		
Regional lymph nodes			84.846	0.000
PN0	133(80.1%)	33(19.9%)		
PN1	77(56.6%)	59(43.4%)		
PN2	35(35.4%)	64(64.6%)		
PN3	4(11.4%)	31(88.6%)		
Distant metastasis			27.611	0.000
No	233(62.1%)	142(37.9%)		
Yes	16(13.1%)	45(86.9%)		



**Fig. 2** Kaplan-Meier curves with univariate analyses (log-rank) for patients with simultaneously low TWIST expression versus high TWIST expression tumors. a: in stage I gastric cancer ( $p < 0.05$ ). b: in

stage II gastric cancer ( $p < 0.05$ ). c: in stage III gastric cancer ( $p < 0.05$ ). d: stage IV gastric cancer ( $p = 0.07$ )

sion and regional lymph node stage, and showed that Twist expression was correlated significantly with regional lymph nodes stage. Upregulation of Twist and its prognostic value have been described in several human cancers. In human breast cancer, increased Twist expression has been shown to correlate with metastasis development and shorter survival [33]. Increased Twist expression has also been suggested to associate with poor outcome and shorter patient survival in patients with melanoma [34]. Therefore, we analyzed the association between Twist expression and prognosis of gastric cancer according to TNM stage. We showed that in stage I, II and III tumors, the 5-year survival rate of patients with high expression of Twist were significantly lower than that in patients with low expression. The factors with possible prognostic effects in gastric carcinoma were analyzed by Cox regression analysis. We revealed that lymph node and distant metastases, TNM stage, and expression of Twist were independent prognostic factors in patients with gastric carcinoma. These results suggest that Twist could be used as a prognostic marker in

predicting development of distant metastasis in patients with gastric carcinoma.

The mechanisms that produce Twist overexpression remain unknown. Previous reports indicate that Twist is regulated by Wnt/ $\beta$ -catenin signaling in mouse mammary cell differentiation; this effect could contribute to mammary carcinogenesis [35]. In addition, the ectopic expression of Frzb/sFRP3, a secreted Wnt antagonist, resulted in decreased expression of Twist in PC-3 cells [36]. Transfection of sLRP5, a dominant negative factor of Wnt/LRP5 signaling, produced downregulation of Twist in osteosarcoma Saos-2 cells [37]. Furthermore, 70–80% of colorectal cancers have defects in the Wnt pathway [38], which implies that Twist could be regulated by this pathway.

In conclusion, increasing evidence has demonstrated that Twist is an oncogene in multiple tumor types, and is associated in most of them with a more aggressive phenotype, possibly via its role in EMT regulation. Our study demonstrated that high levels of Twist expression in primary tumors may further promote the development of

metastasis; and Twist might be a useful prognostic marker for predicting development of distant metastasis in gastric carcinoma.

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