Available online at www.tox.or.kr

Immunohistochemical Analysis of TBX3 and β-catenin in Gastric Cancers

Jae Hwi Song[1], **Jung Hwan Yoon**[1], **Young Hwi Kang**[1], **Zhang Cao**[1], **Suk Woo Nam**[1], **Jung Young Lee**[1] **& Won Sang Park**[1]

[1]Department of Pathology, College of Medicine, The Catholic University of Korea, 505 Banpo-dong, Seocho-gu, Seoul 137-701, Korea
Correspondence and requests for materials should be addressed to W. S. Park (wonsang@catholic.ac.kr)

Accepted 19 November 2009

Abstract

TBX3 has demonstrated oncogenic activity as a downstream target of the Wnt/β-catenin signaling pathway. In this study, the aim was to determine whether overexpression of the TBX3 protein is involved in the development and/or progression of gastric cancers. We analyzed the expression pattern of the TBX3 and β-catenin proteins in a series of 186 sporadic gastric cancers. Altered expression of the TBX3 and β-catenin proteins was observed in 54 (29.0%) and 48 (25.8%) of the 186 gastric cancers. Statistically, overexpression of the TBX3 and β-catenin proteins was not associated with the clinical and pathological parameters studied including: histological type, tumor location, tumor size, and the 5-year survival ($P > 0.05$). However, TBX3 overexpression was closely associated with lymph node metastasis and aberrant β-catenin expression ($P < 0.05$). In addition, overexpression of the TBX3 protein was confirmed by Western blot analysis of primary gastric cancer tissues and cell lines. These data suggest that TBX3 overexpression may play a role in the development and progression of sporadic gastric cancers.

Keywords: TBX3, Wnt signaling pathway, Gastric cancer, Expression

Gastric cancers occur with a high frequency in Asia; they are one of the leading causes of cancer deaths worldwide[1]. In Korea, gastric cancer accounts for an estimated 20.7% of all malignancies, 24.2% in males and 16.2% in females[2]. Therefore, gastric cancer remains a significant health burden worldwide. Surgical removal is the treatment of choice in patients with advanced gastric cancer, whereas endoscopic mucosal resection can be curative in cases with early gastric cancer. Detection and removal of gastric cancer at an early stage is likely to reduce the associated mortality. Although the clinical outcome of gastric cancer has gradually improved, the prognosis of patients with advanced stage disease is still poor. Thus, a better understanding of the biology and development of molecular testing for early detection of advanced gastric cancer is crucial to attempt to improve patient outcomes.

β-catenin is a multifunctional protein that plays an important role in the transduction of Wnt signals and in intercellular adhesion by linking the cytoplasm domains of cadherin[3]. In general, the cytoplasm levels of β-catenin are kept low by interaction with protein complexes that include: adenomatous polyposis coli (APC), axin, protein phosphatase 2A, and glycogen synthase kinase 3β. It is believed that such complexes phosphorylate β-catenin, thereby inducing the ubiquitination-dependent proteolysis of β-catenin. The alteration of these genes results in the accumulation of cytoplasm and nuclear translocation of β-catenin. There are several lines of evidence that suggest that the Wnt signaling pathway related genes are involved in human cancer development and/or progression[4]. In cancer cells, only one of these genes is mutated in a given tumor sample; this finding suggests a common pathway for the activation of these genes[5]. For example, colon tumors with mutations in the *APC* gene have a wild-type *β-catenin* gene, and vice versa, tumors with mutations in the *β-catenin* gene have a wild-type *APC*. Most of the *β-catenin* mutations are activating mutations that occur at one of four phosphorylation sites in exon 3[4,6]. In addition, aberrant cytoplasm overexpression or the nuclear accumulation of β-catenin is frequently found in sporadic gastric cancers, suggesting that constitutive activation of the Wnt canonical pathway contributes to human gastric carcinogenesis[7]. Several target genes upregulated by β-catenin have been identified including *c-Myc* and *cyclin D1*, which

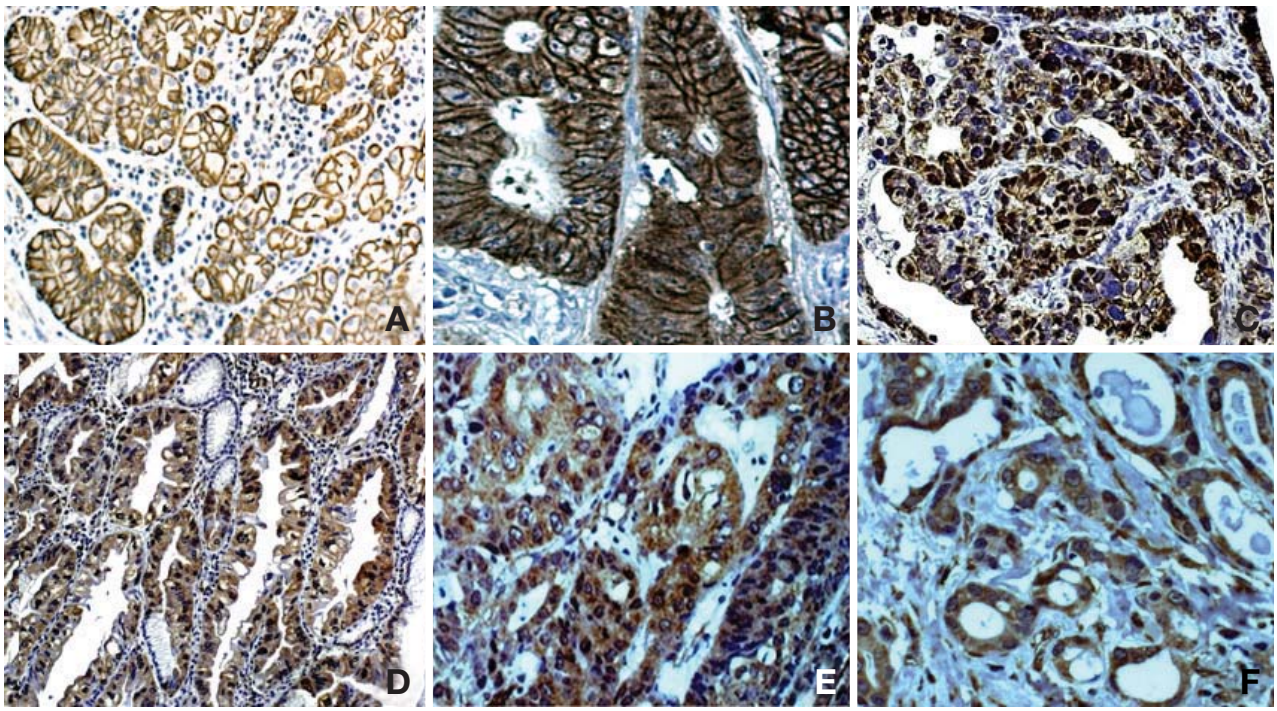

Figure 1. Representative photomicrographs of immunohistochemical staining of gastric tissue, demonstrating expression of β-catenin (A, B, and C) and TBX3 (D, E, and F) proteins. A, normal mucosa; B, C, E, F, gastric cancer; D, gastric adenoma adjacent to gastric cancer. Original magnifications, A, ×100; B, C, D, E, and F, ×200.

are involved in cellular proliferation and oncogenesis[8]. However, there have been few studies on the expression patterns and prognostic value of these proteins in gastric cancers[9,10].

TBX3, a member of the T-box family of transcription factors, is related to Brachyury and other TBX family members by a shared DNA-binding motif known as the T-domain[11]. A single nucleotide deletion and splice-site mutation that cause the ulnar-mammary syndrome disrupt the T-domain and are thought to function as null alleles[12]. The overexpression of TBX3 has been shown to have oncogenic potential by inhibiting the induction of $p19^{ARF}$ and p53 and interfering with apoptosis caused by excessive levels of Myc[13]. In addition, TBX3 has been reported to be a negative regulator of apoptosis in normal bladder epithelial cells[14]. Furthermore, a recent report showed that TBX3 is a mediator of β-catenin with regard to cell proliferation and survival, suggesting that TBX3 is a downstream target of the Wnt/β-catenin signaling pathway[15]. Thus, we hypothesized that activation or overexpression of the *TBX3* gene may contribute to the development of gastric cancers via the activation of the Wnt signaling pathway.

To investigate whether overexpression of the TBX3 protein is involved in the development of gastric cancers, and to clarify not only the association between β-catenin and TBX3 expression but also their prognostic significance in patients with gastric cancer, we performed an analysis of expression of the TBX3 and β-catenin proteins in 186 gastric carcinomas by immunohistochemistry.

Immunohistochemistry of β-catenin and TBX3 Expression

The β-catenin protein was expressed in the membranes of the corresponding gastric mucosal epithelial cells. The cancer cells demonstrated nuclear and cytoplasm staining of β-catenin, whereas the membrane staining was reduced or lost in some tumor cells (Figure 1). Cases with aberrant β-catenin staining showed abnormal patterns in the majority of cells examined. As shown Figure 1, aberrant expression was observed in 48 (25.8%) of the 186 gastric cancers. By contrast, cases with normal β-catenin staining showed strong membrane staining throughout. There was no statistically significant correlation between the β-catenin expression and the clinical/pathological parameters, including the histological type, presence of lymph node metastasis, tumor location, tumor size, and the 5-year survival ($P > 0.05$) (Table 1, Figure 2).

Table 1. Relationship between the expression of β-catenin and TBX3 proteins and the clinical/pathological parameters.

Parameter	β-catenin		TBX3	
	+	−	+	−
Lauren				
Intestinal	16	45	18	43
Diffuse	26	67	28	65
Mixed	6	26	8	24
Lymph node metastasis[≠]				
<7	17	51	20	48
7~15	27	85	24	79
>15	4	2	10	5
Tumor location				
Proximal	8	11	7	12
Distal	40	127	47	120
Tumor size				
<6.5 cm	28	73	31	70
≥6.5 cm	20	65	23	52
TBX3[†]				
+	27	27		
−	21	111		
Total	48	138	54	132

[≠], Chi-square test, $P=0.0025$; [†], Chi-square test, $P=0.00000141$

The TBX3 protein was immunonegative or weakly expressed in the nucleus and cytoplasm of the gastric mucosal epithelial cells. Immunopositivity for the TBX3 protein was detected in 54 (29.0%) out of the 186 gastric cancers (Figure 1). For the gastric cancer cells, TBX3 overexpression was found in 18 (29.5%), 28 (30.1%) and 8 (25.0%) of the following samples: 61 intestinal-type, 93 diffuse-type and 32 mixed-type gastric cancers, respectively. TBX3 was expressed in 54 (29.6%) out of 182 cases with lymph node metastasis. Statistically, there was no significant relationship between the TBX3 protein expression and clinical/pathological parameters including histological type, tumor location and tumor size (Table 1). However, TBX3 immunopositivity was significantly associated with the number of lymph node metastases ($P=0.0025$, Table 1). The 5-year survival rate of patients with TBX3 positive tumors was 44.4%, while that of the patients with TBX3 negative tumors was 51.6% (Figure 2); there was no significant association between TBX3 overexpression and the 5-year survival ($P=0.3355$).

In addition, overexpression of the TBX3 protein was detected in 27 (56.3%) out of 48 gastric cancers with aberrant expression of the β-catenin protein. Statistically, there was a significant positive correlation between the expression of TBX3 and β-catenin ($P=0.00000141$) (Table 1). However, these proteins did not dramatically distinguish between good and poor outcome among the patients with gastric cancer ($P=0.7600$).

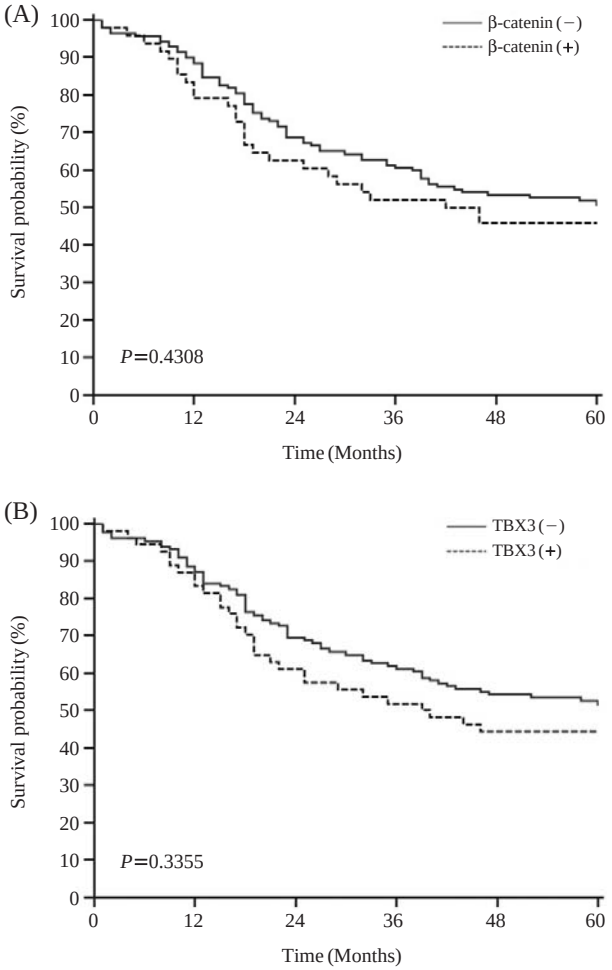

Figure 2. Kaplan-Meier survival analysis of patients with gastric cancer. Comparative survival analysis of immunohistochemically classified tumors demonstrating (—) negative and (---) positive staining for β-catenin (A) and TBX3 (B) proteins showed no significant correlation between β-catenin and TBX3 staining and the 5-year survival, respectively ($P=0.4308$ & $P=0.3355$).

Expression Pattern of TBX3 in Gastric Cancer Cell Lines and Tissues

When primary gastric cancer tissues were examined by Western blot analysis, expression of the TBX3 protein was increased in three of the four cancer tissues compared to the corresponding gastric mucosal tissues. When we reviewed the histology of these cases, cancer without overexpression of the TBX3 protein exhibited considerable heterogeneity with inflammatory cell infiltration and desmoplasia. In addition, ex-

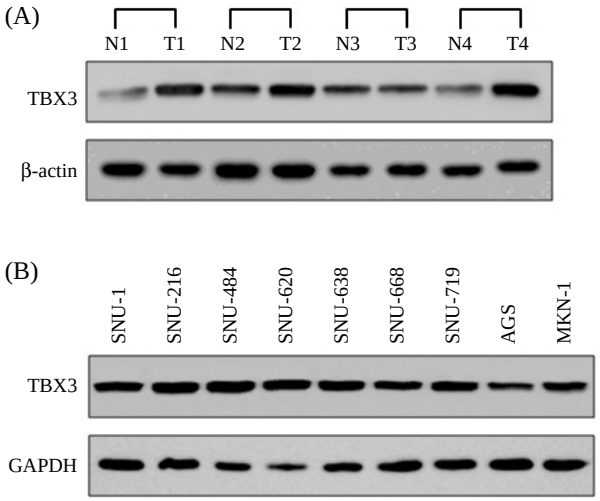

Figure 3. TBX3 expression in primary gastric cancer tissues and cell lines by Western blot analysis. The TBX3 protein was overexpressed in three out of four cancer tissues (A) compared to the corresponding gastric mucosal tissues, and showed strong expression in nine gastric cancer cell lines (B).

pression of TBX3 was increased in all of the gastric cancer cell lines relative to the expression in normal gastric mucosa (Figure 3).

Discussion

Gastric cancer is generally believed to be caused by a multistep progression of events triggered by chronic *Helicobacter pylori* (*H. pylori*) infection. Atrophic gastritis, intestinal metaplasia and dysplasia represent different stages of the gastric carcinogenesis cascade[16]. The oncogenic *H. pylori* strain selectively activates β-catenin in gastric epithelia; the nuclear accumulation of β-catenin is increased in the gastric epithelium harvested from gerbils infected with the *H. pylori* strain as well as from persons carrying the cag^+ strain[17]. After its translocation into the nucleus, β-catenin binds to members of the Tcf/Lef family thereby activating target genes. The identified target genes include *c-Myc* and *cyclin D1*[8]. Recently, the *TBX3* gene was identified as a downstream target of the Wnt pathway; it was associated with aberrant β-catenin/Tcf-induced cell proliferation and the inhibition of apoptosis[15].

The transcriptional factors TBX2 and TBX3 play an important role in cell proliferation, fate and identity during development[18] and are negative regulators of specific target genes including tumor suppressor gene p14, p19 and p21[19-21]. TBX3 protein is overexpressed in several cancers including ovarian, breast, and cervical cancer[22,23]. Recent study has demonstrated that TBX3 is a downstream target of Wnt/β-catenin in liver cancer, and that Wnt/β-catenin upregulates TBX expression[15]. Thus, we explored the effects of aberrant expression of β-catenin on TBX3 expression, to investigate whether changes in the expression of the TBX3 protein are involved in the development of gastric cancers. The results of the immunohistochemical experiments showed that aberrant expression of β-catenin and TBX3 proteins were present in 25.8% and 29.0% of the gastric cancers, respectively, and overexpression of the TBX3 protein was confirmed in human gastric cancer tissues and cell lines by Western blot analysis (Figure 3). Statistically, their expression was not associated with the clinical/pathological parameters studied, including tumor size, tumor location, histological type, and the 5-year survival (Table 1, Figure 2). Previous study has reported similar immunohistochemical results for β-catenin[24]. Thus, it is likely that the same molecular events or expression pattern for these genes occur at different locations and among different histological types.

The results of this study showed that the expression of TBX3 was closely associated with the number of lymph node metastases and aberrant β-catenin expression in gastric cancers (Table 1). How TBX3 contributes to the progression of gastric cancer is speculative. One possibility is that TBX3 combined with Myc or oncogenic Ras can lead to immortalization and efficient transformation of mouse embryo fibroblasts[13,25]. Another possible mechanism is that overexpression of TBX3 in melanoma cells can downregulate endogenous E-cadherin expression, whereas depletion of TBX3 increases E-cadherin mRNA and protein levels and decreases melanoma invasiveness *in vitro*[26]. The third possible mechanism might relate to the negative regulation of the tumor suppressor gene by TBX3[19-21].

In this present study, we found that TBX3 overexpression was not an independent prognostic marker for patients with advanced gastric cancer. Therefore, we investigated whether a combination of β-catenin and TBX3 expression would be a valuable marker for prediction of outcome in patients with advanced gastric cancer and found that these proteins were not useful prognostic markers. Thus, in future studies we plan to investigate markers that can be used to predict sensitivity to chemotherapy in patients with advanced gastric cancer.

Although identification of the underlying mechanisms is needed for improved understanding of gastric carcinogenesis, the findings of this study suggest that TBX3 may contribute to the development and progression of gastric cancer by its affect on invasive cancer cells and that the overexpression of TBX3 might be a

common phenomenon among solid tumors. Even though the clinical/pathological significance of TBX3 protein expression is not fully understood, TBX3 might be useful as a biomarker for the detection of advanced gastric cancer.

In conclusion, the aim of this study was to determine the pathological and clinical significance of TBX3 in gastric cancer by examining TBX protein expression. Overexpression of the TBX3 protein was detected in gastric carcinoma cells and was closely associated with the number of lymph node metastases and aberrant expression of β-catenin. These data suggest that overexpression of the *TBX3* gene may play an important role in the development and progression of gastric cancer. TBX3 overexpression might be a good marker for the detection of advanced gastric cancer. Additional functional studies of TBX3 are needed to confirm these initial observations and advance our understanding of the pathogenesis of gastric cancer.

Materials & Methods

Samples

One hundred eighty six formalin-fixed paraffin-embedded gastric cancer specimens were examined in this study. Histologically, 61 cases were the intestinal-type, 93 were the diffuse-type and 32 were the mixed-type of gastric cancer. The mean size of the tumors was 6.5 cm and 167 cases were located in the distal section (antrum and corpus) of the stomach. Two pathologists screened the histological sections and selected areas with representative tumor cells. Two and one tissue core samples from each cancer and a normal area were taken and placed in a new recipient paraffin block using a commercially available microarray instrument (Beecher Instruments, Micro-Array Technologies, Silver Spring, MD, USA), according to established methods[27]. The study was approved by the institutional review board of The Catholic University of Korea, College of Medicine. There was no evidence of familial cancer in any of the patients.

Immunohistochemistry of TBX3 and β-catenin

To investigate whether TBX3 overexpression is associated with aberrant expression of β-catenin, we analyzed the TBX3 and β-catenin expression in gastric cancers using immunohistochemistry. For the immunohistochemical analysis, 2 μm sections were cut the day before use and stained according to standard protocols. To maximize the immunohistochemistry signal, two strategies were used: antigen retrieval in citrate buffer and signal amplification with biotinylated tyramide. For the former, heat-induced epitope retrieval was conducted by immersing the slides in Coplin jars filled with 10 mmol/L of citrate buffer (pH 6.0) and boiling the buffer for 30 min in a pressure cooker (Nordic Ware, Minneapolis, MN, USA) inside of a microwave oven at 700 W; the jars were then cooled for 20 min. For the latter, the Renaissance TSA indirect kit (NEN Life Science, Boston, MA, USA), which included streptavidin-peroxidase and biotinylated tyramide, was used. After rinsing with PBS, the slides were treated with 1% H_2O_2 in PBS for 15 min at room temperature to remove endogenous peroxidase activity. After washing with TNT buffer (0.1 mol/L Tris-HCl, pH 7.4, 0.15 mol/L NaCl and 0.05% Tween 20) for 20 min, the slides were treated with TNB buffer (0.1 mol/L Tris-HCl, pH 7.4, 0.15 mol/L NaCl and 0.5% blocking reagent). The sections were incubated overnight at 4°C with the anti-β-catenin (1/100 dilution; Santa Cruz Biotechnology, Santa Cruz, CA, USA) and anti-TBX3 (1/100 dilution; Abcam, Cambridge, UK) antibodies. Detection was carried out using biotinylated goat anti-mouse antibodies (Sigma, St. Louis, MO, USA), followed by incubation with the peroxidase-linked avidin-biotin complex. Diaminobenzidine was used as a chromogen, and the slides were counterstained with Mayer's hematoxylin. For β-catenin, nuclear or cytoplasm staining (regardless of presence or absence of membranous staining) or loss of membrane staining was considered aberrant expression. Two pathologists reviewed the results independently. Using Matsumoto's method, the TBX3 immunostaining was interpreted on a 0 to 3 semi-quantitative scoring system for both the intensity of staining and the percentage of positive cells[28]. Briefly, the intensity of TBX staining was grouped into four categories: no staining (0), weak staining above background (1), moderate staining (2), and intense staining (3). The labeling frequency was scored as 0 (< 5%), 1 (6% to 25%), 2 (25% to 50%), and 3 (> 50%). An index of the sum of the scores was obtained by totaling the intensity scores and percentages. If the final score was greater than four, the case was considered positive; otherwise, the tumor was considered negative. As negative controls, the slides were treated with replacement of the primary antibodies with non-immune serum.

Western Blot Analysis

We also examined TBX3 expression by Western blot analysis using anti-TBX3 antibody (Abcam, Cambridge, UK) in nine gastric cancer cell lines and four frozen tissue samples. Preparation of total cell lysates from gastric cancer cell lines and tissue samples was performed using standard RIPA buffer. Equal amounts (20 μg) of protein were separated by polyacrylamide

gel electrophoresis and transferred to nitrocellulose membranes

Statistical Analysis

The Chi-square test for association was used to test the relationship between β-catenin and TBX3 expression and the clinical/pathological parameters associated with the gastric cancers. A P value < 0.05 was considered statistically significant. The predictive value of the clinical parameters for survival was evaluated by the Kaplan-Meier method.

Acknowledgements

This work was supported by the Korea Science and Engineering Foundation (KOSEF) grant funded by the Korea government (MOST) (R13-2002-005-05001-0).

References

1. Jemal, A. *et al*. Cancer statistics, 2008. *CA Cancer J Clin* **58**:71-96 (2008).
2. Shin, H. R., Jung, K. W., Won, Y. J. & Park, J. G. 139 KCCR-affiliated Hospitals, 2002 Annual Report of the Korea Central Cancer Registry: Based on Registered Data from 139 Hospitals. *Cancer Research and Treatment* **36**:103-114 (2004).
3. Peifer, M. & Polakis, P. Wnt signaling in oncogenesis and embryogenesis-a look outside the nucleus. *Science* **287**:1606-1609 (2000).
4. Polakis, P. The many ways of Wnt in cancer. *Curr Opin Genet Dev* **17**:45-51 (2007).
5. Lustig, B. & Behrens, J. The Wnt signaling pathway and its role in tumor development. *J Cancer Res Clin Oncol* **129**:199-221 (2003).
6. Park, W. S. *et al*. Frequent somatic mutations of the beta-catenin gene in intestinal-type gastric cancer. *Cancer Res* **59**:4257-4260 (1999).
7. Ebert, M. P. *et al*. Increased beta-catenin mRNA levels and mutational alterations of the APC and beta-catenin gene are present in intestinal-type gastric cancer. *Carcinogenesis* **23**:87-91 (2002).
8. Kolligs, F. T., Bommer, G. & Göke, B. Wnt/beta-catenin/tcf signaling: a critical pathway in gastrointestinal tumorigenesis. *Digestion* **66**:131-144 (2002).
9. Müller, W., Noguchi, T., Wirtz, H. C., Hommel, G. & Gabbert, H. E. Expression of cell-cycle regulatory proteins cyclin D1, cyclin E, and their inhibitor p21 WAF1/CIP1 in gastric cancer. *J Pathol* **189**:186-193 (1999).
10. Sanz-Ortega, J. *et al*. Comparative study of tumor angiogenesis and immunohistochemistry for p53, c-ErbB2, c-myc and EGFr as prognostic factors in gastric cancer. *Histol Histopathol* **15**:455-462 (2000).
11. Kispert, A. & Hermann, B. G. The Brachyury gene encodes a novel DNA binding protein. *EMBO J* **12**: 4898-4909 (1993).
12. Bamshad, M. *et al*. Mutations in human TBX3 alter limb, apocrine and genital development in ulnar-mammary syndrome. *Nat Genet* **16**:311-315 (1997).
13. Carlson, H., Ota, S., Song, Y., Chen, Y. & Hurlin, P. J. Tbx3 impinges on the p53 pathway to suppress apoptosis, facilitate cell transformation and block myogenic differentiation. *Oncogene* **21**:3827-3835 (2002).
14. Ito, A., Asamoto, M., Hokaiwado, N., Takahashi, S. & Shirai, T. Tbx3 expression is related to apoptosis in rat bladder both hyperplastic epithelial cells and carcinoma cells. *Cancer Lett* **219**:105-112 (2005).
15. Renard, C. A. *et al*. Tbx3 is a downstream target of the Wnt/beta-catenin pathway and a critical mediator of beta-catenin survival functions in liver cancer. *Cancer Res* **67**:901-910 (2007).
16. Yuasa, Y. Control of gut differentiation and intestinal-type gastric carcinogenesis. *Nat Rev Cancer* **3**:592-600 (2003).
17. Franco, A. T. *et al*. Activation of beta-catenin by carcinogenic Helicobacter pylori. *Proc Natl Acad Sci* **102**: 10646-10651 (2005).
18. Naiche, L. A., Harrelson, Z., Kelly, R. G. & Papaioannou, V. E. T-box genes in vertebrate development. *Annu Rev Genet* **39**:219-239 (2005).
19. Lingbeek, M. E., Jacobs, J. J. & van Lohuizen, M. The T-box repressors TBX2 and TBX3 specifically regulate the tumor suppressor gene p14ARF via a variant T-site in the initiator. *J Biol Chem* **277**:26120-26127 (2002).
20. Prince, S., Carreira, S., Vance, K. W., Brahams, A. & Goding, C. R. Tbx2 directly represses the expression of the p21(WAF1) cyclin-dependent kinase inhibitor. *Cancer Res* **64**:1669-1674 (2004).
21. Brummelkamp, T. R. *et al*. TBX-3, the gene mutated in Ulnar-Mammary Syndrome, is a negative regulator of p19ARF and inhibits senescence. *J Biol Chem* **277**: 6567-6572 (2002).
22. Lomnytska, M., Dubrovska, A., Hellman, U., Volodko, N. & Souchelnytskyi, S. Increased expression of cSHMT, Tbx3 and utrophin in plasma of ovarian and breast cancer patients. *Int J Cancer* **118**:412-421 (2006).
23. Lyng, H. *et al*. Gene expressions and copy numbers associated with metastatic phenotypes of uterine cervical cancer. *BMC Genomics* **7**:268 (2006).
24. Gulmann, C. *et al*. Adenomatous polyposis coli gene, beta-catenin, and E-cadherin expression in proximal and distal gastric cancers and precursor lesions: an immunohistochemical study using tissue microarrays. *Appl Immunohistochem Mol Morphol* **11**:230-237 (2003).
25. Fan, W., Huang, X., Chen, C., Gray, J. & Huang, T. TBX3 and its isoform TBX3+2a are functionally distinctive in inhibition of senescence and are overexpressed in a subset of breast cancer cell lines. *Cancer*

Res **64**:5132-5139 (2004).
26. Rodriguez, M., Aladowicz, E., Lanfrancone, L. & Goding, C. R. Tbx3 represses E-cadherin expression and enhances melanoma invasiveness. *Cancer Res* **68**:7872-7881 (2008).
27. Kononen, J. *et al*. Tissue microarrays for high-throughput molecular profiling of tumor specimens. *Nat Med* **4**:844-847 (1998).
28. Matsumoto, K., Shariat, S. F., Ayala, G. E., Rauen, K. A. & Lerner. S. P. Loss of coxsackie and adenovirus receptor expression is associated with features of aggressive bladder cancer. *Urology* **66**:441-446 (2005).