TC1 (C8orf4) Correlates with Wnt/β-Catenin Target Genes and Aggressive Biological Behavior in Gastric Cancer

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Abstract

Purpose: We have recently reported that TC1 (C8orf4), a small protein present in vertebrates, functions as a novel regulator of the Wnt/β-catenin pathway. TC1 up-regulates β-catenin target genes that are implicated in the aggressive behavior of cancers. Our aim was to investigate the clinical and pathobiological relevance of TC1 in gastric cancer.

Experimental Design: The expression of TC1 was analyzed using tissue microarray in correlation with clinicopathologic variables and β-catenin target genes in 299 gastric cancers. The biological effects of TC1 on Matrigel invasiveness and the proliferation of cancer cells were analyzed. TC1 expression was analyzed in gastric cancer cells after serial peritoneal implantation in nude mice.

Results: TC1 expression was present in 111 carcinomas (37.1%), correlating with tumor stage ($P < 0.002$), poor differentiation ($P < 0.001$), lymphatic infiltration ($P < 0.005$), and lymph node metastasis ($P < 0.006$). TC1 also correlated with poor survival in diffuse type carcinomas ($P < 0.0001$), and even in patients with lymph node metastasis ($P < 0.0014$). TC1 also correlated with the expression of β-catenin target genes including laminin γ2, metalloproteinase-7 and metalloproteinase-14, cyclin D1, c-Met, and CD44. TC1 enhanced Matrigel invasiveness and proliferation, supporting its role in the aggressive biological behavior of cancers. The expression of TC1 increased in MKN45 cells after serial peritoneal seeding in nude mice.

Conclusions: Our data suggests that TC1 coordinates the up-regulation of Wnt/β-catenin target genes that are implicated in the aggressive biological behavior of cancers. The strong clinical relevance, even in patients with lymph node metastasis, suggested that TC1 could be a potential therapeutic target of advanced gastric cancers.

Gastric cancer is one of the leading fatal malignancies worldwide (1). Chronic *Helicobacter pylori* gastritis, ethnic, and dietary factors have been associated with gastric cancers (2–5). The biological behavior and clinical outcome of gastric cancers vary considerably. The molecular pathways involved in its biological variety have not been characterized well. Therefore, searching for the molecular regulators of carcinogenesis and/or clinical progression has been a major goal of gastric cancer research.

TC1 (*C8orf4*) was one of the up-regulated genes in high-grade cancers in our previous expression profiling study of gastric cancers (6), suggesting that it might be implicated in the poor differentiation and/or aggressive biological behavior of cancers. TC1 was first described as one of the genes elevated in expression in thyroid cancers (7). It is present in vertebrates and encodes a protein of 106 amino acids without an identified functional domain (7, 8). It has been implicated in cancer and signal transduction (9, 10). However, its clinical relevance and precise biological functions have not heretofore been elucidated.

Recently, we have reported that TC1 functions as a novel regulator of the Wnt/β-catenin signaling pathway (11), which has been widely implicated in regulating cell proliferation and differentiation in cancers and in development (12–16). TC1 interacts with Chibby (Cby) and thereby enhances the signaling pathway by relieving the antagonistic function of Cby on β-catenin-mediated transcription (11, 17). TC1 enhances the expression of β-catenin target genes that are implicated in the aggressive biological behavior of cancer. The proposed function of TC1 in the Wnt/β-catenin pathway regulation suggests that it could regulate the downstream genes in cancers and have a significant clinical relevance.

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Received 11/9/05; revised 2/19/06; accepted 3/24/06.

Grant support: National Research Laboratory Project (M10400000305-05J0000-30510) and Molecular and Cellular Function Discovery Project (M10401000003-05N0100-00310) from the Korean Ministry of Science and Technology. R.T. Moon is an investigator of the Howard Hughes Medical Institute.

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doi:10.1158/1078-0432.CCR-05-2440

To test this hypothesis, we have done an extensive tissue microarray analysis of gastric cancers for the comparative analysis of TC1, β-catenin target genes of biological significance, and comprehensive clinicopathologic variables. We also analyzed the effects of TC1 on the Matrigel invasiveness of cancer cells. TC1 had strong correlations with aggressive biological behaviors and poor clinical outcome in gastric cancers. It also correlated with target genes of the Wnt/β-catenin pathway that are implicated in cancers. Our data suggest that TC1 might be a potential therapeutic target and/or an indicator determining appropriate therapies in cancers.

Materials and Methods

Cells and plasmids. AGS, NKN45, and HeLa cells were grown in DMEM supplemented with 10% fetal bovine serum and 100 units/mL of streptomycin/penicillin at 37°C in humidified atmosphere with 5% CO_2. KATO-III cells were grown in RPMI 1640 supplemented with 10% fetal bovine serum.

Full-length human TC1 cDNA was cloned in pcDNA3 vector (Invitrogen, Carlsbad, CA), as described previously (11). For bacterial expression, pET-TC1 was similarly cloned using the pET28 vector (Novagen, Madison, WI). All plasmids were confirmed by DNA sequencing.

To facilitate gene transfer in mammalian cells, Lenti-TC1, a full-length TC1-expressing lentiviral vector was constructed by inserting a TC1 open reading frame into the LentiM1.2 vector (VectorCore A, Inc., Deajeon, Korea), which was designed to express targeting protein promoted from mCMV promoter and eGFP-Zeocin fusion protein from the internal ribosome entry segment promoter. LentiM1.4-eGFP vector was used as a control. Lentiviruses were prepared according to the standard protocol (18).

Anti-TC1 antibody production and Western blotting. Bacterially expressed TC1 protein was purified using His-bind resin column according to the manufacturer's instructions (Novagen). Rabbit anti-TC1 antiserum was produced and affinity-purified as described previously (11, 19). For Western blotting, the protein was separated on 15% SDS-PAGE, blotted onto nitrocellulose membrane and probed with the anti-TC1 antiserum, followed by goat anti-rabbit second antibody (Amersham Biosciences, Piscataway, NJ), and visualized using the enhanced chemiluminescence method (Amersham Biosciences).

Tissue microarray and immunohistochemical staining. The gastric cancer tissue microarray has been previously described (SuperBioChips, Seoul, Korea; ref. 20). Affinity-purified anti-TC1 and anti-Cby antisera were applied for immunohistochemical staining. Negative controls were taken using antigen-preabsorbed antisera. Other primary antibodies were purchased commercially and applied in pretitrated dilutions: anti-c-Myc (mouse monoclonal 9E11, ×100; Novocastra, Newcastle, United Kingdom), anti-cyclin D1 (mouse monoclonal, P2D11F11, ×100; Novocastra), anti-c-Met (rabbit antiserum ×100; Santa Cruz Biotechnology, Santa Cruz, CA), anti-CD44 (mouse monoclonal, DF1485, ×50; DakoCytomation, Carpinteria, CA), anti-laminin γ2 (LAMC2; goat antiserum, ×50; Santa Cruz Biotechnology), anti–matrix metalloproteinase-7 (MMP7; goat antiserum, ×100; Santa Cruz Biotechnology), and anti-MMP14 (goat antiserum, ×100; Santa Cruz Biotechnology).

Immunohistochemical staining was done using Benchmark autostainer (Ventana Medical Systems, Inc., Tucson, AZ). Microarray slides were independently examined by two pathologists (H. Koo and I. Lee), without access to the clinical data. In case of discrepancy, if present, slides were reviewed together to reach a consensus. Kaplan-Meier life table curves were prepared using SPSS software (SPSS, Inc., Chicago, IL). Correlations of clinicopathologic variables and/or protein expressions were analyzed using χ^2 test and/or t test (SPSS).

Matrigel invasion assay. To analyze the biological effect of TC1 on the Matrigel invasion, 4×10^5 AGS cells were treated with either Lenti-TC1 or Lenti-control at a titer of 4×10^7 TU/mL and 6 mg/mL polybrene. The medium was changed after incubation for 10 hours, and cells were harvested after 24 hours for the invasion analysis. For the loss-of-function analysis, TC1 was knocked down in HeLa cells by transfecting either one of two synthetic TC1-short interfering RNAs (siRNA), 5′-acacagaccaagaatcactagaaag-3′ or 5′-tcatcatgtccacgtcgctacgagt-3′ (Stealth, Invitrogen), using LipofectAMINE 2000 according to the manufacturer's instructions. For controls, Stealth RNAi-negative control medium GC (Invitrogen) was applied instead. The efficiency of TC1 transfection and knockdown was analyzed using real-time and/or semiquantitative RT-PCR as described previously (6, 11). β-Catenin was used as internal control.

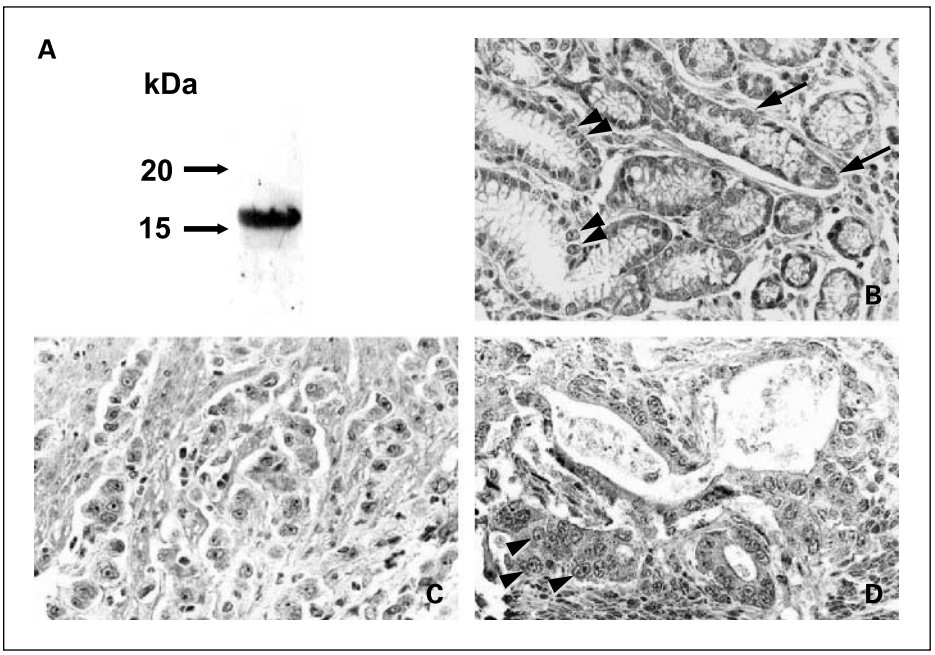

Fig. 1. TC1 protein expression in gastric cancers. *A,* Western blot of bacterially expressed TC1 protein using affinity-purified antiserum. *B,* normal gastric mucosa showing cytoplasmic staining of TC1 in parietal cells (*arrows*). There is minimal TC1 expression in the cytoplasm and nuclei of epithelial cells in the proliferative zone (*arrowheads*; immunohistochemical staining, ×100). *C,* cytoplasmic staining of cancer cells (×200), *D,* nuclear staining is also noted in certain cancers (×200).

Invasion assay was done using Matrigel Invasion Chambers (Becton Dickinson Biosciences, Bedford, MA) according to the manufacturer's protocols. Lenti-TC1 or Lenti control-transfected AGS cells, 4×10^4, were placed in the top chamber in 500 μL of DMEM containing 0.1% fetal bovine serum. In the bottom chamber, the medium contained 15% fetal bovine serum as chemoattractant. Alternatively, TC1-siRNA or control RNA-transfected HeLa cells were analyzed similarly. After incubation for 20 hours at 37°C, cells on the top surface of the filter were wiped off with a cotton-tipped swab, and the filter was fixed in methanol, and stained using DiffQuick stain. The invasion rate was determined by counting cells at the bottom of the chamber. Experiments were repeated in triplicate and values were analyzed using ANOVA test.

Proliferation assay. KATO-III cells transfected with Lenti-TC1 or Lenti-control were analyzed using WST-1 proliferation assay kit according to the manufacturer's instructions (Roche, Mannheim, Germany). Alternatively, KATO-III and HeLa cells were transfected with siTC1-siRNAs or control RNA for the loss-of-function analysis. In each experiment, 8×10^4 cells/well were plated in 96-well plates, and the proliferation was measured in quadruplicate after 6, 24, 48, and/or 72 hours. The fold changes were analyzed using ANOVA test.

TC1 expression in MKN45 cells after peritoneal passages in nude mice. The expression of TC1 was analyzed in MKN45 cells after serial passages into nude mice peritoneal cavity. This study was approved by the Institutional Review Board, and followed the guidelines of the Animal Study Committee. Exponentially growing MKN45 cells were harvested, and 1×10^5 cells/0.5 mL of Hank's balanced salt solution were injected into the peritoneal cavity of nude mice. After 3 weeks, mice were killed and ascites fluid was obtained aseptically and cultured in dishes. The implantations were repeated for up to seven passages. The expression of TC1 was analyzed using semiquantitative RT-PCR and competitive hybridization on cDNA microarrays as described previously (6, 11).

Results

TC1 expression in gastric carcinoma and mucosa. The affinity-purified antiserum reacted with bacterially expressed TC1 protein on Western blotting (Fig. 1A). In normal mucosa, TC1 immunostaining was present in parietal cells (Fig. 1B). Minimal TC1 expression was noted in epithelial cells of the proliferative zone (4). No immunostaining was present using antigen-preabsorbed antibody (data not shown).

We then analyzed the expression of TC1 in 299 gastric cancers including 159 diffuse type, 110 intestinal type, and 30 mixed type carcinomas. They included 204 males and 95 females, mean age 54.2 (Table 1). All patients underwent curative subtotal gastrectomy and were followed-up for 5 years (19). TC1 expression was evident in 111 carcinomas (37.1%). It was mostly in the cytoplasm (Fig. 1C), but nuclear staining was also partly present (Fig. 1D). The expression of TC1 was relatively low, compatible with our previous report that TC1 is maintained at low levels in most cells (11). It was shown that TC1 was up-regulated by a proteasome inhibitor, suggesting a posttranslational regulation through the proteasome degradation pathway (11).

TC1 correlates with aggressive behavior and poor survival in gastric cancer. As summarized in Table 1, TC1 expression correlated strongly with advanced gastric cancers ($P < 0.001$), high tumor-node-metastasis stage ($P < 0.002$), depth of wall invasion ($P < 0.001$), size ($P < 0.002$), poor differentiation ($P < 0.001$), lymphatic infiltration ($P < 0.005$), and lymph node metastasis ($P < 0.006$). It did not correlate with distant metastasis; however, the number of patients with liver

Table 1. TC1 and clinicopathologic variables

Factor	Cases	TC1 expression		*P**
		TC1 − (*n* = 188)	TC1 + (*n* = 111)	
Gender				
Male	204	122	82	>0.05
Female	95	66	29	
Age (y)				
Mean ± SE	299	53.3 ± 0.94	55.84 ± 1.24	>0.05†
Stage				
Early gastric cancers	92	75	17	<0.001
Advanced gastric cancers	207	113	94	
Tumor-node-metastasis stages				
I	128	95	33	0.002
II	58	35	23	
III	68	37	31	
IV	45	21	24	
Depth of invasion				
T_1	92	75	17	<0.001
T_2	141	79	62	
T_3	62	32	30	
T_4	4	2	2	
Tumor size (cm)				
≤5	177	124	53	0.002
>5	119	62	57	
Location				
Antrum	159	101	58	>0.05
Body/Cardia	140	84	53	
Lauren classification				
Intestinal	110	71	39	>0.05
Diffuse	159	100	59	
Mixed	30	17	13	
Differentiation				
Well	28	17	11	0.001
Moderated	79	53	26	
Poorly	129	66	63	
Muci	15	14	1	
Sig	48	38	10	
Lymph node metastasis				
N_0	112	80	32	0.006
N_1	97	64	33	
N_2	57	30	27	
N_3	33	14	19	
Lymphatic infiltration				
Negative	213	145	68	0.005
Positive	86	43	43	
Distant metastasis				
Absent	283	181	102	>0.05
Present	16	7	9	
Vascular invasion				
Negative	281	178	103	>0.05
Positive	18	10	8	
Lymphoid stroma				
Absent	266	163	103	>0.05
Present	14	9	5	

* χ^2 test.
† *t* test.

Table 2. Gene expression and survival

Genes	Positive (%)			Survival (*P*)*			
	Total	Alive	Died	Total	Diffuse	Metastasis†	Diffuse and metastasis
TC1	37.2	32.7	50.6	0.0028	0.0001	0.0113	0.0014
LAMC2	49.3	44.1	66.7	0.0005	0.0004	0.0032	
MMP14	41.3	36.7	55.4	0.0035	0.0008	0.0279	
c-Myc	63.7	61.4	71.2	0.1054			
CCND1	43.2	42.2	47.4	0.3384			
CD44	21.9	20.4	26.4	0.3740			
MMP7	31.5	33.5	28.6	0.4745			
c-Met	58.0	58	56.8	0.8251			

*Inverse correlation with survival in 5-year follow-up using Kaplan-Meier life tables. Only significant numbers are given.
† Survival of patients with lymph node metastasis.

metastasis was too small to be conclusive. Stomach cancers with liver metastasis are not frequently resected surgically. Taken together, our data indicate that TC1 is implicated in the aggressive biological behavior of gastric cancers.

In accordance with the pathologic variable analysis, there was a strong inverse correlation between TC1 expression and survival ($P < 0.0028$, Table 2). The inverse correlation was even stronger in diffuse type carcinomas ($P < 0.0001$, Fig. 2A), which tend to have more aggressive clinical courses than intestinal type carcinomas (21). However, no significant correlation was present in intestinal type cancers. A strong correlation was still present in patients who had diffuse type cancers with lymph node metastasis at the surgical resection ($P < 0.0014$, Fig. 2B).

TC1 correlates with Wnt/β-catenin target genes. Previously, we reported that TC1 enhances the Wnt/β-catenin pathway target genes in cultured cells (11). To investigate the target genes in gastric cancers, we analyzed the expression of c-Myc, cyclin D1, MMP7 (matrilysin), MMP14, CD44, c-Met, and LAMC2, which are associated with invasiveness of cancers (22–28). TC1 correlated strongly with all target genes except for c-Myc, suggesting the regulation by TC1 through the Wnt/β-catenin pathway in cancers (Table 3). Cyclin D1, LAMC2, MMP7, and MMP14 correlated strongly among themselves (Table 3).

Among the target genes, LAMC2 and MMP14 showed strong correlations with poor survival as TC1 did ($P < 0.0005$ and $P < 0.0035$, Table 2), suggesting that their up-regulation was a significant factor for the poor clinical outcome in TC1-expressing cancers. Similar to TC1, the correlations were even higher in diffuse type cancers ($P < 0.0004$ and $P < 0.0008$) and were also present in patients with lymph node metastasis at surgical resection ($P < 0.0032$ and $P < 0.0279$). As summarized in Table 4, LAMC2 and MMP14 correlated with such pathologic variables as tumor stage, size, depth of wall invasion, and lymphatic infiltration. LAMC2 also correlated with lymph node and distant metastasis. c-Myc and CD44 correlated with advanced gastric cancers. c-Myc also correlated with tumor size, depth of wall invasion, and poor differentiation.

TC1 enhances Matrigel invasiveness. The clinicopathologic data suggested that TC1 might regulate the invasiveness and

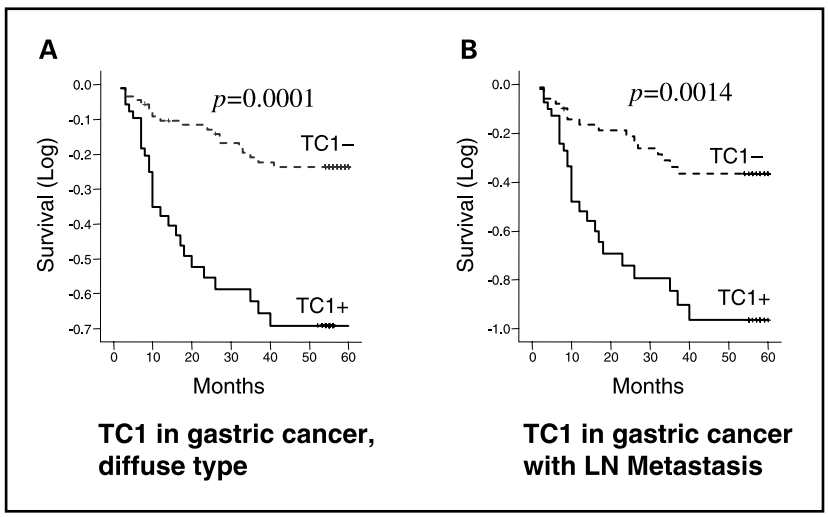

Fig. 2. Kaplan-Meier survival curve. *A,* inverse correlation of TC1 expression with survival in diffuse type stomach cancers. *B,* diffuse type cancer patients with lymph node metastasis.

Table 3. Correlations of TC1 and β-catenin target genes

Genes	Correlations (*P*)							
	TC1	*CCND1*	*LAMC2*	*MMP7*	*MMP14*	*CD44*	*c-Met*	*c-Myc*
TC1	—	<0.001	<0.001	<0.001	<0.001	0.003	0.001	
CCND1		—	<0.001	<0.001	0.003			
LAMC2			—	<0.001	<0.001		0.001	
MMP7				—	<0.001			
MMP14					—	0.004	<0.001	
CD44						—	0.001	0.004
c-Met							—	<0.001
c-Myc								—

NOTE: Correlation analysis using χ^2 test. Only significant numbers are given.

metastasis of cancers. To investigate this possibility, we analyzed the effect of TC1 on Matrigel invasion, a correlate of invasion and metastatic potential *in vivo*, using AGS cells. After 24 hours, most cells transfected with Lenti-TC1 or Lenti-control expressed green fluorescent protein on fluorescence microscopy (data not shown). TC1 expression was considerably up-regulated as shown by semiquantitative RT-PCR (Fig. 3A). The Matrigel invasion rate of TC1-Lenti–transfected AGS cells increased significantly compared with the control ($P < 0.001$, Fig. 3B).

We then analyzed the requirement of endogenous TC1 for the Matrigel invasiveness. HeLa was used for the knockdown experiment, because it has shown reproducible data in Matrigel invasion studies (29) and expresses relatively high levels of TC1 (data not shown). Upon siRNA transfection, TC1 mRNA decreased significantly as shown by real-time PCR for each siRNA (Fig. 3C). The Matrigel invasion rate of TC1 knockdown cells decreased markedly compared with the control RNA transfection ($P < 0.001$ for both siRNAs, Fig. 3D).

TC1 enhances cancer cell proliferation. Along with invasiveness, the enhanced proliferation is another hallmark of malignancy. TC1 was reported to increase the proliferation rate of a human thyroid cell line and anchorage-independent growth in soft agar (9). Upon Lenti-TC1 transfection, the proliferation of KATO-III cells was enhanced compared with control cells ($P < 0.001$, Fig. 4A). Alternatively, TC1 knockdown using TC1-siRNAs showed significant down-regulation of proliferation in KATO-III ($P < 0.003$ and $P < 0.037$ for siRNA nos. 1 and 2, respectively) and HeLa cells ($P < 0.001$ for both siRNAs) compared with control RNA transfection (Fig. 4B and C), suggesting that endogenous TC1 is required for proliferation.

TC1 expression enhances after serial peritoneal passages in nude mice. To further investigate the pathobiological relevance of TC1 expression *in vivo*, we analyzed the expression of TC1 in MKN45 cells after serial passages in nude mice peritoneal cavity. MKN45 cells have been shown to form i.p. xenografts well in nude mice (30). Under the experimental conditions,

Table 4. Clinicopathologic correlations of target genes

Factor	*LAMC2*	*MMP14*	*MMP7*	*CCND1*	*c-Myc*	*CD44*	*c-Met*
Stage (advanced gastric cancers/early gastric cancers)	<0.001	<0.001			0.005	0.009	
Tumor-node-metastasis stage	0.001	0.031					
Depth of invasion	<0.001	0.003			0.022		
Tumor size	0.004	0.008			0.039		
Location							
Lauren classification (intestinal/diffuse)			0.001*				
Differentiation			0.004*		0.001		
Lymph node metastasis	0.006		0.008*				
Lymphatic infiltration	0.002	0.001					
Distant metastasis	0.017						
Vascular invasion							
Lymphoid stroma							

NOTE: *P* values by χ^2 test.
*Inverse correlation.

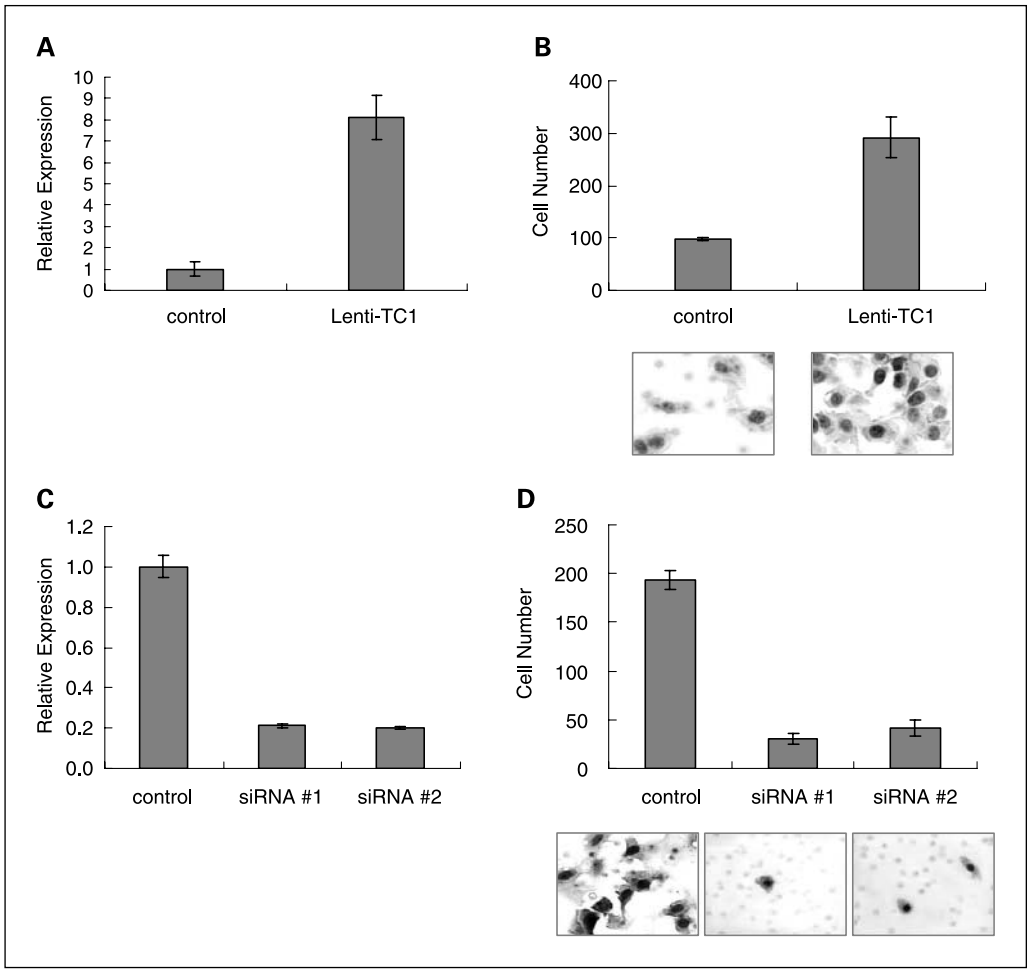

Fig. 3. TC1 effect on Matrigel invasion. *A,* semiquantitative RT-PCR for TC1 expression in AGS cells transfected with either Lenti-TC1 or Lenti-control. The expression was measured by densitometry in duplicate using β-actin as an internal control. *B,* Matrigel invasion assay of AGS cells. Lenti-TC1 or Lenti-control–transfected cells were incubated for 20 hours, 4×10^4 in each assay chamber, and cells which invaded the Matrigel-coated membranes were counted in triplicate. Representative figures of invaded cells are shown under the corresponding bars. *C,* TC1 knockdown in HeLa cells transfecting with either siRNA 1 or 2. After 24 hours of transfection, TC1 expression was measured using real-time PCR. The control was transfected with control RNA. *D,* Matrigel invasion assay of HeLa cells transfected with mock RNA, and siRNA 1 and 2.

MKN45 cells form ascites and peritoneal tumors in 3 weeks. As the passages progressed, ascites tended to be detected earlier and the tumor burden seemed to increase, including the infiltration into abdominal organs (data not shown). We then measured TC1 expression in cells of passage 7 using semiquantitative RT-PCR. MKN45-7p showed significantly enhanced TC1 expression compared with original MKN45 cells (Fig. 5). Upon competitive hybridization on cDNA microarray, TC1 expression in MKN45-7p was elevated 2.03 ± 0.11 times of the control.

Discussion

We have shown that TC1 correlated with aggressive biological behaviors and poor clinical outcome in gastric cancers. TC1 expression correlated strongly with nearly all pathologic variables of aggressive biological behavior of cancers including advanced stage, depth of wall invasion, size, poor differentiation, lymphatic infiltration, and lymph node metastasis. In accordance with the pathologic variable analysis, there was a strong correlation with poor survival in diffuse type cancers. The correlation was still present in patients with lymph node metastasis, suggesting that TC1 could be a potential therapeutic target of advanced gastric cancers and/or could be applied for monitoring the efficiency of therapy.

We have also done an extensive correlation analysis with the Wnt/β-catenin pathway target genes. TC1 correlated with β-catenin target gene expression in gastric cancers, compatible with the proposed role as a regulator of the Wnt/β-catenin signaling pathway in cancer (11). According to the correlation pattern, β-catenin target genes seem to consist of two groups in gastric cancers. LAMC2, MMP14, MMP7, and cyclin D1 correlated among themselves and with TC1 very strongly, suggesting a coordinated regulation as a group in gastric cancers. LAMC2 and MMP14 showed strong correlation with lymphatic infiltration and poor clinical outcome independently, suggesting that their up-regulation contributed significantly to the aggressive biological behavior of TC1-expressing cancers. As far as we are aware of, this is the first report of poor prognostic association of LAMC2 and/or MMP14 in stomach cancers. It is of note that MMP14 is an enzyme activating LAMC2, and thus, they function synergistically to promote tumor cell migration and invasion (31, 32). Together, it is suggested that TC1 might activate the molecular network involved in cancer invasion in a coordinated way.

Another group of target genes, CD44, c-Met, and c-Myc, correlated with others selectively. CD44 was reported to up-regulate and activate c-Met in transformed cells (33), supporting a cooperative function in the group. c-Myc was

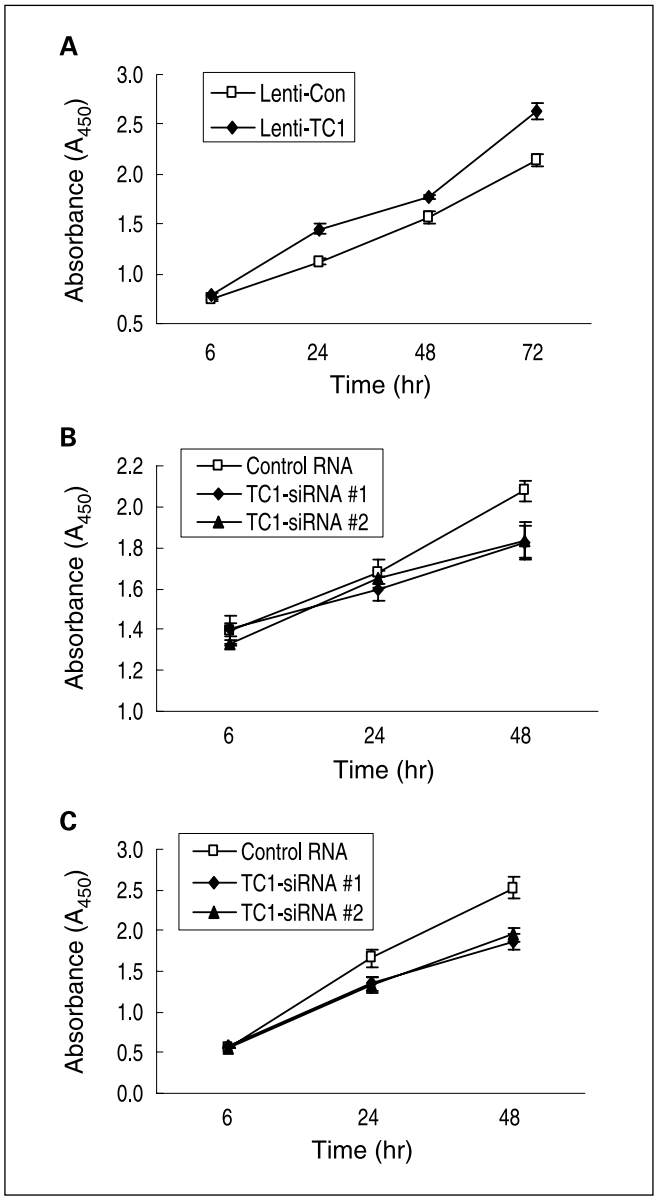

Fig. 4. TC1 effect on cell proliferation. *A,* gain-of-function analysis. KATO-III cells transfected with Lenti-TC1 or Lenti-control vectors were analyzed using WST-1 proliferation assay kit in quadruplicate. In each well, 8×10^4 cells/well were plated and the proliferation was measured in quadruplicate after 6, 24, 48, and 72 hours. The fold changes were analyzed using ANOVA test. *B* and *C,* loss-of-function analyses. The proliferation of KATO-III (*B*) and HeLa cells (*C*) were measured after TC1 knockdown using two siRNAs.

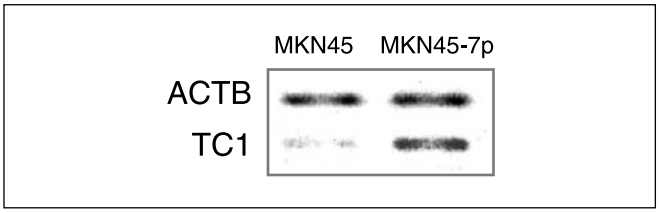

Fig. 5. Semiquantitative RT-PCR for TC1 in MKN45 and MKN45-7p, passage 7 in nude mice peritoneal implantation. β-actin was used as internal control.

the only gene that did not correlate with TC1 in cancers, although it was shown that TC1 up-regulated the expression at both RNA and protein level (11). In ovarian endometrioid adenocarcinomas, it was also reported that c-Myc expression did not correlate significantly with the β-catenin activity (34). The biological significance of the poor correlation in the cancers is not clear.

Enhanced invasiveness and proliferation are hallmarks of malignancy that are associated with the biological behavior of cancers. The Matrigel invasion assay simulates a part of the process involved in tissue invasion and metastasis *in vivo*. Our Matrigel invasion assay data was compatible with the clinicopathologic correlations with lymphatic infiltration, lymph node metastasis, and poor survival. The loss-of-function analysis showed that TC1 was required for the Matrigel invasiveness of non–gastric cancer cells as well. Further investigations are required for the pathobiological role of TC1 in other cancers. TC1 also enhanced the proliferation of gastric cancer cells. TC1 expression was enhanced in gastric cancer cells after serial peritoneal passages in nude mice, supporting the pathobiological relevance of TC1 in the spread of gastric cancers.

Taken together, our data suggest that TC1 is a major regulator of the Wnt/β-catenin pathway that promotes the aggressive biological behavior of cancers. The strong inverse correlation with survival in gastric cancers, especially in high-grade cancers and in patients with lymph node metastasis, suggests that TC1 may have potential as a therapeutic target of advanced cancers and might be a marker for determining how aggressively individual cancers should be treated at diagnosis.

Acknowledgments

We thank Brian M. Davis for the lentiviral construct, Hyunseok Lee for statistical analysis, and Seol Baik for immunohistochemical staining of gastric cancers.

References

1. Plummer M, Franceschi S, Munoz N. Epidemiology of gastric cancer. IARC Sci Publ 2004;157:311–26.
2. Correa P. *Helicobacter pylori* and gastric carcinogenesis. Am J Surg Pathol 1995;157:S37–43.
3. Crew KD, Neugut AI. Epidemiology of upper gastrointestinal malignancies. Semin Oncol 2004;31:450–64.
4. Jang J, Lee S, Jung Y, et al. Malgun (clear) cell change in *Helicobacter pylori* gastritis reflects epithelial genomic damage and repair. Am J Pathol 2003;162:1203–11.
5. Lee I, Lee H, Kim M, et al. Geographic difference of *Helicobacter pylori* gastritis: Korean and Japanese gastritis is characterized by male-, and antrum-predominant acute foveolitis in comparison with Americans. World J Gastroenterol 2005;11:94–8.
6. Kim B, Bang S, Lee S, et al. Expression profiling and subtype-specific expression of stomach cancer. Cancer Res 2003;63:8248–55.
7. Chua EL, Young L, Wu WM, et al. Cloning of TC-1 (C8orf4), a novel gene found to be overexpressed in thyroid cancer. Genomics 2000;69:342–7.
8. Nicod M, Michlig S, Flahaut M, et al. A novel vasopressin-induced transcript promotes MAP kinase activation and ENaC downregulation. EMBO J 2002;21:5109–17.
9. Sunde M, McGrath KC, Young L, et al. TC-1 is a novel tumorigenic and natively disordered protein associated with thyroid cancer. Cancer Res 2004;64:2766–73.
10. Friedman JB, Brunschwig EB, Platzer P, et al. C8orf4 is a transforming growth factor B induced transcript downregulated in metastatic colon cancer. Int J Cancer 2004;111:72–5.
11. Jung Y, Bang S, Choi K, et al. TC1 (C8orf4) enhances the Wnt/β-catenin pathway by relieving antagonistic activity of Chibby. Cancer Res 2006;66:723–8.
12. Moon RT, Bowerman B, Boutros M, et al. The promise and perils of Wnt signaling through β-catenin. Science 2002;296:1644–6.
13. Giles RH, van Es JH, Clevers H. Caught up in a Wnt

storm: Wnt signaling in cancer. Biochim Biophys Acta 2003;1653:1–24.

14. Nelson WJ, Nusse R. Convergence of Wnt, β-catenin, and cadherin pathways. Science 2004;303:1483–7.

15. Moon RT, Kohn AD, De Ferrari GV, et al. WNT and β-catenin signalling: diseases and therapies. Nat Rev Genet 2004;5:691–701.

16. Karim R, Tse G, Putti T, et al. The significance of the Wnt pathway in the pathology of human cancers. Pathology 2004;36:120–8.

17. Takemaru K, Yamaguchi S, Lee YS, et al. Chibby, a nuclear β-catenin-associated antagonist of the Wnt/Wingless pathway. Nature 2003;422:905–9.

18. Dull T, Zufferey R, Kelly M, et al. A third-generation lentivirus vector with a conditional packaging system. J Virol 1998;72:8463–71.

19. Song K, Jung Y, Jung D, et al. Human Ku70 interacts with HP1α. J Biol Chem 2001;276:8321–7.

20. Lee HS, Lee HK, Kim HS, et al. Tumour suppressor gene expression correlates with gastric cancer prognosis. J Pathol 2003;200:39–46.

21. Lee KH, Lee JW, Cho JK, et al. A prospective correlation of Lauren's histological classification of stomach cancer with clinicopathological findings including DNA flow cytometry. Pathol Res Pract 2001;197:223–9.

22. He TC, Sparks AB, Rago C, et al. Identification of c-MYC as a target of the APC pathway. Science 1998;281:1509–12.

23. Tetsu O, McCormick F. β-Catenin regulates expression of cyclin D1 in colon carcinoma cells. Nature 1999;398:422–6.

24. Crawford HC, Fingleton BM, Rudolph-Owen LA, et al. The metalloproteinase matrilysin is a target of β-catenin transactivation in intestinal tumors. Oncogene 1999;18:2883–91.

25. Takahashi M, Tsunoda T, Seiki M, et al. Identification of membrane-type matrix metalloproteinase-1 as a target of the β-catenin/Tcf4 complex in human colorectal cancers. Oncogene 2002;21:5861–7.

26. Wielenga VJ, Smits R, Korinek V, et al. Expression of CD44 in Apc and Tcf mutant mice implies regulation by the WNT pathway. Am J Pathol 1999;154:515–23.

27. Boon EM, van der Neut R, van de Wetering M, et al. Wnt signaling regulates expression of the receptor tyrosine kinase met in colorectal cancer. Cancer Res 2002;62:5126–8.

28. Hlubek F, Jung A, Kotzor N, et al. Expression of the invasion factor laminin γ2 in colorectal carcinomas is regulated by β-catenin. Cancer Res 2001;61:8089–93.

29. Ertongur S, Lang S, Mack B, et al. Inhibition of the invasion capacity of carcinoma cells by WX-UK1, a novel synthetic inhibitor of the urokinase-type plasminogen activator system. Int J Cancer 2004;110:815–24.

30. Kaneko K, Yano M, Yamano T, et al. Detection of peritoneal micrometastases of gastric carcinoma with green fluorescent protein and carcinoembryonic antigen promoter. Cancer Res 2001;61:5570–4.

31. Ogawa T, Tsubota Y, Maeda M, et al. Regulation of biological activity of laminin-5 by proteolytic processing of γ2 chain. J Cell Biochem 2004;92:701–14.

32. Koshikawa N, Minegishi T, Sharabi A, et al. Membrane-type matrix metalloproteinase-1 (MT1-MMP) is a processing enzyme for human laminin γ2 chain. J Biol Chem 2005;280:88–93.

33. Suzuki M, Kobayashi H, Kanayama N, Nishida T, Takigawa M, Terao T. CD44 stimulation by fragmented hyaluronic acid induces upregulation and tyrosine phosphorylation of c-Met receptor protein in human chondrosarcoma cells. Biochim Biophys Acta 2002;1591:37–44.

34. Zhai Y, Wu R, Schwartz DR, et al. Role of β-catenin/T-cell factor-regulated genes in ovarian endometrioid adenocarcinomas. Am J Pathol 2002;160:1229–38.

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Clin Cancer Res 2006;12:3541-3548.