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TBX19 is overexpressed in colorectal cancer and associated with lymph node metastasis

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Abstract

The *T-box 19* (*TBX19*) gene encodes a transcription factor characterized by a highly conserved DNA-binding motif (T-box). Recent studies have revealed that *TBX19* has been identified as one of the genes activated by *KRAS* mutations, and is upregulated in colon adenoma. These results indicate that *TBX19* may work as an oncogene in colorectal cancer (CRC). However, the expression and role of *TBX19* have yet to be investigated. Here, we investigated *TBX19* mRNA and protein expressions in colon cancer cells or surgically resected CRC. We found that *TBX19* mRNA expression was significantly increased in tumorous tissues compared to that in non-tumorous tissues, and increased *TBX19* mRNA expression was associated with positive lymph node metastasis in our cohort. The expression of *TBX19* mRNA was not correlated with that of TBX19 protein in tissue sample taken from the CRC patients. Moreover, TBX19 showed positive staining even in the normal colonic tissues and the adjacent non-tumorous tissues. These results suggest that the expression of TBX19 protein is not correlated with the expression of *TBX19* mRNA. In addition, our results promote further investigations into the impact of *TBX19* upregulation on colorectal carcinogenesis, as well as the underlying mechanisms.

Key words : TBX19, colorectal cancer, lymph node metastasis, diagnostic biomarker, cytoplasmic granular signal

Introduction

Colorectal cancer (CRC) is the third most common type of cancer that occurs in both men and women all over the world[1,2)]. One of the most important molecular pathogeneses of colorectal carcinogenesis is the adenoma-carcinoma sequence[1)]. The adenoma-carcinoma sequence is stepwise genetic aberrations, including *APC*, *KRAS* and *TP53* mutations, in CRC patients[3)]. *APC* mutations are an early event in this multistep process, followed by *KRAS*-activating mutations and *TP53*-inactivating mutations[3)]. While chemotherapy and molecular targeting drugs for CRC treatment have progressed recently, genetic aberrations among *APC*, *KRAS*, and *TP53* are not therapeutic targets[4,5)]. It is particularly necessary to develop therapeutic agents for *KRAS* mutations as they are frequently detected in CRC patients, as well as other malignant tumors, to improve cancer mortality[6)]. Although there have been several studies on the development of targeted drugs for *KRAS* mutations[7-9)], they have not yet been used in a clinical setting.

The T-box (*TBX*) gene family encodes a large family of transcription factors and plays a fundamental role in early embryogenesis during the develop-

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mental process[10]. Several studies have revealed aberrations of *TBX* genes in inherited human disorders, such as *TBX1* mutation in DiGeorge syndrome, *TBX3* mutation in Ulnar-Mammary syndrome, *TBX5* mutation in Holt-Oram syndrome, and *TBX22* mutation in cleft palate with ankyloglossia[11]. In addition, recent studies have also found that *TBX* genes may be associated with cancer development in various malignant tumors[12]. *TBX2* and *TBX3*, which are downstream targets of the Wnt/beta-catenin pathway, are frequently mutated in ovarian cancer and are amplified in breast cancer. Furthermore, they are associated with hepatocellular carcinoma, pancreatic cancer, and malignant melanoma[13-18].

In the present study, we focused on the analysis of *TBX19*, which is expressed in the rostral ventral diencephalon and pituitary gland[19]. *TBX19* mutations lead to a lack of adrenocorticotrophin resulting in adrenal insufficiency[20]. On the other hand, *TBX19* has been identified as one of the genes activated by *KRAS* mutation, and is upregulated in colon adenoma[21,22]. These results indicate that *TBX19* might work as an oncogene in CRC, but the expression and role of *TBX19* in CRC remain unknown. Here, we investigated *TBX19* mRNA and protein expressions in surgically resected CRC tissues, and examined the biological significance.

Materials and Methods

Clinical samples of patients

A total of 89 surgical specimens obtained from CRC patients who had undergone surgical resection at Fukushima Medical University Hospital between January 2008 and December 2010 were used for the experiments. All 89 cases are used for comprehensive gene expression analysis, 5 cases are used for protein expression analysis by western blotting, and 54 cases are used for immunohistochemical (IHC) staining. In addition, 3 cases of adenoma were used for IHC staining. Information regarding age, sex, TNM stage, and pathological diagnosis, including lymphatic and venous invasion, were retrospectively collected. The carcinomas at the time of primary tumor resection were staged according to the Union for International Cancer Control UICC classification (the 7th classification)[23,24]. Written informed consent was obtained from all patients. This study was approved by the ethics committee of Fukushima Medical University.

Comprehensive gene expression analysis

TBX19 expression data were obtained using custom microarray analysis as previously described[25,26]. In brief, the surgical specimen was homogenized and mixed with ISOGEN reagent (NIPPON GENE, Tokyo, Japan). Total RNA was subjected to purification of polyA(A)+RNA using MicroPoly(A) Purist Kit (Thermo Fisher Scientific, Waltham, MA, USA). The human common reference RNA was prepared by mixing equal amounts of poly(A)+ RNA extracted from 22 human cancer cell lines (A431, A549, AKI, HBL-100, HeLa, HepG2, HL60, IMR-32, Jurkat, K562, KP4, MKN7, NK-92, Raji, RD, Saos-2, SK-N-MC, SW-13, T24, U251, U937, and Y79).

Synthetic polynucleotides (80-mers) representing 31,797 human transcripts (MicroDiagnostic, Tokyo, Japan) were arrayed on aminosilane-coated glass slides with a custom-made arrayer. RNA (2 μg) was subjected to reverse transcription with SuperScript II (Thermo Fisher Scientific). Sample RNA was labeled using Cyanine 5-dUTP (Perkin-Elmer, Boston, MA, USA) and reference RNA was labeled using Cyanine 3-dUTP. Hybridization was performed with a labeling and hybridization kit (MicroDiagnostic). Signals were measured with a GenePix 4000B scanner (Axon Instruments, Union City, CA, USA) and then processed into primary expression ratios. The primary expression ratios were then converted into log2 values and compiled into a matrix. We assigned an expression ratio of 1 (log ratio of 0) for spots that exhibited fluorescence intensities under the detection limits, and we included these in the signal calculation of the mean averages. Data were processed by MDI gene expression analysis software package (MicroDiagnostic).

Cell line culture

The colon cancer cell lines used in this study were originally obtained from the American Type Culture Collection (Rockville, MD, USA) and were cultured in the recommended media with 10% fetal bovine serum. These monolayer cells were maintained in a 37°C incubator with 5% CO_2. Cells were checked regularly under a light microscope and subcultured once they had reached 80% to 90% confluence.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR)

Total RNA was extracted from cells using

TRIzol reagent (Thermo Fisher Scientific) according to the manufacturer's instructions as previously described[27]. Complementary DNA (cDNA) was synthesized from 5 μg of total RNA with a random hexamer using the SuperScript III First-Strand Synthesis System (Thermo Fisher Scientific). These cDNAs were used for the measurement of gene expression with a 7500 Real-time PCR system (Thermo Fisher Scientific) using TaqMan probes. The assessors were blinded to patient information and performed experiments in triplicate. Taqman expression assays were purchased from Thermo Fisher Scientific ; *TBX19* (Hs01113611_m1) and β-actin (Hs99999903_m1). β-actin was used as an internal control. Relative *TBX19* gene expression was calculated using the 2-ΔΔCT method, according to the supplier's protocol (Thermo Fisher Scientific)[28].

Western blotting

Cancer cell lines and surgical specimens were homogenized in a 100 mM Tris-HCl (pH 7.6) buffer containing 0.15 M NaCl, 5 mM EDTA, 1% Triton X-100, 5% glycerol by Polytron PT3100 homogenizer (Kinematica AG, Luzern, Switzerland). After centrifugation at 17,400 xg for 15 min at 4°C, the supernatants were collected. Then, 20 ug of each protein sample was run on SDS-polyacrylamide gels (5-15% gradient ; Thermo Fisher Scientific) and blotted onto Immun-Blot PVDF membranes (Bio-Rad Laboratories, Hercules, CA, USA). The blotted membranes were incubated with the indicated primary antibodies overnight at 4°C. Rabbit monoclonal anti-TBX19 (HPA005800, Sigma-Aldrich, St. Louis, MO, USA) was used at 1 : 500 dilution, while mouse monoclonal anti-β-actin antibody (sc-69879, Santa Cruz Biotechnology, USA) was used as a loading control at 1 : 2,500 dilution. The blotted membranes were then incubated with the appropriate horseradish peroxidase (HRP)-conjugated goat-anti-mouse IgG (sc-2005, Santa Cruz Biotechnology) secondary antibody at a dilution of 1 : 5,000. Signals were detected by ImageQuant LAS4000 (GE Healthcare Bio-sciences, Pittsburgh, PA, USA) using SuperSignal West Pico Chemiluminescent Substrate (Thermo Fisher Scientific).

Immunohistochemical staining and evaluation

IHC staining was carried out on paraffin-embedded histological sections (4 μm thick) using a polymer peroxidase method. Briefly, after deparaffinization and rehydration, the sections were treated with 0.3% hydrogen peroxide in methanol for 30 min to block endogenous peroxidase activity. Following rinsing in phosphate-buffered saline (PBS) (Thermo Fisher Scientific), the sections were incubated with anti-TBX19 antibody (HPA005800, 1 : 2,000 dilution ; Sigma-Aldrich) at 4°C overnight. Three further washes (5 min per wash) in PBS was followed by treatment with a peroxidase-labeled polymer, conjugated to goat anti-rabbit immunoglobulins (Dako EnVision+System-HRP Labelled Polymer ; ready-to-use ; #K4003 ; Dako ; Agilent Technologies), as the secondary antibody for 30 min at room temperature. The staining was visualized with diaminobenzidine, followed by counterstaining with hematoxylin. Expression of these proteins was evaluated as positive when the nucleus of tumorous tissues and the total field of view were observed at 400×magnification. We evaluated the staining of each specimen. The rate of positively stained cells was counted among three randomly selected fields (200 μm×200 μm) in the tumorous and non-tumorous tissues. Cytoplasmic granular signals were counted in the tumorous tissues.

Statistical Analysis

Data were presented as the mean±SD. Statistical analyses were performed using GraphPad Prism 6 software (GraphPad Software, La Jolla, CA, USA). The Mann-Whitney U test and Wilcoxon matched pairs test were used for comparison of the means of the two groups and Kruskal-Wallis test was used for comparison of more than two groups. Log-rank test was used for survival comparisons. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Expression analysis of TBX19 from the comprehensive analysis data

Firstly, the mRNA level of *TBX19* in the CRC specimen was determined by using comprehensive gene expression analysis data. The expression ratios of *TBX19* were compared between 89 tumorous tissue samples and 60 non-tumorous tissue samples, which revealed that *TBX19* had a significantly higher expression in tumorous tissues compared to non-tumorous tissues ($P<0.0001$, Mann-Whitney's U-test) (Fig. 1A). Of note, among available 40 pairs of non-tumorous and tumorous tissues, upregulation of *TBX19* was also observed in the tumorous tissues compared to the non-tumorous tissues ($P<0.0001$, Wilcoxon matched pairs test) (Fig. 1B).

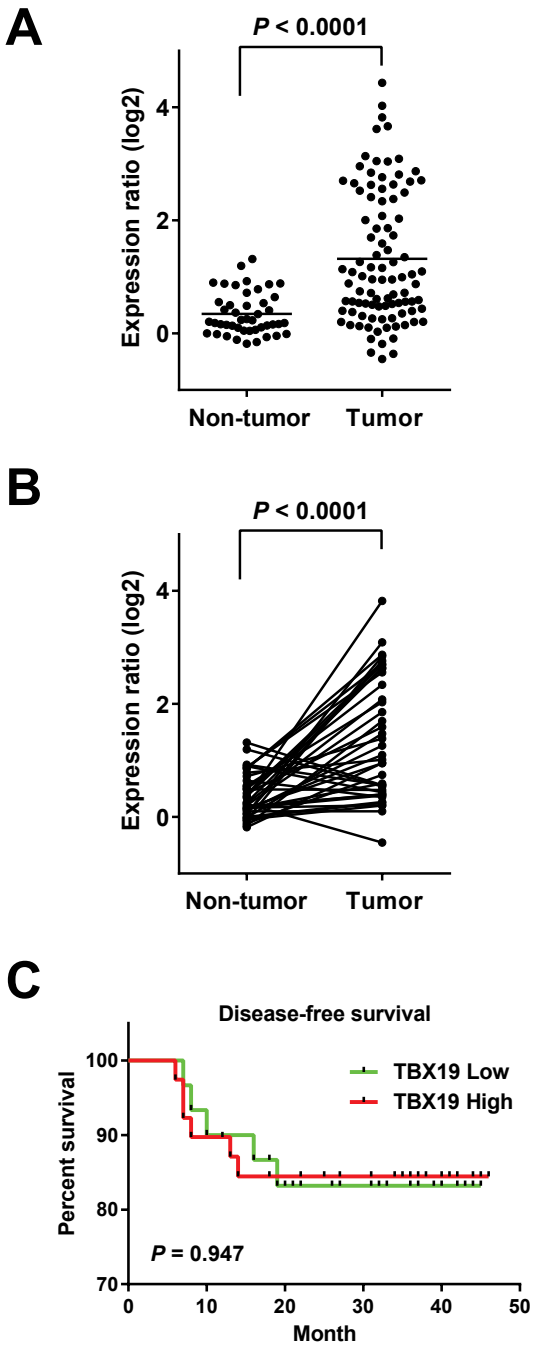

Fig. 1. The mRNA expression of *TBX19* in CRC specimens. (A) Expression differences of *TBX19* between 89 tumorous and 60 non-tumorous tissues from our CRC cohort. Dot plot represents *TBX19* expression from microarray analysis. The expression level on the log2 scale is shown. Horizontal bars indicate mean expression values. $P<0.0001$, the Mann-Whitney U-test. (B) *TBX19* expression differences in each of 40 pairs tumorous and non-tumorous tissues. Dot plot represents *TBX19* expression from microarray analysis. The expression level on the log2 scale is shown. $P<0.0001$, Wilcoxon matched pairs test. (C) Kaplan-Meier survival of 89 cases in our cohort stratified by *TBX19* tumor expression. $P=0.947$, log-rank test.

Next, we analyzed *TBX19* expression levels with clinicopathological factors in the CRC specimens (Table 1). The case with positive lymph node metastasis showed significantly higher expression of *TBX19* ($P=0.012$). However, *TBX19* expression level was not found to be associated with age, gender, TNM stage, histology, tumor depth, lymphatic invasion, venous invasion, or distant metastasis. Kaplan-Meier analysis demonstrated no association between increased *TBX19* levels and relapse-free survival ($P=0.9473$, log-rank test) (Fig. 1C).

Table 1. Clinicopathological factors and *TBX19* mRNA expression

	$n=89$	*TBX19* mRNA expression ratio	*P*-value
Age			
<65	35	1.2±1.0	0.54
≥65	54	1.3±1.1	
Gender			
Male	60	1.2±1.0	0.284
Female	29	1.4±1.1	
TNM classification[a]			
I	18	1.1±1.2	0.366
II	31	1.2±1.0	
III	27	1.5±1.0	
IV	13	1.4±1.1	
Histology			
tub1[b]	38	1.4±1.0	0.096
tub2[c]	40	1.3±1.0	
Other[d]	11	0.8±1.0	
T factor			
T1	8	1.0±0.8	0.62
T2	17	1.5±1.2	
T3	62	1.3±1.1	
T4	2	0.5±0.03	
Lymphatic invasion			
Absent	21	1.1±1.1	0.178
Present	68	1.3±1.0	
Venous invasion			
Absent	16	1.3±1.2	0.802
Present	73	1.3±1.0	
Lymph node metastasis			
Negative	54	1.1±1.0	0.012
Positive	35	1.6±1.0	
Distant metastasis			
M0	77	1.2±1.0	0.552
M1	12	1.4±1.1	

Values are expressed as the mean±standard deviation. [a]UICC TNM 7th classification[23,24], [b]Well differentiated tubular adenocarcinoma. [c]Moderately differentiated tubular adenocarcinoma. [d]Solid-type pooly differentiated adenocarcinoma, mucinous adenocarcinoma, papillary adenocarcinoma or adenosquamous carcinoma. *P*-values were calculated using Mann-Whitney test or a Kruskal-Wallis test, where appropriate.

TBX19 mRNA and protein expression

To further confirm that *TBX19* mRNA expression is upregulated in CRC, we investigated *TBX19* expression in 7 colon cancer cell lines. *TBX19* mRNA expression was investigated by real-time PCR, and TBX19 protein expression was investigated by western blotting for the 7 colon cancer cells (Fig. 2A). Consistent with *TBX19* mRNA expression, TBX19 protein expression was upregulated in the LS180, LS174T, and SW837 cells. Then, we analyzed TBX19 protein expression by western blotting in three representative non-tumorous/tumorous CRC tissues that showed high *TBX19* mRNA expression (Fig. 2B). However, TBX19 protein was not highly expressed in the tumorous tissues compared to the non-tumorous tissues. These results indicate that the upregulation of *TBX19* mRNA did not result in upregulation of TBX19 protein expression.

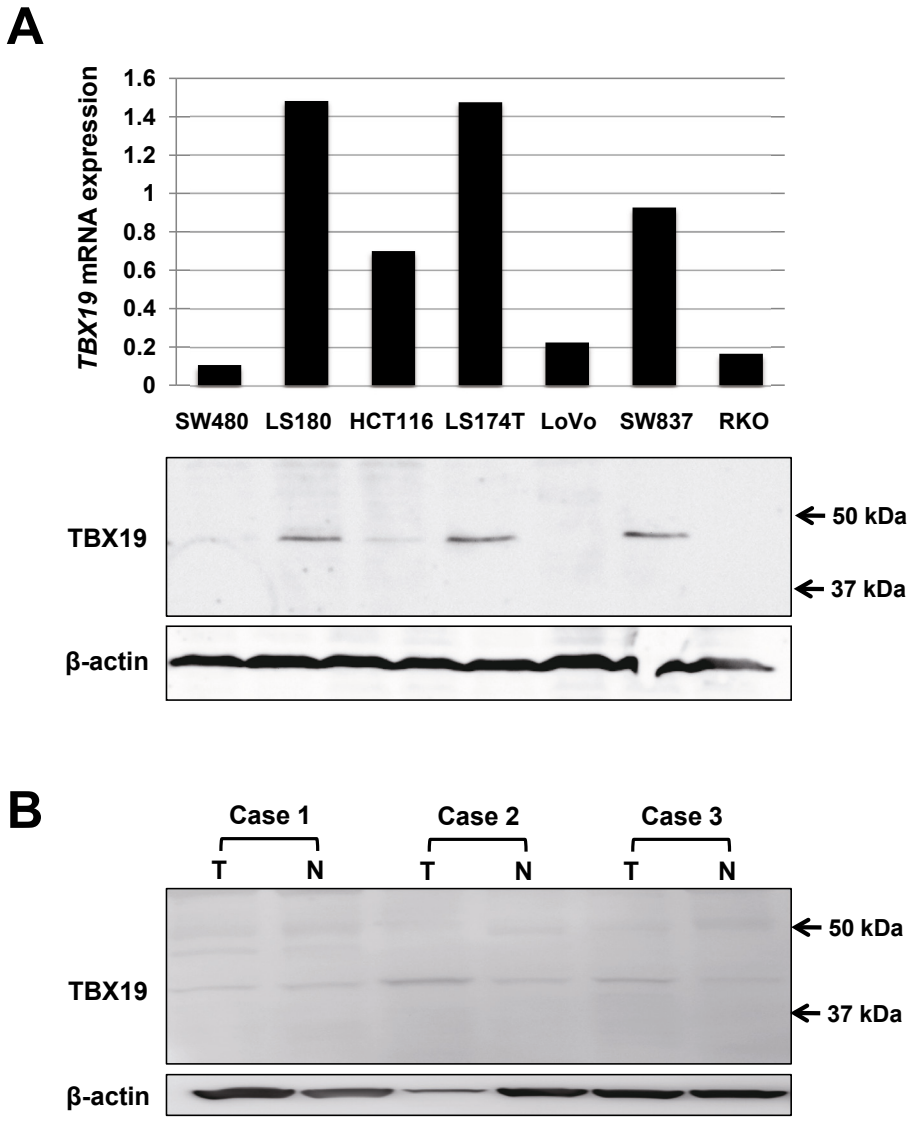

Fig. 2. The expression of TBX19 in colon cancer cell lines and CRC specimens. (A) *TBX19* expression analyses of mRNA by real-time PCR and protein by western blot in 7 colon cancer cells. Relative *TBX19* mRNA expression levels are shown in the upper panel (normalized to β-actin). TBX19 protein expressions are shown in the lower panel. β-actin was used as a loading control. (B) TBX19 protein expression analysis by western blot in 3 CRC patients. β-actin was used as a loading control.

IHC staining for TBX19

Next, we performed IHC staining for TBX19 in 3 adenoma and 54 CRC specimens. While normal colonic mucosa exhibited weak TBX19 staining (Fig. 3A), all 3 adenoma showed positive TBX19 staining (Fig. 3B). In the CRC specimens, TBX19 expression was observed in the nucleus of tumorous and adjacent non-tumorous cells (Fig. 3C). When we assessed TBX19 staining intensity, the tumorous tissue samples showed a higher percentage of positive TBX19 cells compared to the non-tumorous tissue samples (Fig. 3D). However, this staining intensity was not associated with any clinicopathological factors in our cohort (Table 2). We additionally performed TBX19 staining for metastatic liver cancer from CRC. The metastatic liver tumor showed positive staining for TBX19, but normal liver tissue showed negative TBX19 staining (Fig. 3E). These results suggested that TBX19 was specifically upregulated in CRC cells.

To further understand TBX19 staining, we focused on the cytoplasmic granular signals in CRC cells (Fig. 3F). While the cytoplasmic granular signals in cytoplasmic tumor cells were not observed in the normal tissue, they were observed in 15 of the 54 (27.8%) CRC tissues. The signals tended to be observed in undifferentiated histological types, such

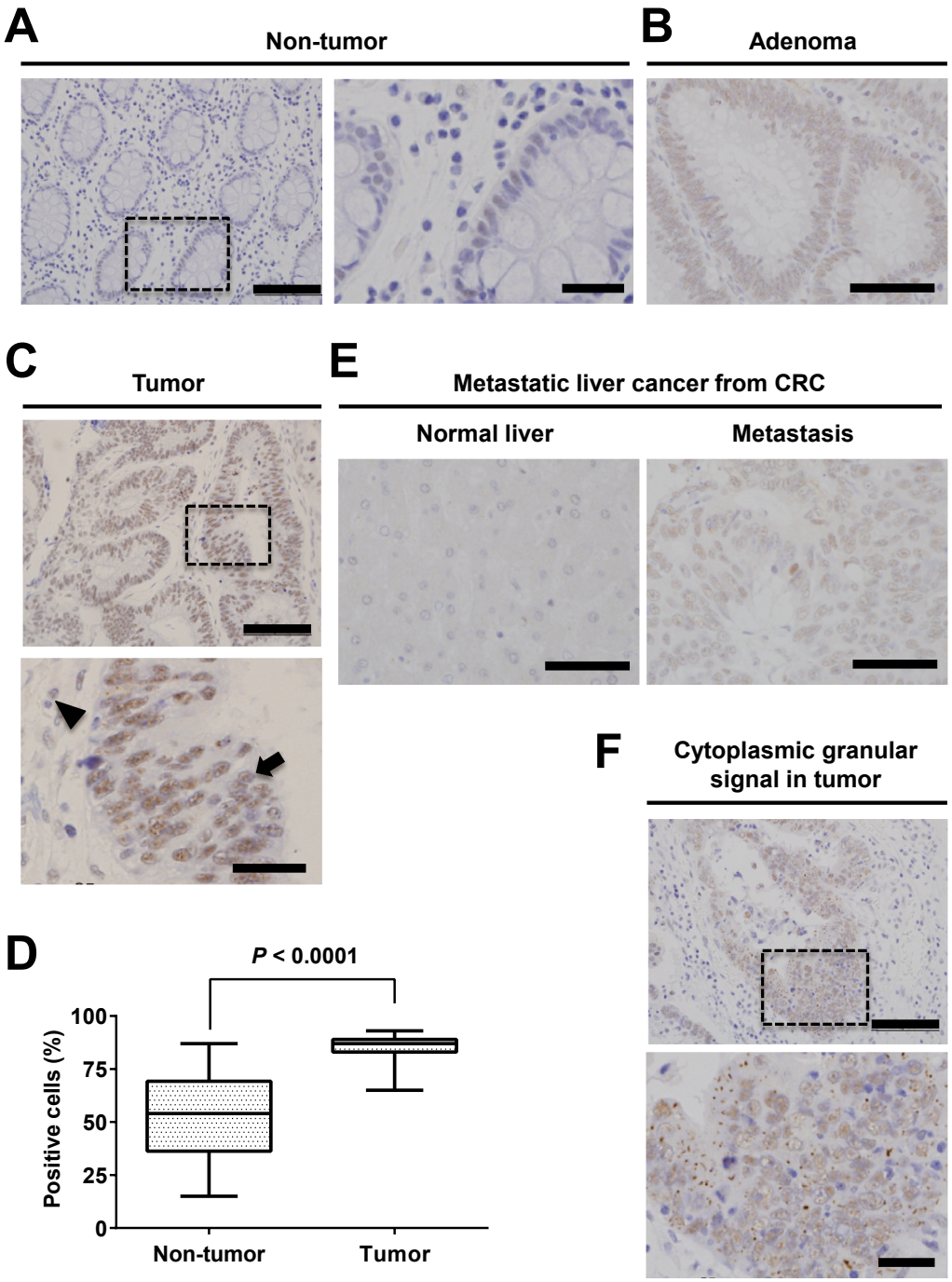

Fig. 3. Immunohistochemical staining of TBX19. Representative images of TBX19 staining in non-tumorous tissue (A), adenoma (B), CRC tissue (C and F), and metastatic liver cancer from CRC (E). (A) Weak positive staining for TBX19 in non-tumorous tissue. The boxed area is shown at higher magnification. Scale bars=100 μm (left) and 25 μm (right). (B) Positive staining for TBX19 in adenoma. Scale bar=50 μm. (C) Positive staining for TBX19 in tumorous cells (arrow) and weak positive staining for TBX19 in adjacent non-tumorous cells (arrow head). Scale bars=100 μm (upper) and 25 μm (lower). (D) Difference in percentage of TBX19-positive staining cells between 89 tumorous and 60 non-tumorous tissues from our cohort. $P<0.0001$, the Mann-Whitney U-test. (E) Weak positive staining for TBX19 in normal liver tissue and positive staining for TBX19 in metastatic liver cancer from CRC. Scale bars=50 μm. (F) Cytoplasmic granular signal of TBX19 in CRC. Scale bars=100 μm (upper) and 25 μm (lower).

as poorly differentiated or mucinous adenocarcinoma (Table 3). These results further suggest that *TBX19* mRNA was upregulated in CRC and associated with worse CRC outcomes.

Table 2. Clinicopathological factors and TBX19 IHC expression

		TBX19 IHC	
	$n=54$	% of positive cells	P-value
Age			
<65	22	90.0±5.0	0.699
≥65	32	87.4±9.2	
Gender			
Male	36	88.2±7.9	0.673
Female	18	89.0±7.7	
TNM classification[a]			
I	15	87.6±10.3	0.805
II	17	87.8±9.1	
III	15	88.7±4.8	
IV	7	91.1±1.8	
Histology			
tub1[b]	19	89.3±7.1	0.283
tub2[c]	23	86.3±9.5	
Other[d]	12	91.2±3.5	
Lymph node metastasis			
Absent	33	87.8±9.4	0.478
Present	21	89.4±4.3	
Distant metastasis			
Absent	47	88.0±8.3	0.787
Present	7	91.1±1.8	

Values are expressed as the mean±standard deviation. [a]UICC TNM 7th classification[23,24], [b]Well differentiated tubular adenocarcinoma. [c]Moderately differentiated tubular adenocarcinoma. [d]Solid-type pooly differentiated adenocarcinoma, mucinous adenocarcinoma, papillary adenocarcinoma or adenosquamous carcinoma. P-values were calculated using Mann-Whitney test or a Kruskal-Wallis test, where appropriate.

Discussion

In the present study, we found that *TBX19* mRNA was upregulated in CRC and increased expression of *TBX19* mRNA was associated with positive lymph node metastasis in CRC patients. On the contrary, TBX19 protein expression was not upregulated in CRC and was not associated with any clinicopathological factors in our cohort. Therefore, our study shows that, while upregulated *TBX19* mRNA may have a pivotal role in colon tumorigenesis, the role of TBX19 protein in colonic tumorigenesis is still unknown and need a further consideration. Furthermore, our results also suggest that the expression of TBX19 protein was not correlated with *TBX19* mRNA expression. This was further confirmed by the result that the CRC patients, which showed high *TBX19* mRNA expression, did not exhibit higher TBX19 protein expression in the tumorous tissues than in the non-tumorous tissues by Western blotting in our small number of cases. Even in the normal colonic tissues and the adjacent non-tumorous tissues showed weak positive staining for TBX19, suggesting that TBX19 protein may have a role in keeping normal mucosal homeostasis and in affecting CRC tumor development. Therefore, the evaluation of TBX19 in CRC by IHC staining requires further investigation. Of course, the evaluation of sensitivity and specificity for anti-TBX19 antibody remains to be required. When observing IHC staining, we were interested in the cytoplasmic granular signals in the tumor cells and found that they tended to associate with tumor differentiation in our cohort. To date, because the mechanism for the formation of granular signals in tumor cells has not yet been fully understood, further morphological and functional studies are required.

Consistent with previous reports, TBX19 was also upregulated in adenoma[21,22]. Because adenoma is considered to be a type of pre-cancerous tumor, we believe that TBX19 plays a role in the ade-

Table 3. Clinicopathological factors and cytoplasmic granular signal

		Cytoplasmic granular signal		
	Total (n=54)	Positive (n=15)	Negative (n=39)	P-value
Age				
<65	22	5 (33.3%)	17 (43.6%)	0.551
≥65	32	10 (66.7%)	22 (56.4%)	
Gender				
Male	36	10 (66.7%)	26 (66.7%)	1.000
Female	18	5 (33.3%)	13 (33.3%)	
TNM classification[a]				
I	15	2 (13.3%)	13 (33.3%)	0.086
II	17	6 (40.0%)	11 (28.2%)	
III	15	5 (33.3%)	10 (25.6%)	
IV	7	2 (13.3%)	5 (12.8%)	
Histology				
tub1[b]	19	3 (20.0%)	16 (41.0%)	0.013
tub2[c]	23	5 (33.3%)	18 (46.2%)	
Other[d]	12	7 (46.7%)	5 (12.8%)	
Lymph node metastasis				
Absent	33	9 (60.0%)	24 (61.5%)	1.000
Present	21	6 (40.0%)	15 (38.5%)	
Distant metastasis				
Absent	47	13 (86.7%)	34 (87.2%)	1.000
Present	7	2 (13.3%)	5 (12.8%)	

[a]UICC TNM 7th classification[23,24], [b]Well differentiated tubular adenocarcinoma. [c]Moderately differentiated tubular adenocarcinoma. [d]Solid-type pooly differentiated adenocarcinoma, mucinous adenocarcinoma, papillary adenocarcinoma or adenosquamous carcinoma. P-values were calculated using Mann-Whitney test or a Kruskal-Wallis test, where appropriate.

noma-carcinoma sequence. A previous *in vitro* experiment revealed that *TBX19* is one of the downstream genes that are activated by *KRAS* mutations[21]. In fact, *TBX19* was highly expressed in most colorectal adenomas with activated *KRAS* mutations[22]. *KRAS* mutations are one of the steps in the adenoma-carcinoma sequence that accumulate several genetic aberrations in CRC. Therefore, because *KRAS* mutations occur in both colorectal adenoma and cancer, it is understandable that *TBX19* expression is upregulated in CRC as well as in adenoma cases. In CRC treatment, the mutational status of *KRAS* is only used as a predictive marker for the effectiveness of anti-EGFR antibodies, cetuximab, or panitumumab[29]. This is due to *KRAS* being a downstream effector of *EGFR*. In addition, and most importantly, *KRAS* mutations have not yet to be a direct druggable target. *KRAS* mutations are the most frequently occurring aberrations in human cancer, including CRC[6,30]. In spite of huge efforts to develop molecular targeting drugs for *KRAS* mutations[7], no useful agents have yet been included in clinical treatment strategies for CRC. With recent progress in the understanding of *KRAS* biology, *KRAS* has been considered as druggable by either targeting the mutations directly or targeting the downstream pathways, such as RAF-MAPK and PIK3K[31]. Of note, because *TBX19* is also downstream of *KRAS*, further experimental or mice studies investigating the functional role of *TBX19* may provide a therapeutic opportunity for *KRAS*-driven cancers.

In conclusion, the current study reports the *TBX19* expression status in CRC. Our results suggest that *TBX19* as a candidate therapeutic target for CRC.

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Conflict of Interest

The authors declare that there is no conflict of interest.

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