

Expression of TMED3 is independently associated with colorectal cancer prognosis

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Abstract. Colorectal cancer (CRC) is the third most common cancer type and one of the deadliest cancers worldwide. Transmembrane p24 trafficking protein 3 (TMED3) has previously been indicated to suppress CRC metastasis, but its clinical significance has remained undetermined. In the present study, the expression of TMED3 was indicated to be elevated at the mRNA and protein levels in CRC tumor samples relative to that in para-cancerous healthy tissue samples ($P < 0.05$). Furthermore, Kaplan-Meier survival analysis revealed a significant negative association between elevated TMED3 protein levels and overall survival of patients with CRC ($P < 0.001$, log-rank test). Multivariate Cox regression analysis additionally determined that elevated TMED3 expression in primary CRC tumors was an independent predictor of poor prognosis ($P < 0.05$). These results revealed that elevated TMED3 expression in CRC was associated with patient survival outcomes, suggesting that TMED3 may be a potential prognostic biomarker for this cancer type.

Introduction

Colorectal cancer (CRC) remains one of the most common malignancies worldwide, with over 1 million cases diagnosed in 2014. While patients with early stage CRC have a 5-year survival rate of 90%, those with advanced disease have a

survival rate of just 13% (1). The mechanistic basis of CRC development and progression remains to be fully elucidated and numerous patients exhibit various associated risk factors contributing to disease onset (2). CRC also remains the fourth deadliest cancer type, although there have been significant reductions in average mortality rates for patients with CRC in recent decades owing to diagnostic and therapeutic innovations (3). Poor outcomes of CRC are most frequently a result of tumor metastasis or the acquisition of a drug-resistant form of the disease, and such phenotypes arise from cancer-associated gene dysregulation (2,4-6). In this light, a better understanding of the genes associated with CRC development may be of value. In addition, it is essential that novel diagnostic and prognostic biomarkers of CRC are identified in order to better screen for this deadly disease and to predict its progression in affected individuals.

Transmembrane p24 trafficking protein 3 [also known as transmembrane Emp24 protein transport domain containing 3 (TMED3)] is an important protein associated with both innate immune functionality and protein trafficking within the vesicles of cells (7). There are 10 known TMED family proteins (8), and most of them have been studied in tumor-associated contexts (9-12), whereas TMED3 has only been studied in select instances wherein its role in the development and progression of prostate (13), colon (14) and liver (15) cancers was examined. TMED3 belongs to a family of p24 proteins involved in selecting cargo in coat protein complex vesicles in the secretory endoplasmic reticulum-Golgi network (16). Given the large diversity of cargo and the existence of only 10 TMED p24 proteins, it is likely that each is able to affect multiple secretion events in direct and indirect context-dependent manners. Furthermore, p24 proteins may exist as monomers or dynamic complexes where one is able to affect the stability of others (17-20). They appear to be non-redundant (21) and affect multiple signaling pathways in mammalian cells (10,22,23). In flies and mammals, specific TMED proteins control WNT secretion (14,24-26). These studies all suggested that TMED3 is related to these tumorigenic processes. Previous studies have reported that TMED3 act as metastatic suppressors in human colorectal cancer cells through the WNT-TCF pathway (14,27). The importance of this gene in CRC, however, has remained largely elusive, with its association with patient prognosis being completely

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Abbreviations: CRC, colorectal cancer; TMED3, transmembrane p24 trafficking protein 3; FFPE, formalin-fixed paraffin-embedded; IHC, immunohistochemistry; CA19-9, carbohydrate antigen 19-9; CEA, carcinoembryonic antigen; OS, overall survival; RFS, recurrence-free survival

Key words: transmembrane p24 trafficking protein 3, colorectal cancer, biomarker, multivariate Cox regression analysis, prognosis

undetermined. Thus, in the present study, TMED3 expression was examined at the protein level in CRC and normal para-cancerous tissue samples, and furthermore, the relationship of TMED3 with clinicopathological findings and survival outcomes in these patients was assessed.

Patients and methods

Patients and samples. Between June 2006 and March 2009, a total of 176 formalin-fixed paraffin-embedded (FFPE) pairs of tumor and normal para-cancerous tissue samples were collected from patients with stage I-III CRC undergoing curative surgery at Changhai Hospital, Second Military Medical University (Shanghai, China). These samples were archived for immunohistochemistry (IHC) analyses and the CRC diagnosis was confirmed by pathological examination of all samples. Patients were not included in the present study cohort if they had received prior anti-cancer therapy, suffered from abnormal cardiac, lung, liver or renal function, had been previously diagnosed with other cancers or died due to other causes. The clinicopathological characteristics of this patient cohort are compiled in Table I.

An additional cohort of independent samples was obtained between April and October 2013 from 63 patients with stage I-III CRC undergoing curative surgery at this same institution. These samples were stored for reverse transcription-quantitative (RT-q) PCR analyses. The clinicopathological characteristics of this patient cohort are provided in Table II.

All patients provided written informed consent and this study conformed to the Declaration of Helsinki and was approved by the Institutional Ethics Committee of Changhai Hospital, Second Military Medical University (Shanghai, China; ethics approval no. 2017-148-01).

RT-qPCR. TRIzol (Invitrogen; Thermo Fisher Scientific, Inc.) was used to extract total RNA from individual samples, and total RNA (1 μ g) was used for RT with PrimeScript™ RT reagent kit (Takara Bio, Inc.) according to the manufacturer's protocol, using a thermal cycler (i-Cycler; Bio-Rad Laboratories, Inc.). The RT reaction was conducted in 40 μ l reaction buffer at 37°C for 15 min and terminated by heating at 85°C for 5 sec, followed by cooling at 4°C. qPCR was performed with a 7500 Real-time PCR system (Applied Biosystems; Thermo Fisher Scientific, Inc.) and SYBR Premix Ex Taq™ (Takara Bio, Inc.) according to the manufacturer's protocol. PCR was performed at 95°C for 10 sec, followed by 40 cycles of 95°C for 5 sec and 60°C for 34 sec. Dissociation was initiated at 95°C for 15 sec followed by 60°C for 1 min and 95°C for 15 sec. GAPDH served as an internal standard. The comparative 2^{- $\Delta\Delta$ C_q} method was used to determine the relative gene expression (28). Primers were as follows: TMED3, forward 5'-GGGTTCTGTACCTGAGGAAA-3' and reverse 5'-CACCGAGGGTGAGCAGAT-3'; GAPDH, forward 5'-TGTGGCATCAATGGATTGG-3' and reverse 5'-ACACCATGTATCCGGGTCAAT-3'.

Immunohistochemistry (IHC). IHC was performed as in previous studies (29). In brief, sections were heated and then probed with anti-TMED3 antibody (1:50; cat. no. ab151056; Abcam) for 60 min at 37°C, followed by a 15 min incubation with HRP-conjugated goat anti-rabbit polyclonal antibody solution (ready to use; cat. no. SP-9001; OriGene Technologies,

Inc.) at room temperature for 15 min. Hematoxylin was then used to counterstain samples and diaminobenzidine was used for sample development. A total of three independent pathologists blinded to the patient characteristics then examined and scored individual samples. H-scores (30) were then assigned at x200 magnification, with samples receiving scores of 0, 1, 2 or 3 corresponding to negative, weak, intermediate or strong staining, respectively. Numbers of cells per field of view with a particular staining intensity were then quantified, with H-scores being assigned based on the following formula: H-score = (% of cells with staining strength 1x1) + (% of cells with staining strength 2x2) + (% of cells with staining strength 3x3). The final scores ranged from 0-300, with 0 corresponding to 100% of cells being negative for the antigen of interest and 300 corresponding to 100% strong staining for that antigen. Median H-score values were used to stratify patients into low- and high-expressing groups.

Follow-up of cases. The patients enrolled in the present study were followed up from the date of surgery until death or most recent follow-up, with those remaining alive as of the last follow-up being censored. None was lost to follow-up. Overall survival (OS) was determined based on the period of time between surgery and death, while recurrence-free survival (RFS) was determined as the period of time between surgery and CRC recurrence, with patients not exhibiting recurrence being censored on the date of death or most recent follow-up. The 7th edition of the tumor-node-metastasis (TNM) system was used to stage patients' tumors based on the criteria defined by the American Joint Committee on Cancer Staging (31). All patients with stage III CRC as well as those with stage II disease and either poorly differentiated or pT4 tumors were administered 5-fluorouracil-based adjuvant chemotherapy. In all patients, RFS and OS were calculated monthly through to December 2017.

Online database. In the present study, the gene expression database for normal and tumor tissues (GENT) (<http://medicalgenome.kribb.re.kr/GENT/>; <http://genome.kobic.re.kr/GENT/>) was used.

Statistical analysis. SPSS 24.0 (IBM Corp.) was used for all statistical testing. Differences in TMED3 mRNA expression and H-scores between the CRC tissues and their corresponding normal para-cancerous tissues were analyzed for statistical significance using the paired t-test. The relationship between TMED3 expression and clinicopathological characteristics of patients was assessed using the χ^2 test. Differences in patient overall survival (OS) and recurrence-free survival (RFS) as a function of TMED3 expression were compared via the Kaplan-Meier method, with the significance determined with the log-rank test. A Cox proportional hazards model was used to determine the independent factors of OS and RFS based on variables selected after the univariate analysis. P<0.05 (two-tailed) was considered to indicate statistical significance.

Results

Assessment of TMED3 expression in CRC tissue samples. In the GENT database, significant increases in the expression

Table I. Association between TMED3 protein expression and clinicopathologic characteristics of patients with CRC in the first study cohort (n=176).

Characteristic	Patients (n)	TMED3 protein level		P-value
		High (n=88)	Low (n=88)	
Sex				0.879
Female	77	38	39	
Male	99	50	49	
Age (years)				0.868
<60	51	25	26	
≥60	125	63	62	
Tumor location				0.649
Rectum	79	38	41	
Colon	97	50	47	
Degree of differentiation				0.724
Well + moderate	134	66	68	
Poor	42	22	20	
Tumor size (cm)				0.006
<5	70	26	44	
≥5	106	62	44	
Local invasion				0.013
pT1-T2	134	60	74	
pT3-T4	42	28	14	
Lymph node metastasis				0.003
N0+N1	109	45	64	
N2	67	43	24	
TNM stage				0.282
I + II	105	49	56	
III	71	39	32	
Adjuvant chemotherapy				0.245
No	51	29	22	
Yes	125	59	66	
CA19-9 (kU/l)				0.546
<40	82	39	43	
≥40	94	49	45	
Serum CEA level (ng/ml)				0.544
<10	78	41	37	
≥10	98	47	51	

Pearson's Chi-squared test was used for comparison between subgroups. TMED3, transmembrane p24 trafficking protein 3; CEA, carcinoembryonic antigen; CA, carbohydrate antigen.

of TMED3 at the mRNA level were observed in numerous cancer types, including CRC (Fig. 1A). This thus suggested a potential role for TMED3 as a regulator in the development and/or progression of CRC. This finding was then further confirmed in an independent cohort of patients with CRC (Table II), in which elevated TMED3 expression in CRC

Table II. Clinicopathological characteristics of the second study cohort (n=63) for assessing TMED3 mRNA level.

Characteristic	N
Sex	
Female	30
Male	33
Age (years)	
<60	20
≥60	43
Tumor location	
Rectum	40
Colon	23
Degree of differentiation	
Well + moderate	50
Poor	13
Tumor size (cm)	
<5	38
≥5	25
Local invasion	
pT1-T2	22
pT3-T4	41
Lymph node metastasis	
N0+N1	39
N2	24
TNM stage	
I + II	36
III	27
Adjuvant chemotherapy	
No	26
Yes	37
CA19-9 (kU/l)	
<40	21
≥40	42
Serum CEA level (ng/ml)	
<10	27
≥10	35

CEA, carcinoembryonic antigen; CA, carbohydrate antigen.

tissue samples relative to the levels in normal para-cancerous tissues from the same patients was observed ($P<0.01$; Fig. 1B). Next, an IHC-based approach was used in order to assess TMED3 protein levels in 176 pairs of FFPE CRC and normal para-cancerous tissue sections (Table I). These analyses revealed that TMED3 was localized to both the nucleus and the cytoplasm of tumor cells and confirmed the elevated expression of this protein specifically in tumor cells relative to normal para-cancerous cells (Figs. 1C and 2).

Association between TMED3 levels and clinicopathological characteristics of patients with CRC. Next, the relationship between TMED3 expression levels and clinicopathological

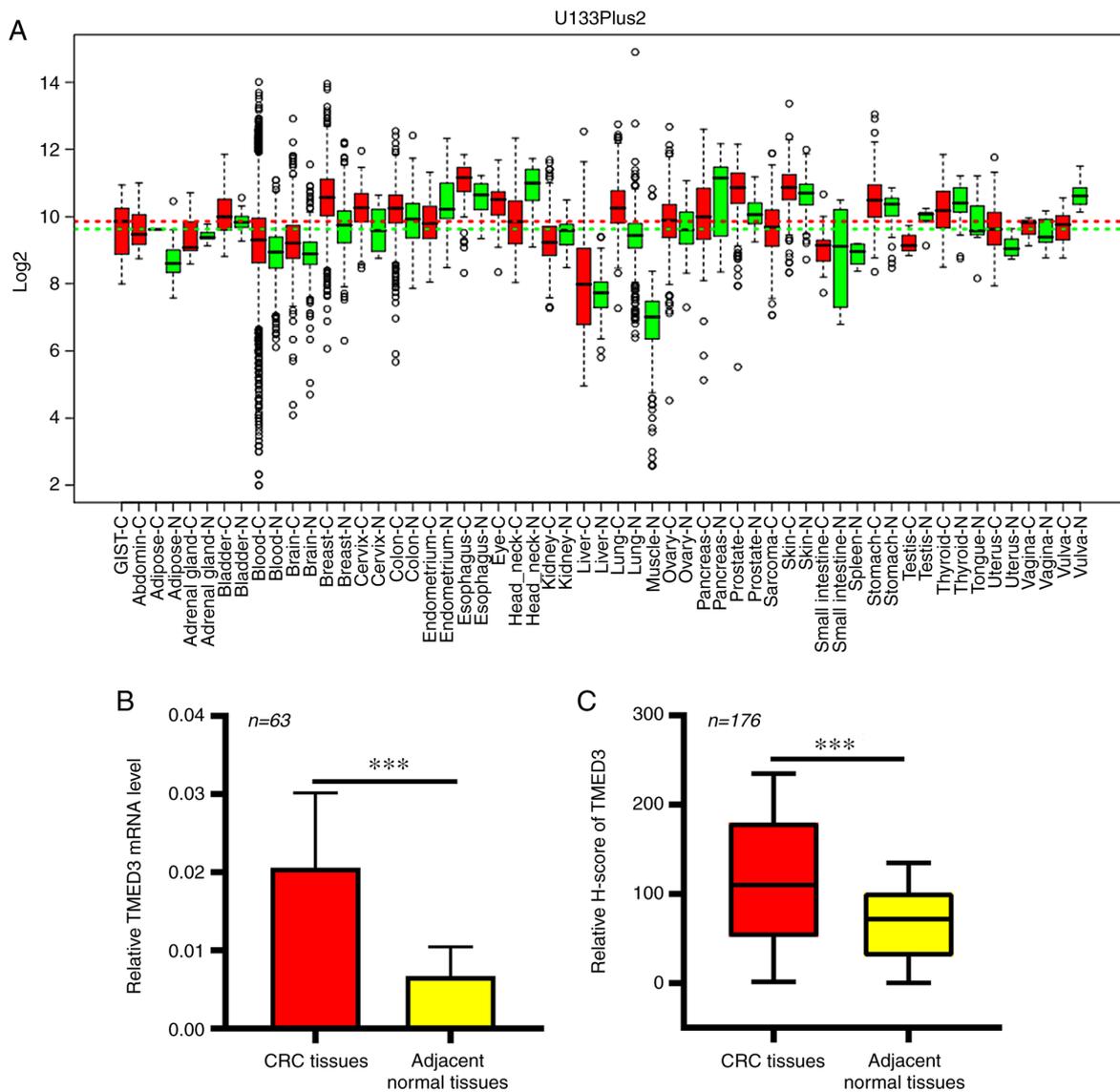


Figure 1. TMED3 expression in CRC and para-cancerous healthy tissues. (A) Expression of TMED3 at the mRNA level in various cancer types from the GENT database, with cancer samples indicated in red and normal tissues in green. (B) TMED3 mRNA expression was assessed via reverse transcription-quantitative PCR in 63 pairs of CRC and healthy para-cancerous tissues. (C) TMED3 H-scores in 176 pairs of CRCs and healthy para-cancerous tissues. *** $P < 0.001$. TMED3, transmembrane p24 trafficking protein 3; CRC, colorectal cancer; N, normal; C, cancer; GIST, gastrointestinal.

characteristics of patients with CRC was examined. The 176 patients were divided into a TMED3-high ($n=88$) and a TMED3-low ($n=88$) group according to the median TMED3 expression levels in this cohort. Elevated TMED3 expression was indicated to be significantly associated with larger tumor size ($P=0.006$), depth of local invasion ($P=0.013$) and lymph node metastasis ($P=0.003$; Table I). By contrast, TMED3 expression levels were not significantly associated with patient sex, age, tumor location, tumor differentiation grade, TNM stage, adjuvant chemotherapy status, carbohydrate antigen 19-9 (CA19-9) levels or serum carcinoembryonic antigen levels. These results thus suggested that TMED3 expression may be significantly linked to CRC metastasis.

Association between TMED3 expression and postoperative survival of patients with CRC. The association between the expression of TMED3 and postoperative survival outcomes in the 176 patients with CRC was then explored. The results

indicated that the TMED3-low group had a significantly lower median OS time than the TMED3-high group (78.10 vs. 70.70 months; 95% CI, 1.115-2.715, $P=0.014$; Fig. 3A). Similarly, TMED3 expression was associated with RFS of the patients with CRC, with the TMED3-low group having a significantly longer median RFS time than the TMED3-high group (72.30 vs. 52.70 months, 95% CI, 1.059-2.433, $P=0.026$; Fig. 3B).

Identification of factors associated with prognosis of patients with CRC. Next, logistic regression analysis using the Cox proportional hazards model was used to identify factors associated with OS and RFS outcomes for patients with CRC. According to the univariate analysis, tumor size, degree of differentiation, local invasion status, lymph node metastasis, TNM staging and TMED3 expression levels were all significantly associated with OS ($P < 0.05$; Table III). These same factors were also associated with post-operative RFS in the patients with CRC ($P < 0.05$). However, patient sex,

Table III. Univariate and multivariate analyses of factors influencing patient survival in the study cohort (Table I).

A, Overall survival									
Variable	Comparison	Univariate analysis				Multivariate analysis			
		HR	95% CI	P-value	HR	95% CI	P-value		
Sex	Male/female	1.236	0.298	2.131	0.901	-	-	-	-
Age (years)	≥60/<60	0.522	0.679	1.655	0.537	-	-	-	-
Tumor location	Colon/rectum	1.412	0.264	1.974	0.141	-	-	-	-
Tumor size (cm)	≥5/<5	1.139	1.241	1.987	0.044	1.109	0.887	1.397	NS
Degree of differentiation	Poor/well + moderate	1.593	1.295	3.692	0.015	1.761	1.286	2.962	0.039
Local invasion	pT3-4/pT1-2	1.588	1.887	4.153	0.011	1.471	1.388	1.655	0.043
Lymph node metastasis	N2/N0+N1	1.954	1.216	2.366	0.039	1.204	1.229	1.912	0.041
TNM stage	III/I + II	2.181	1.451	2.957	0.021	1.769	1.545	2.697	0.019
CA19-9 (kU/l)	≥37/<37	1.041	0.361	1.483	0.172	-	-	-	-
CEA (ng/ml)	≥5/<5	1.368	0.849	1.933	0.051	-	-	-	-
TMED3 protein level	High/low	2.128	1.821	3.928	0.021	1.875	1.305	2.641	0.036

B, Recurrence-free survival									
Variable	Comparison	Univariate analysis				Multivariate analysis			
		HR	95% CI	P-value	HR	95% CI	P-value		
Sex	Male/female	1.334	0.513	1.726	0.417	-	-	-	-
Age (years)	≥60/<60	1.231	0.321	1.631	0.322	-	-	-	-
Tumor location	Colon/rectum	1.279	0.550	1.675	0.926	-	-	-	-
Tumor size (cm)	≥5/<5	1.515	1.317	2.975	0.035	0.812	0.663	1.781	NS
Degree of differentiation	Poor/well + moderate	1.912	1.527	2.701	0.024	1.207	1.162	2.718	0.046
Local invasion	pT3-4/pT1-2	1.564	1.115	2.565	0.017	0.991	0.869	1.718	NS
Lymph node metastasis	N2/N0+N1	1.805	1.247	2.992	0.043	1.238	1.186	2.118	0.039
TNM stage	III/I + II	2.155	1.443	2.787	0.032	1.032	0.789	1.878	NS
CA19-9 (kU/l)	≥37/<37	1.291	0.847	2.386	0.735	-	-	-	-
CEA (ng/ml)	≥5/<5	1.317	0.907	2.483	0.493	-	-	-	-
TMED3 protein level	High/low	1.438	1.302	2.643	0.028	1.776	1.374	2.661	0.048

HR, hazard ratio; 95% CI, 95% confidence interval; TMED3, transmembrane p24 trafficking protein 3; NS, not significant; CEA, carcinoembryonic antigen; CA, carbohydrate antigen.

age and CA19-9 levels had no significant effect on the RFS and OS of the patients with CRC ($P>0.05$). A multivariate analysis was next performed, with all factors identified in the univariate analyses being incorporated into this multivariate model. This analysis revealed that elevated TMED3 protein levels were associated with reduced OS [hazard ratio (HR), 1.875; 95% CI, 1.305-2.641; $P=0.036$] and RFS (HR, 1.776; 95% CI, 1.374-2.661; $P=0.048$) of patients with CRC. Taken together, these results indicated that TMED3 may be an independent predictor of survival outcomes for patients with CRC.

Discussion

TMED3 is a protein that is well-known to have key roles in vesicular trafficking, particularly in the context of early

secretory pathways (12). To date, only three studies have performed a comprehensive examination of the significance of TMED3 in human cancer, proving its relevance in prostate (13), colon (14) and liver cancer (15). These studies, however, have provided contradictory results that suggest that the role of TMED3 is strongly dependent on the tumor type. Vainio *et al* (13) first observed high TMED3 mRNA expression levels in prostate cancer and found this expression to correlate with that of the oncogenes ETS transcription factor ERG and androgen receptor, leading to the conclusion that TMED3 may represent a potential therapeutic target in this cancer type. Duquet *et al* (14) performed a study on TMED3 in a xenograft model of colon cancer, indicating that TMED3 suppressed tumor metastasis via regulating WNT/TCF pathway signaling, thus suggesting that in this context, TMED3 suppresses tumor progression. In contrast to these results, however,

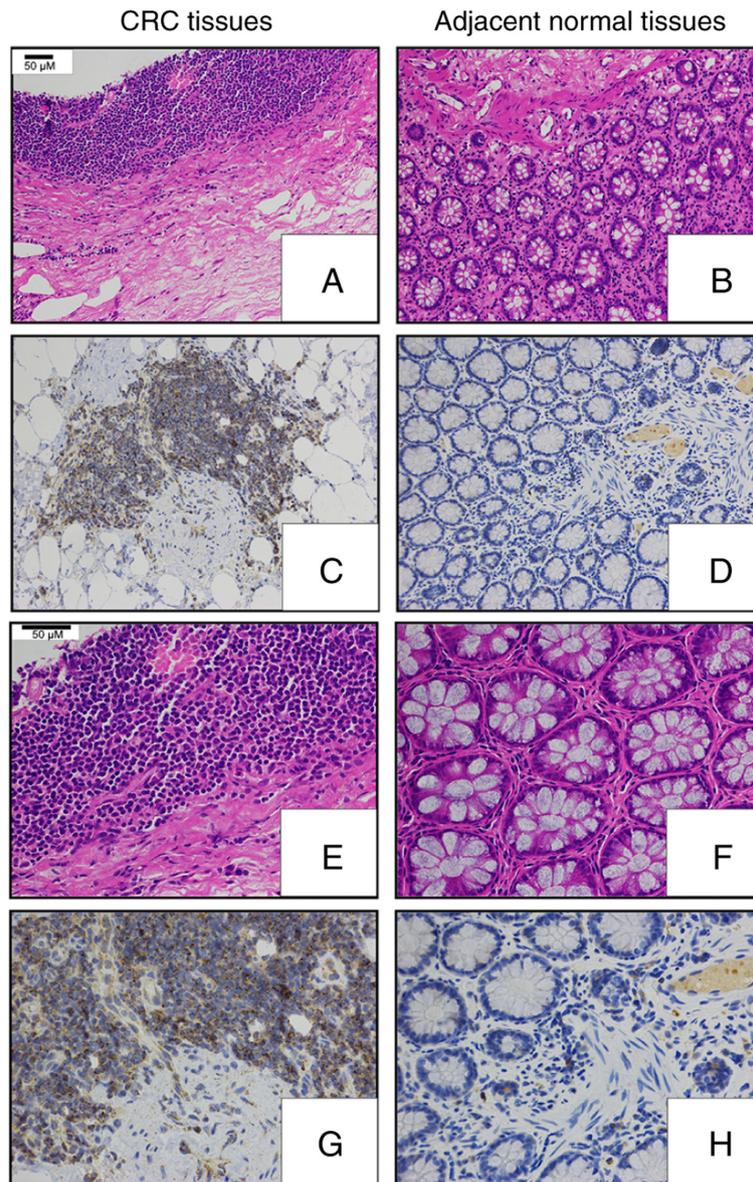


Figure 2. Assessment of TMED3 expression via IHC. (A and B) Representative H&E staining images of (A) CRC and (B) normal para-cancerous tissue. (C and D) Representative IHC staining images for detecting TMED3 in (C) CRC and (D) normal para-cancerous tissue. (E-H) Magnified images of (E) panel A, (F) panel B, (G) panel C and (H) panel D (scale bars, 50 μ M). TMED3, transmembrane p24 trafficking protein 3; CRC, colorectal cancer; IHC, immunohistochemistry.

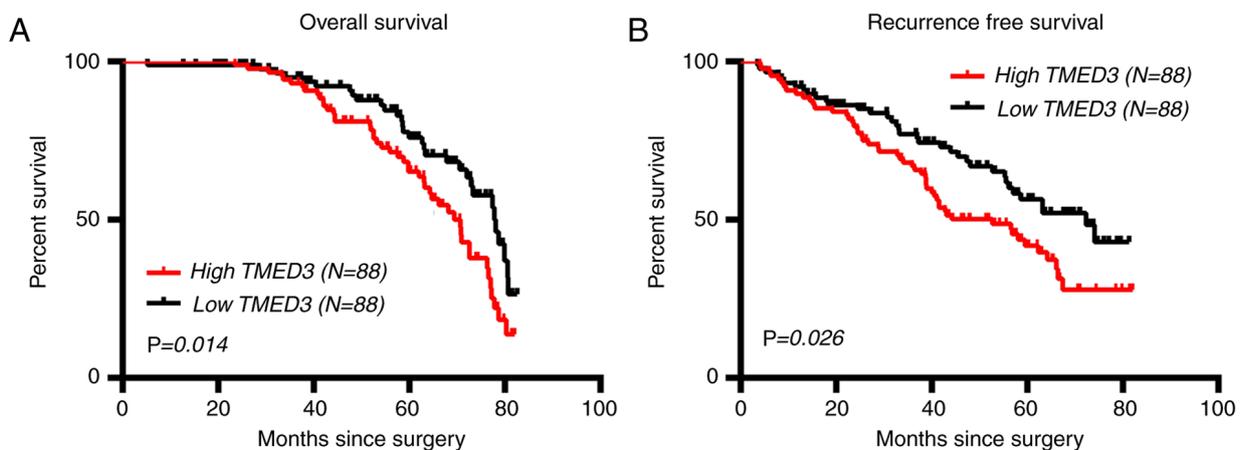


Figure 3. Association between TMED3 expression and postoperative prognosis of patients with CRC. The 176 patients with were divided into two groups (TMED3-high and -low) and (A) OS and (B) RFS outcomes were compared between the groups using a 2-sided log-rank test, revealing a significant inter-group difference in terms of patient prognosis. TMED3, transmembrane p24 trafficking protein 3; CRC, colorectal cancer; OS, overall survival; PFS, progression-free survival.

Zheng *et al* (15) determined that elevated TMED3 expression in hepatocellular carcinoma (HCC) was associated with more aggressive disease and unfavorable patient prognosis, with this protein promoting enhanced tumor cell invasion at least in part via modulating IL-11/STAT3 signaling. Duquet *et al* (14) performed an extensive functional examination of the role of TMED3 in murine models and colon cancer cell lines, while the role of this protein in clinical tissue samples was not directly assessed.

In the present study, TMED3 levels were significantly higher in CRC tissue samples relative to those in normal para-cancerous tissues. This was the case at both the mRNA level, as confirmed via RT-qPCR analysis of 63 patient sample pairs, and at the protein level, as confirmed via IHC assessment of 176 patient sample pairs. In all cases, significantly elevated TMED3 levels were observed in tumor tissue samples relative to those in the matched controls ($P < 0.001$). These results confirmed that TMED3 expression was significantly elevated in CRC tissues at the mRNA and protein level.

Next, the association between the expression of TMED3 and the clinicopathological characteristics of patients with CRC were explored in the IHC cohort ($n=176$). When patients were stratified according to TMED3 expression levels, it was indicated that high TMED3 expression was significantly associated with larger tumor size ($P=0.006$), depth of local invasion ($P=0.013$) and lymph node metastasis ($P=0.003$), suggesting that elevated TMED3 expression is associated with more aggressive and malignant behaviors of CRC. Postoperative survival outcomes were then assessed in these 176 patients, revealing that TMED3-low patients had significantly longer OS and RFS relative to TMED3-high CRC patients. Furthermore, univariate and multivariate models were used to explore the relationship between specific variables and postoperative patient survival. The analysis revealed that elevated TMED3 expression was an independent predictor of reduced CRC patient survival. This suggests that TMED3 may have a role in the development of CRC or in its progression, thus making it a potential prognostic biomarker for CRC. Further research regarding the relevance of TMED3 in CRC and whether it is a viable therapeutic target is thus warranted and such analyses may have the potential to further improve the OS and RFS of patients with CRC. Previous studies have confirmed that TMED3 expression levels are linked to the prognosis of patients with HCC (15), but the present study was the first, to the best of our knowledge, to examine its prognostic value in CRC. The present results are consistent with those of Zheng *et al* (15), but not with those of Duquet *et al* (14), possibly due to small sample sizes. The present results are, however, the first to highlight the prognostic relevance of TMED3 expression levels in CRC.

In conclusion, the present results revealed that TMED3 expression levels are predictive of survival outcomes for patients with CRC, with elevated expression of this protein being independently predictive of poor OS. As such, TMED3 represents a valuable novel prognostic biomarker that may be used for monitoring and/or the treatment of patients with CRC. However, as the present study had a relatively small sample size, it has the potential to be susceptible to bias. Therefore, future studies with a larger independent sample of patients with CRC are required in order to validate these results.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

RFW and HTY contributed to the study design. YGH performed data analysis. LQH contributed to the collection of tissue samples and patient data. YGH and HTY wrote the manuscript. RFW, HTY, LQH and YGH confirmed the authenticity of the raw data. All authors read and approved the final version of the manuscript.

Ethics approval and consent to participate

The Ethics Committee of Changhai Hospital, Second Military Medical University (Shanghai, China) approved the study. Written informed consent was obtained from all patients.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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