

# ***TNNT2* as a potential biomarker for the progression and prognosis of colorectal cancer**

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**Abstract.** Colorectal cancer (CRC) is the third most common cancer worldwide. At present, there are limited effective biomarkers of CRC. The present study aimed to identify potential signatures associated with the tumorigenesis and prognosis of CRC using publicly available databases, and further validate the identified biomarkers in CRC cell lines. Identification of differentially expressed mRNAs between CRC and paraneoplastic samples was conducted based on data from The Cancer Genome Atlas (TCGA; 471 tumor samples and 41 normal samples). Survival analysis was performed to explore the prognostic value of troponin 2 (*TNNT2*) in the TCGA training set, which was further validated in an external dataset, GSE17531. Functional enrichment analysis was conducted to determine the possible biological functions using GSEA 3.0. Reverse transcription-quantitative PCR (RT-qPCR) and western blotting were utilized to detect the mRNA and protein expression levels of *TNNT2* between CRC and normal colorectal cells. Immunohistochemistry was performed to detect the protein expression of *TNNT2* in CRC and normal tissues. *TNNT2* was significantly upregulated in CRC samples compared with adjacent normal samples in the TCGA dataset. Increased expression of *TNNT2* was associated with inferior prognosis in the TCGA training dataset and GSE17531 validation dataset. Functional enrichment analysis revealed that the ErbB signaling pathway and glycerophospholipid metabolism pathway were significantly activated in the *TNNT2* high expression group. Overexpression of *TNNT2* mRNA and *TNNT2* protein in CRC tumor cells was confirmed by RT-qPCR and western blotting, respectively.

Immunohistochemistry indicated increased protein expression levels of *TNNT2* in CRC tissues in comparison with normal tissues. *TNNT2* was associated with the tumorigenesis and prognosis of CRC, which may be useful for novel biomarker identification and targeted therapeutic strategy development.

## **Introduction**

Colorectal cancer (CRC) is the third most common cancer and the second leading cause of cancer-associated mortalities worldwide (1). The incidence rate of CRC in China has been nearly three-fold lower than that in Europe (2,3); however, its incidence has increased rapidly in recent years (4,5). CRC is more frequent in patients ranged 40-70 years old; however, an increasing number of patients are diagnosed with CRC under 40 years of age (6). Currently, the underlying mechanism of CRC carcinogenesis remains unclear. Due to improvement in early detection and surgical treatment, the prognosis of patients with CRC with localized tumors has been significantly enhanced (7). However, the prognosis for patients with advanced tumors remains poor (8). Moreover, although clinical staging is useful to some extent to predict CRC prognosis, patient outcomes can vary significantly even for patients with similar clinical features (9). Heterogeneity in somatic or germline changes may substantially affect patient prognosis.

Altered mRNA expression of genes involved in tumor proliferation, autophagy and transcription has been reported to play an important role in predicting the onset or survival outcome of CRC (10-12). However, the number of studies investigating mRNAs in order to predict the onset and prognosis of CRC remains limited. A previous study reported that cyclooxygenase-2 mRNA upregulation was associated with CRC carcinogenesis using reverse transcription-quantitative PCR (RT-qPCR) based on 60 CRC tumor tissues and normal tissues (13). Another study reported that increased expression of rabaptin-5-associated exchange factor mRNA was associated with poor survival of CRC using RT-qPCR and survival analysis based on 187 CRC tumor tissues (14). Wang *et al* (15) established a 31-gene expression classifier to predict CRC recurrence using a gene expression microarray based on 281 CRC samples. However, these studies evaluated only a single clinical outcome (progression or prognosis), and lacked laboratory-based

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validation experiments, restricting the possible application of these reported mRNAs in clinical practices. Therefore, it is imperative to identify and validate key mRNAs associated with the carcinogenesis and prognosis of CRC in order to further facilitate the development of new targeted therapies.

Substantial developments in high-throughput transcriptome sequencing and microarray technologies have provided opportunities to identify novel mRNA biomarkers associated with the tumorigenesis and prognosis of CRC. In the present study, differential expression analysis was performed to explore critical genes in CRC. Survival analysis was performed to evaluate the prognostic value of *TNNT2*. Functional enrichment analysis was employed to explore the potential biological processes involved in CRC tumorigenesis and prognosis. Epidermal growth factor receptor (EGFR) is a key factor for the ErbB signaling pathway involved in tumor growth (16,17). In addition, Fatty acid synthase (*Fasn*) is the key metabolic enzyme that accounts for the glycerophospholipid metabolism pathway to regulate the proliferation and migration in various tumors (18,19). Therefore, EGFR and *Fasn* were selected as a biomarker for the two respective signaling pathways. RT-qPCR and western blotting were conducted to measure the mRNA and protein expression levels of *TNNT2* between CRC tumor and normal cells. The protein expression of *TNNT2* in CRC and normal tissues was detected via immunohistochemistry.

In summary, the present study investigated the expression of *TNNT2* at the mRNA and protein level in CRC and clarified the correlation between the *TNNT2* expression and clinicopathological parameters.

## Materials and methods

**Data sources.** The data of CRC tumor and adjacent normal tissue samples were obtained from The Cancer Genome Atlas (TCGA; [www.cancergenome.nih.gov](http://www.cancergenome.nih.gov)) and the Gene Expression Omnibus (GEO; <https://www.ncbi.nlm.nih.gov/geo/>; Access number: GSE17537) databases. The TCGA-CRC dataset contained a total of 512 CRC samples, including 471 tumor samples and 41 adjacent normal samples. The age of the samples is from 41 to 90 with the median age of 68. Numbers of female and male patients is 212 and 235, respectively. The GEO dataset contained 55 CRC tumor samples. The age of those 55 patients is from 23 to 94 with the median age of 61. Numbers of female and male patients is 29 and 26, respectively.

**Differential expression analysis.** Differential expression analysis was performed on the TCGA-CRC dataset using edgeR package in R v. 3.5.3 software (20). First, genes were excluded with average counts <10. Then the samples were divided into normal tissue group (N) and tumor tissue group (T) according to the sample type. A false discovery rate (FDR) <0.05 and  $|\log_2$  fold change (FC)| >1 were set as the criteria for screening differentially expressed genes.

**Gene set enrichment analysis (GSEA).** GSEA (version 2.2) was conducted for functional enrichment analysis (21). The selected gene set was Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway. P<0.05 was set as the threshold for screening significantly enriched KEGG pathways.

Table I. Primer sequences for reverse transcription-quantitative PCR.

Gene	Primer sequence (5'→3')
<i>TNNT2</i>	F: TTCACCAAAGATCTGCTCCTCGCT R: TTATTACTGGTGTGGAGTGGGTGTGG
EGFR	F: TCCCTCAGCCACCCATATGTAC R: GTCTCGGGCCATTTTGGAGAATTC
<i>Fasn</i>	F: ATGCGGGACAGAGCAACTACGG R: CAGCCTTCTCAGCCAGCACAAA
<i>GAPDH</i>	F: GGTGAAGGTCGGTGTGAACG R: CTCGCTCCTGGAAGATGGTG

F, forward; R, reverse; *TNNT2*, troponin T2.

**Cell culture.** The normal colorectal cell line FHC, colon cancer cell line LoVo, CRC cell line SW620, and colon cancer cell line SW1116 were purchased from BeNa Culture Collection. FHC, LoVo, SW620 and SW1116 cells were cultured in DMEM (Gibco; Thermo Fisher Scientific, Inc.), DMEM/F12 (Gibco; Thermo Fisher Scientific, Inc.), DMEM/F-12K (Gibco; Thermo Fisher Scientific, Inc.) and DMEM/L-15 (Gibco; Thermo Fisher Scientific, Inc.) containing 10% fetal bovine serum (FBS; Hyclone; GE Healthcare Life Sciences) and 1% penicillin/streptomycin (Hyclone; GE Healthcare Life Sciences), respectively. The cells were incubated at 37°C with 5% CO<sub>2</sub>.

**Overexpression of *TNNT2*.** RNA was extracted from LoVo cells using Trizol reagent (Thermo Fisher Scientific, Inc.). cDNA was synthesized by TransScript® Two-Step RT-PCR SuperMix (TransGen Biotech). ORF sequence of *TNNT2* was amplified via RT-PCR according to the manufacturer's instructions of 2xTransTaq® HiFi PCR SuperMix II (TransGen Biotech). *TNNT2* primer sequences were as follows: Forward, 5'-ATGTCTGACATAGAAGAGGTGGTGG-3' and reverse, 5'-CTATTTCCAGCGCCCGGTGACTTTA-3'. PCR reaction condition was as follows: 94°C 3 min, 1 cycle, 94°C 30 sec, 55°C 30 sec, 72°C 3 min, 35 cycles and 72°C 10 min, 1 cycle. Then PCR products were inserted into the pCMV-Myc vector (Biovector Co., Ltd.) to construct the expression vector pCMV-Myc-*TNNT2*. LoVo, SW620 and SW1116 cells were transfected with 16 µg/ml pCMV-Myc-*TNNT2* using Lipofectamine 2000 (Thermo Fisher Scientific, Inc.) for 48 h according to the manufacturer's instructions.

**RT-qPCR.** Total RNA of FHC, LoVo, SW620 and SW1116 cells was extracted using Trizol (Invitrogen; Thermo Fisher Scientific, Inc.). The purity and concentration of RNA were determined via NanoDrop ND-2000 spectrophotometer (NanoDrop Technologies; Thermo Fisher Scientific, Inc.). Then the RNA was reverse transcribed into cDNA using TransScript® Two-Step RT-PCR SuperMix (TransGen Biotech) according to the manufacturer's instructions. RT-qPCR was performed according to the manufacturer's instructions of the SYBR Premix Ex Taq kit (Roche Diagnostics), with GAPDH used as the internal reference. The thermocycling conditions

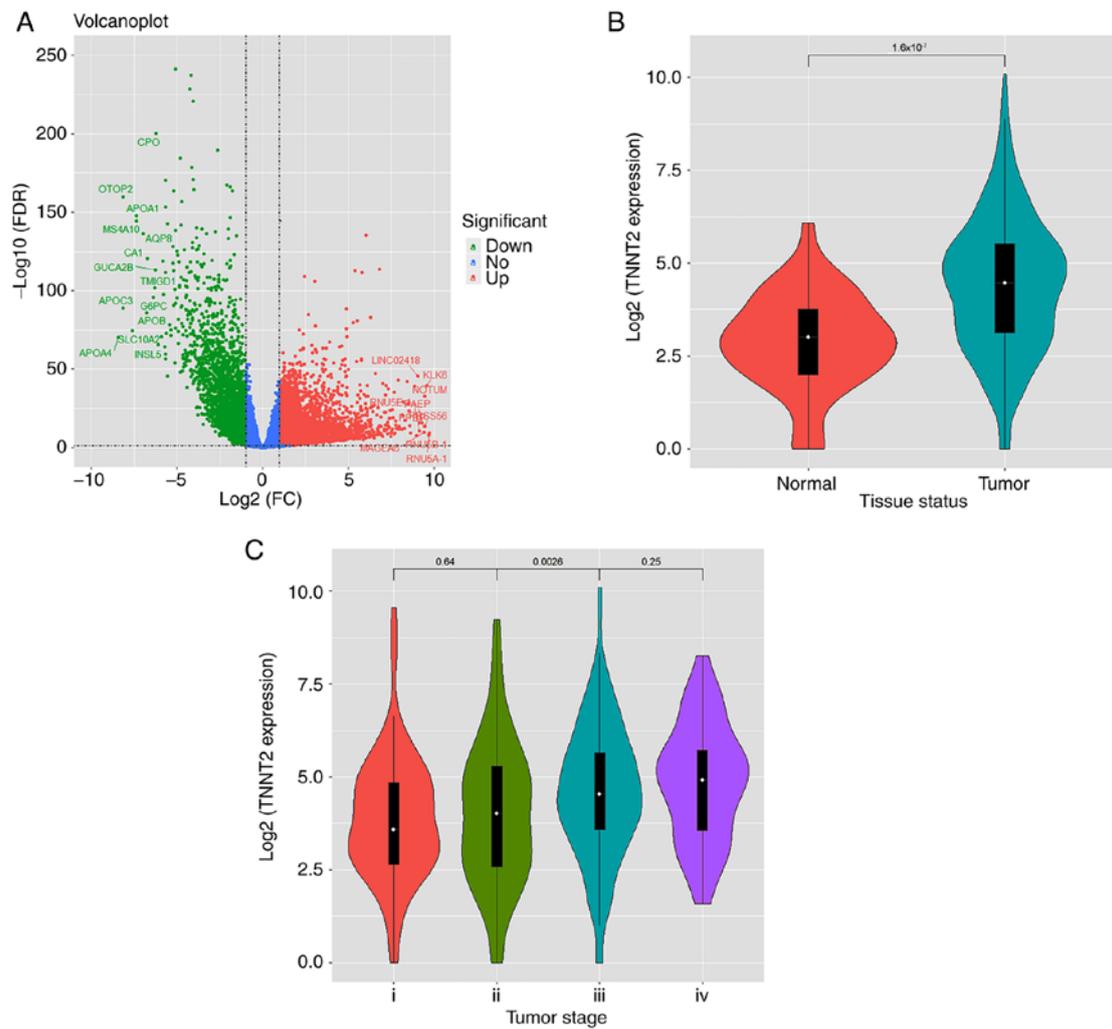


Figure 1. Elevated *TNNT2* expression is associated with the tumorigenesis and progression of CRC. (A) Volcano plot of differentially expressed genes between CRC and paracancerous tissues. Green plots represented significantly downregulated genes in CRC tumor tissues. Red plots represented significantly upregulated genes in CRC tumor tissues. Blue plots represented genes that were not significantly differentially expressed between CRC tumor and normal tissues. (B) Box plot of *TNNT2* mRNA expression levels between CRC tumor and normal tissues. (C) Box plot of *TNNT2* mRNA expression levels among different pathological stages of CRC. *TNNT2*, troponin T2; CRC, colorectal cancer.

were as follows: Step 1, 95°C for 5 min; step 2, 95°C for 30 sec; step 3, 58-65°C for 30 sec; step 4, 72°C for 30 sec; step 5, repeat from step 2 for 34 cycles; step 6, 72°C for 10 min. The relative expression of *TNNT2* was calculated using the  $2^{-\Delta\Delta C_q}$  method. The primer sequences of RT-qPCR are listed in Table I.

**Western blotting.** The cells were lysed using RIPA solution and then total proteins were extracted. The proteins were quantified using bovine serum albumin (BSA; Boster Biological Technology Co., Ltd). The protein samples were electrophoresed on 10% SDS-PAGE and transferred onto PVDF membranes. After blocking with 1X Blotto (Thermo Fisher Scientific, Inc.) for 2 h at room temperature, the membranes were incubated with anti-Cardiac Troponin T antibody (cat. no. ab91605; 1:1,000; Abcam) and anti-GAPDH antibody (cat. no. ab8245; 1:1,000; Abcam) at 4°C overnight. After washing with TBST three times at room temperature, the membranes were incubated with horseradish peroxidase (HRP)-labeled goat anti-rabbit IgG as the secondary antibody (cat. no. ab6728; 1:1,000; Abcam) for 1.5 h at room temperature. The proteins were visualized using Western Lightning™

Chemiluminescence reagent (PerkinElmer, Inc.), and densitometric analysis was conducted using LabWorks™ 4.6 (UVP, LLC). GAPDH was used as the internal control.

**Immunohistochemistry.** The CRC and normal tissues were fixed using 4% paraformaldehyde at 4°C overnight, embedded with paraffin, and sliced into 3-5 μm sections, followed by dewaxing. After antigen retrieval by microwave, the sections were blocked with 10% normal goat serum (Beijing Solarbio Science & Technology Co., Ltd.) and incubated at room temperature for 20 min. Then the primary antibody rabbit anti-cardiac troponin T (cat. no. ab91605; Abcam) were added for an incubation at 4°C overnight. The biotin-labeled IgG (cat. no. Sp-9001; 1:1,000; OriGene Technologies, Inc.) was used as secondary antibody for 30 min at room temperature. After development in DAB and counterstaining with hematoxylin for 5 min at room temperature, the sections were sealed with resinene. Five fields were randomly selected and imaged under an optical microscope (BX51T-PHD-J11, Olympus Corporation). The images were analyzed using Image-Pro Plus (Media Cybernetics, Inc.).

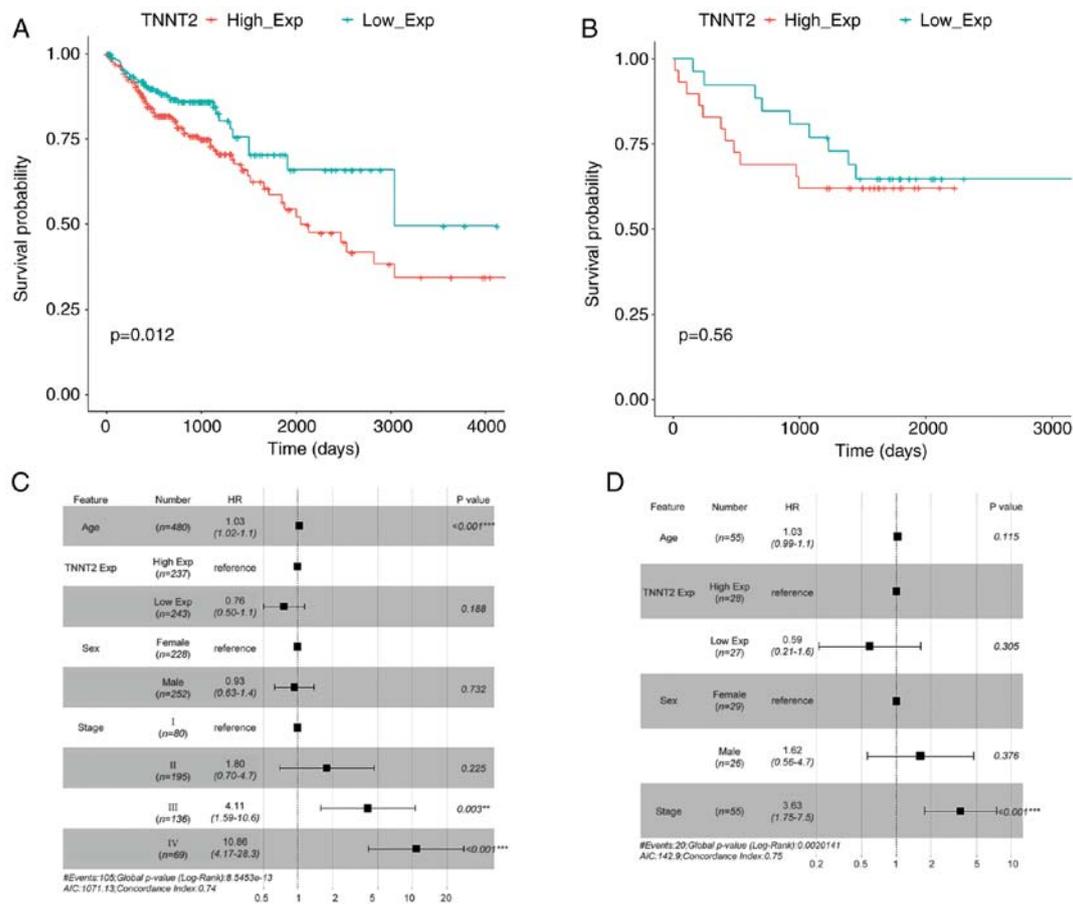


Figure 2. Elevated *TNNT2* expression is associated with poor prognosis in patients with CRC. (A) Kaplan-Meier survival curves for CRC patients between high- and low-*TNNT2* expression groups in TCGA. The blue curve represents the low expression group. The red curve represents the high expression group. (B) Kaplan-Meier survival curves for patients with CRC between high- and low-*TNNT2* expression groups in GSE17537. The blue curve represents the low-*TNNT2* expression group. The red curve represented high-*TNNT2* expression group. (C) Cox regression analysis adjusting for age, sex, staging and *TNNT2* expression level (high or low) in TCGA.  $P < 0.1$  was set as the cutoff criteria for screening genes significantly associated with CRC survival. (D) Cox regression analysis adjusting for age, sex, staging and *TNNT2* expression level (high or low) in GSE17537.  $P < 0.1$  was set as the cutoff criteria for screening genes significantly associated with CRC survival. *TNNT2*, troponin T2; CRC, colorectal cancer.

**Statistical analysis.** Cox regression analysis was performed by using survival and survminer packages in R software (22). Clinical factors, including age, sex, TNM staging system [American Joint Committee on Cancer (AJCC) (23) and the International Union Against Cancer (UICC) (24)] and gene expression levels, were adjusted in the Cox regression model.  $P < 0.1$  was set as the threshold to screen factors significantly associated with CRC survival. Kaplan-Meier analysis was performed by stratifying CRC patients according to median *TNNT2* expression. Data were presented as the mean  $\pm$  SEM,  $n=3$ . The significance was assessed via one-way ANOVA (with Tukey's post hoc analysis). Statistical analysis was performed using GraphPad Prism Version 5.0 software (GraphPad Software, Inc.).

## Results

*TNNT2* is upregulated in CRC samples compared with paracancerous samples. A total of 2,703 downregulated genes and 4,001 upregulated genes between CRC tumor samples and paracancerous samples were identified. The volcano blot is presented in Fig. 1A. Analysis of these differentially expressed genes revealed that *TNNT2* was significantly upregulated in

CRC tumor samples vs. adjacent normal samples, as indicated by Fig. 1B ( $P=1.6 \times 10^{-7}$ ). Furthermore, there was a positive association between *TNNT2* gene expression with the stages of patients with CRC, especially between stage II and stage III, as indicated in Fig. 1C ( $P=0.0026$ ). As the association between *TNNT2* and CRC has not been investigated in previous studies, *TNNT2* was selected as the key candidate gene, and the association of *TNNT2* with the progression and prognosis of CRC was investigated.

*Elevated TNNT2 is associated with poor prognosis in CRC.* CRC samples were divided into a high- and low-expression group according to the median level of *TNNT2* expression which was 4.82 ( $\log_2$ -based) according to the survival package. The survival curve was plotted using survminer package (Fig. 2A). It was revealed that the expression level of *TNNT2* had a significant effect on survival outcome ( $P=0.012$ ). Moreover, a Cox regression model was constructed adjusting for age, sex, staging and *TNNT2* expression level (high or low) (Fig. 2C). It was revealed that compared with the high-*TNNT2* expression group, the low-expression group exhibited a more favorable survival outcome. Additionally, patients with CRC with higher stages had a higher risk of mortality.

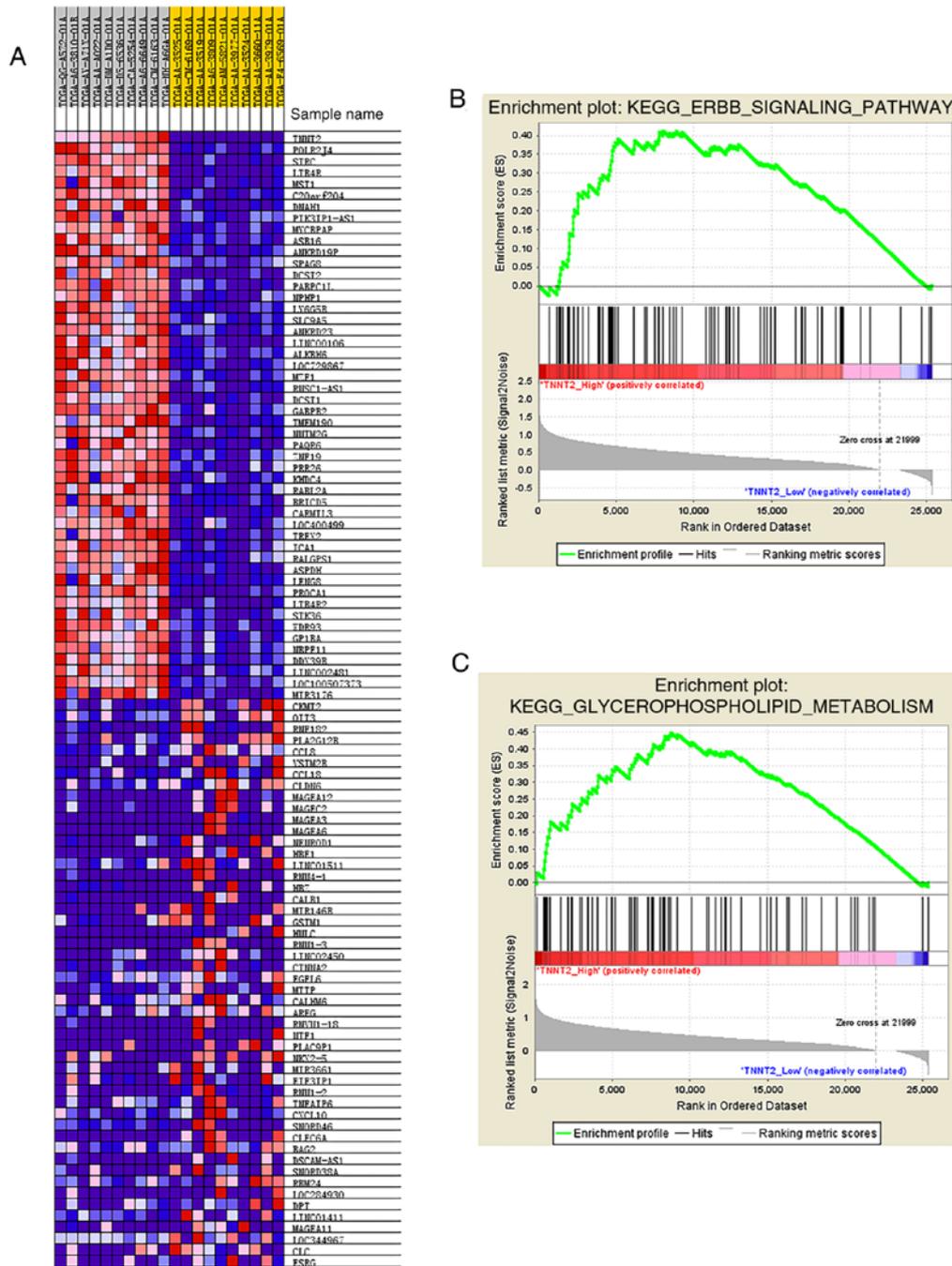


Figure 3. Increased *TNNT2* expression may influence the tumorigenesis and prognosis of CRC via activation of ErbB and glycerophospholipid metabolism pathways. (A) Heatmap of the selected 200 genes according to the 20 CRC samples with the top 10 and the bottom 10 *TNNT2* expression levels among all CRC samples. (B) The ErbB signaling pathway was significantly activated in CRC samples with high *TNNT2* expression. (C) The glycerophospholipid metabolism pathway was significantly activated in CRC samples with high *TNNT2* expression. *TNNT2*, troponin T2; CRC, colorectal cancer.

*Validation of the prognostic effect of TNNT2 in a GEO cohort.* The same method was utilized for the survival analysis to verify the association of *TNNT2* expression with survival in GEO dataset GSE17537. The survival curve suggested that different expression levels of *TNNT2* had an effect on survival in patients with CRC, but the difference was not statistically significant ( $P=0.56$ ). The Cox regression analysis indicated that the *TNNT2* low expression group had a lower risk of mortality compared with the high expression group, and patients at higher stages had a higher risk of mortality (Fig. 2B and D).

*Significantly enriched pathways in CRC samples with high TNNT2 expression.* According to the median expression level of *TNNT2*, CRC samples were stratified into high and low expression groups, and then differential expression analysis was performed. A total of 784 differentially expressed genes were identified, of which 163 genes were downregulated and 621 genes were upregulated. The top 100 differentially expressed genes within down- and upregulated gene sets were selected, respectively. A heatmap was constructed comprising the 200 selected genes in 10 CRC samples with the highest and lowest *TNNT2* expression, respectively (Fig. 3A). According to the

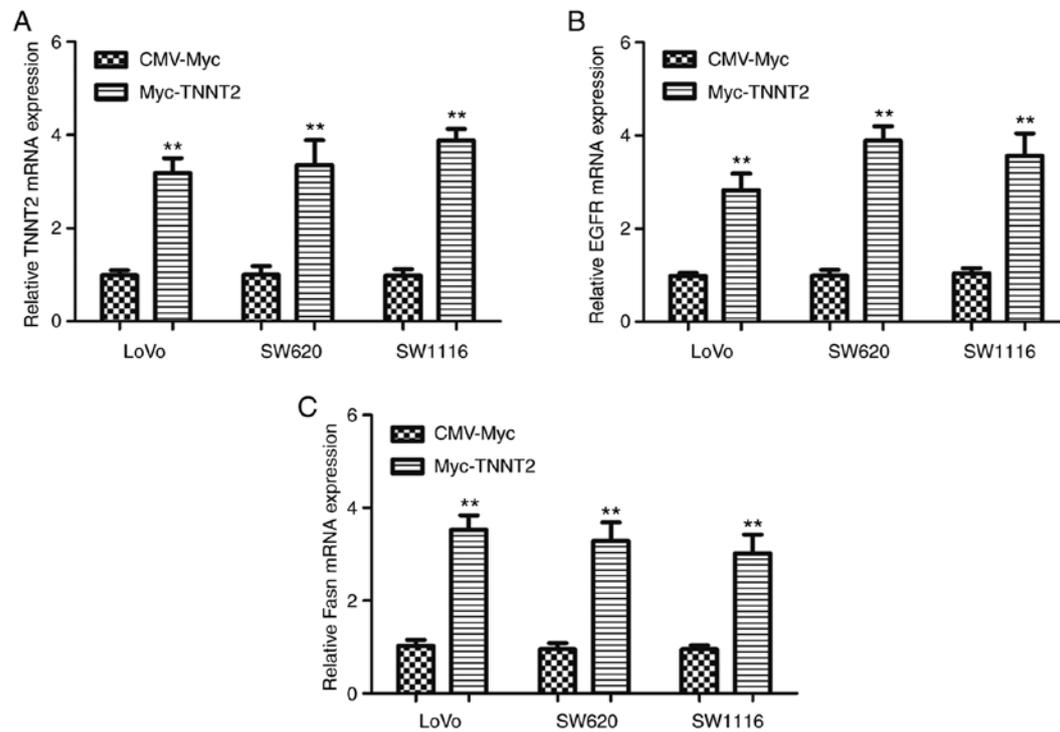


Figure 4. *TNNT2* overexpression upregulates the ErbB signaling pathway and glycerophospholipid metabolism pathway in CRC cell lines. (A) Overexpression of *TNNT2* in LoVo, SW620 and SW1116 cells transfected with pCMV-Myc-*TNNT2* or empty vector, then the expression of *TNNT2* was validated by RT-PCR. (B) *TNNT2* overexpression upregulated *EGFR* expression. (C) *TNNT2* overexpression upregulated *Fasn* expression. \*\* $P < 0.01$  vs. the control group. *TNNT2*, tropinin T2; CRC, colorectal cancer; EGFR, epidermal growth factor receptor; Fasn, fatty acid synthase.

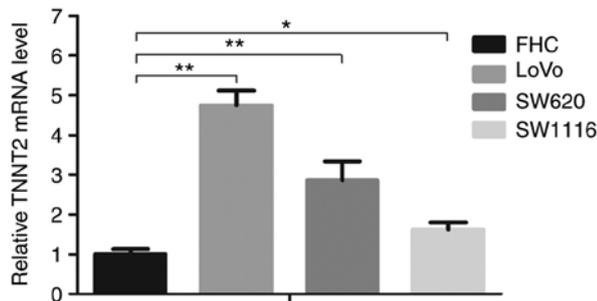


Figure 5. *TNNT2* mRNA expression levels among normal colorectal cell line (FHC) and CRC cell lines (LoVo, SW620 and SW1116). Data were reported as mean  $\pm$  SEM. Significance was assessed by one-way analysis of ANOVA and Tukey's post hoc analysis. \* $P < 0.05$ ; \*\* $P < 0.01$  vs. the control group. *TNNT2*, tropinin T2; CRC, colorectal cancer.

functional enrichment analysis, the 'ErbB signaling pathway' and 'glycerophospholipid metabolism pathway' were significantly activated in the high *TNNT2* expression group (Fig. 3B).

#### *TNNT2* overexpression upregulates the ErbB signaling and glycerophospholipid metabolism pathways in CRC cell lines.

In order to further confirm the effect of *TNNT2* on the ErbB signaling pathway and glycerophospholipid metabolism pathway, RT-PCR were carried out in CRC cell lines transfected with the expression vector pCMV-Myc-*TNNT2*. As indicated in Fig. 4A, *TNNT2* expression was significantly increased in LoVo, SW620 and SW1116 cells following transfection with pCMV-Myc-*TNNT2*. Additionally, the expression of *EGFR* and *Fasn* were significantly increased (Fig. 4B and C). This

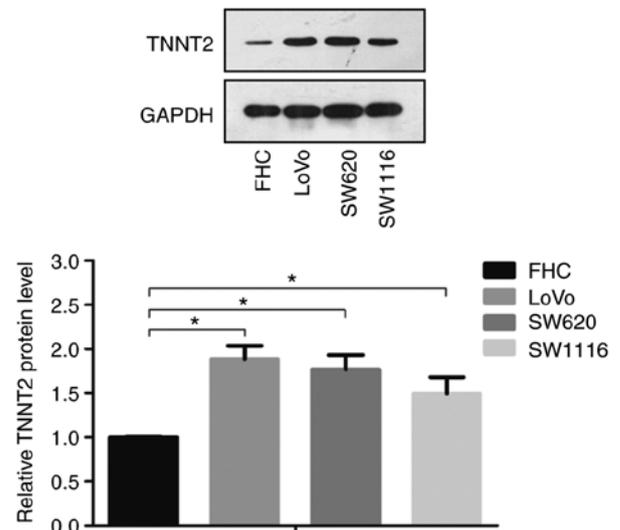


Figure 6. *TNNT2* protein expression levels between a normal colorectal cell line (FHC) and CRC cell lines (LoVo, SW620 and SW1116). The data were reported as mean  $\pm$  SEM. Significance was assessed via one-way ANOVA and Tukey's post hoc analysis. \* $P < 0.05$  vs. the control group. *TNNT2*, tropinin T2; CRC, colorectal cancer.

result demonstrated that high *TNNT2* expression upregulates the ErbB signaling and glycerophospholipid metabolism pathway in CRC cell lines, which was consistent with the present bioinformatics analysis.

*Validation of elevated TNNT2 expression in CRC cell lines and tissues.* The mRNA and protein expression levels of

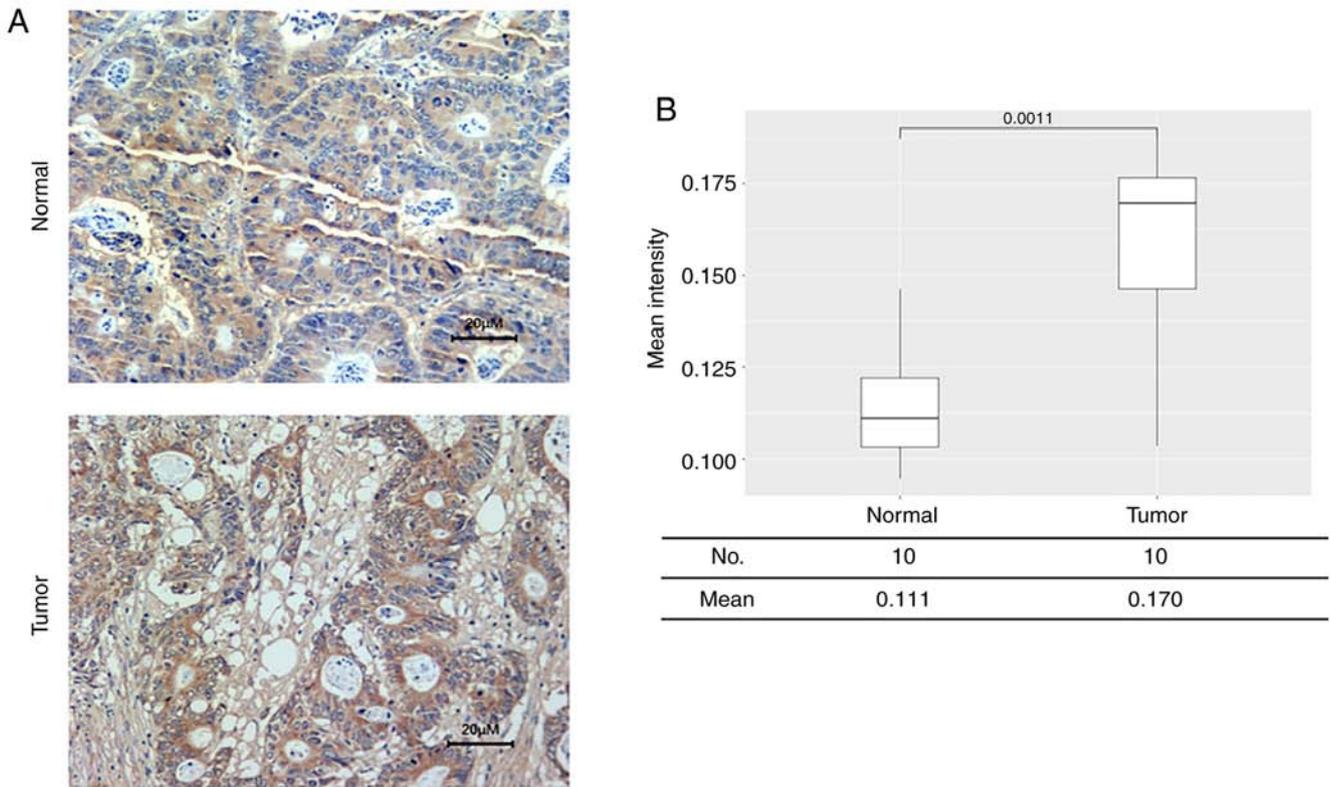


Figure 7. Protein expression levels of *TNNT2* in CRC tissues are significantly higher compared with normal tissues. (A) Immunohistochemical staining of CRC and normal tissues. (B) Mean intensity of CRC and normal tissues ( $P<0.05$ ). *TNNT2*, troponin T2; CRC, colorectal cancer.

*TNNT2* were detected in normal colorectal cell line FHC, colon cancer cell line LoVo, CRC cell line SW620 and colon cancer cell line SW1116. The result revealed that compared with the normal colorectal cells, the mRNA expression level of *TNNT2* was significantly increased in the CRC cells ( $P<0.05$ ; Fig. 5). Moreover, the protein expression level of *TNNT2* in CRC cells was higher compared with in normal colorectal cells ( $P<0.05$ ; Fig. 6). Immunohistochemistry was performed to detect the protein expression of *TNNT2* in CRC and normal tissues. The result indicated increased protein expression level of *TNNT2* in CRC tissues in comparison to that in normal tissues ( $P<0.05$ ; Fig. 7).

## Discussion

In the present study, the potential mRNA signatures associated with CRC risk were explored using datasets from publicly available databases. Differential expression analysis in TCGA cohort indicated that *TNNT2* was significantly upregulated in CRC samples and may be associated with the onset of CRC. Survival analysis was performed to investigate the potential of *TNNT2* as a prognostic biomarker. The carcinogenic and prognostic value of *TNNT2* was further replicated in an external validation dataset, GSE17537. Furthermore, laboratory work was also conducted to confirm the increased mRNA and protein expressions of *TNNT2* in CRC cell lines. The RT-qPCR and western blot analyses revealed that *TNNT2* mRNA and protein expressions were significantly elevated in CRC cell lines compared with normal colorectal cell lines, which further validated the present analysis.

*TNNT2* encodes the tropomyosin-binding subunit of the troponin complex, which controls muscle contraction in response to differential concentrations of intracellular calcium ions (25,26). Several studies have reported the association between abnormal *TNNT2* mRNA splicing and dilated cardiomyopathy risk (27,28). Typically, altered mRNA splicing generates different transcripts, which are then translated into protein isoforms and influence diverse biological processes (29). However, abnormal mRNA splicing may result in significant changes to protein structure and function, thus resulting in tumorigenesis (30). Lokody and Isabel (31) reported that aberrant mRNA splicing promotes the growth of colon tumors via upregulation of PRPF6, a driver of colon carcinogenesis. According to genome-wide profiling of alternative splicing events, Zong *et al* (32) identified 13 genes with differential mRNA splicing patterns, which may predict colorectal cancer prognosis. The present study demonstrated that *TNNT2* was differentially expressed between CRC tumor and adjacent normal tissues, based on both bioinformatics analysis and experimental strategies. Normal colon tissue is composed of smooth muscle. *TNNT2* is expressed at a low level in normal colon tissue, which is consistent with the present results (33). Therefore, it was speculated that *TNNT2* may not serve a physiological role in normal colon tissue. A significant difference in *TNNT2* mRNA expression between different tumor stages was also identified. Thus, it was hypothesized that the altered mRNA and protein expression levels of *TNNT2* in CRC cells may be induced by abnormal *TNNT2* mRNA splicing, which may result in colorectal carcinogenesis.

According to the current functional enrichment analysis, the 'ErbB signaling pathway' was significantly activated in the high *TNNT2* expression group. Upregulation, or mutation of several members of the ErbB pathway including EGFR, ErbB2 and ErbB3, have been identified in numerous cancer types (34). By modulating extracellular matrix components, ErbB receptors serve a notable role in tumor proliferation and metastasis (35). Upregulation of EGFR, a member of the ErbB family, is associated with the onset and prognosis of colorectal cancer, via activating multiple pathways, such as the MAPK and PI3K pathways (36). The current finding that differentially expressed genes in the high *TNNT2* expression group were enriched in the ErbB signaling pathway was in accordance with previous studies and suggested that elevated *TNNT2* expression might influence CRC tumorigenesis and prognosis through regulating the ErbB signaling pathway. In addition, the current study identified that the glycerophospholipid metabolism pathway was also activated in CRC samples with high *TNNT2* expression. Based on global lipid omics analysis, Hung *et al* (37) reported that diverse glycerophospholipid levels were associated with chromosome instability in gastric cancer. Several studies have reported an association between alteration of lipid metabolism and the risk of colorectal cancer (38,39). The results of the present study were consistent with previous studies and suggested that high expression of *TNNT2* may affect CRC tumorigenesis via regulation of glycerophospholipid metabolism.

In conclusion, the present study revealed that elevated *TNNT2* expression is associated with the tumorigenesis and prognosis of CRC, which may facilitate the identification of novel biomarkers and the development of targeted therapeutic strategies.

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#### Availability of data and materials

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

#### Authors' contributions

LJ and LF contributed to the design of the study, wrote the manuscript and analyzed the data. ZZ, SS and RD revised the manuscript and contributed to the design of the study. ZW, YZ and ZR acquired, analyzed and interpreted the data. YL made substantial contributions to the conception and design of the present study and revised the manuscript. All authors read and approved the final manuscript.

#### Ethics approval and consent to participate

Written informed consent was obtained from all participants and the present study was approved by the Ethics Committee

of Fourth Hospital of Hebei Medical University (Shijiazhuang, China; approval no. 2019MEC107).

#### Patient consent for publication

Not applicable.

#### Competing interests

The authors declare that they have no competing interests.

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