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The expressional level of tankyrase-1 gene and its regulation in colorectal cancer in a Saudi population

Hala Abdulaziz M ALWARTHAN[1]; Mohammad Saud AL ANAZI[1]; Narasimha R. PARINE[1]; Ramesa Shafi BHAT[1*]; Ghadah ALAMRO[1]; Ftoon ALJARBOU[1]; Sooad K AL-DAIHAN[1]

[1] Department of Biochemistry, College of Science, King Saud University, P. O. Box 22452, Zip code 11495, Riyadh, Saudi Arabia

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Abstract: Tankyrase1 plays an essential role in cancer progression by regulating telomere length. The study aimed to determine expression of *TNKS1* and its regulation in colorectal cancer (CRC) in 20 samples from Saudi patients. mRNA expression of *TNKS1* in CRC and paired normal tissues was measured by qRT-PCR. Epigenetic modification of *TNKS1* promoter was determined by methylation-specific PCR while somatic mutation was analyzed by Sanger sequencing in exon 10 of the gene. All cancerous and normal tissues expressed *TNKS1*, but level of expression in CRC tissues was significantly associated with tumor stage though no other parameters; age, gender, and tumor location, showed any correlation. Expression of *TNKS1* was markedly higher in earlier (I, II) than later (III, IV) stages of CRC development. Both cancerous and healthy tissues had unmethylated promoter. Sanger sequencing of exon 10 masked any somatic mutation in the samples. Our findings suggest that up-regulation of *TNKS1* was inversely correlated with cancer progression in CRC, indicating that *TNKS1* participates in the initiation of CRC by stabilizing telomere length in the first phase of cancer progression. Mechanisms other than *TNKS1* might play a role in malignant tumor progression and telomere maintenance in the late stages of CRC.

Introduction

Protein-DNA complexes known as telomeres are very important for genome stability and play a critical role in the process of carcinogenesis in humans (Gaspar *et al.*, 2018). These structures are hexanucleotide repeats associated with some proteins and are present at the end of eukaryotic chromosomes (Blackburn, 2005). Tumor cells proliferate indefinitely as they compensate for replicative telomere losses (Bernal and Tussel, 2018). Telomeres are maintained by Tankyrase proteins with poly(ADP-ribose) polymerase activity and are present in both the nucleus and cytoplasm of the cell and are a major component of the human telomeric complex. Tankyrase-1 and 2 are the main components of the tankyrase protein and show 85% amino acid sequence identity with each other.

Tankyrase-1 (TNKS1) can interact with (TRF1), which is a telomeric DNA-binding protein and a negative regulator of telomere length (Yang *et al.*, 2017). Tankyrase-1 can inhibit the binding of TRF1 to telomeres through Tankyrase-mediated ADP-ribosylation of TRF1 protein (Ye and de Lange, 2004). Tankyrase-1 thus acts as a positive regulator of telomere length as overexpression of Tankyrase-1 in the nucleus can release TRF1 from telomeres and can induce telomere elongation (Donigan and de Lange, 2007). Many studies have suggested that Tankyrase-2 acts in similar ways as Tankyrase-1, as both bear the same protein-binding properties and structural homology (Chiang *et al.*, 2008). Altered expression of Tankyrase-1 and Tankyrase-2 is detected in many tumors. Many different cancers, such as fibrosarcoma, ovarian cancer, glioblastoma, pancreatic adenocarcinoma, and breast cancers, have been found to have increased expression of Tankyrase-1 and Tankyrase-2 in the tumor tissue.

This study evaluates mRNA expression level of the Tankyrase-1 gene by real-time PCR in 20 colorectal cancers (CRC) and in 20 adjacent normal tissues in the Saudi population. Epigenetic modification (promoter methylation) of the gene and Sanger sequencing of exon 10 including ankyrin repeat cluster were also evaluated to detect somatic mutation.

Materials and Methods

Subject Recruitment

This study was conducted after reviewed and approval by the Institutional Review Board (IRB # 16/0357, College of

*Address correspondence to: Ramesa Shafi Bhat, rbhat@ksu.edu.sa

Medicine). All cancer cases were recruited at clinicians of the oncology department of King Khalid University Hospital (KKUH) located in Riyadh, Saudi Arabia. Tissues were obtained from 20 Saudi patients with CRC undergoing a routine biopsy procedure as part of their medical treatment. Adjacent normal tissues for all the subjects were also collected. Informed written consent was signed by all patients recruited in the study. Samples were immediately stored in RNAlater solution (Ambion®) to maintain the quality and quantity of RNA.

DNA and RNA Extraction

Total RNA from the collected cancer and normal tissues was extracted with the All Prep DNA/RNA Mini Kit (Qiagen, Germany). Genomic DNA was purified from the whole blood of each patient using the QI Aamp DNA Blood Mini Kit (Qiagen, Germany). All steps recommended by the manufacturer were strictly followed. The concentrations and purity of the extracted DNA and RNA were quantitated using NanoDrop™ Lite Spectrophotometer (Thermo Fisher Scientific, Epsom, UK).

Nucleic acid Extraction

Total RNA was obtained from RNAlater-stabilized colorectal tissues using the All Prep DNA/RNA Mini Kit (Qiagen, Germany) and was subsequently treated with DNase I (DNA-free, Ambion Inc., Austin, TX) to remove genomic DNA. The genomic DNA was also purified from the whole blood samples using the QI Aamp DNA Blood Mini Kit (Qiagen, Germany). All purifications were performed according to the manufacturer's protocol. The nucleic acid concentrations and purity were quantified using NanoDrop™ Lite Spectrophotometer (Thermo Fisher Scientific, Epsom, UK). cDNA synthesis was performed using Reverse Transcription Kit (Applied Bio systems, USA). Gene expression studies were performed using SYBR green method and GAPDH (reference gene) was used as the internal normalizer.

Quantification of TNKS1 Expression Using qRT-PCR

Gene expression was studied using qRT-PCR by quantifying the transcribed mRNA (cDNA). Briefly, PCR reactions were prepared in 96-Well Micro AmpFast Optical Reaction Plate from Applied Biosystems® (Life Technologies, USA). Gene expression primers were designed by using primer 3 NCBI from Invitrogen (Thermo Fisher Scientific, Epsom, UK). Gene expression primer sequences used are listed in Tab. 1. GAPDH from CROGEN was used as the reference gene. The optimization of the primer concentration was performed. Each set of primers worked best at 10 p mol·µl^{-1} concentration. The annealing temperature was optimized and restricted to 60°C, which corresponds to the optimal working conditions for the AmpliTaq Gold® DNA polymerase enzyme (Perkin Elmer). The Syber Green Master Mix in each well contained 6.25 mL Power SYBR® Green PCR Master Mix 2X from Applied biosystems® (Life Technologies, USA), 0.25 µL forward primer, 0.25 µL reverse primer for each gene, 'and 3.75 µL nuclease free water, and 2 µL cDNA was added which gave a total volume of 12.5 µL in each well. Each sample of cDNA was analyzed in triplicate for both the cytokine genes and GAPDH. The plate was covered with sealant and placed in ViiA™ 7 Real-Time PCR System Applied Biosystems® (Life Technologies, USA). In the qPCR thermocycler, optimal conditions for each target gene were applied as 10 min at 95°C (activation), followed by PCR stages: 45 cycles of 15 sec at 95°C (denaturation), 1 min at 60°C (annealing), followed by the melting curve stage, 15 sec at 95°C.

The specificity of primer assays used for the reaction was verified by the presence of a single melting temperature peak. Results were then analyzed using $2^{-\Delta Ct}$ a variation of the Livak method, where ΔCt was calculated, and then the fold change values were measured using normalization method.

Promoter Methylation of TNKS1 Gene

Sodium bisulfite treatment of DNA: Tissue genomic DNA was treated with sodium bisulfite using EpiTect® Bisulfite Conversion Kit from (Qiagen, Germany) as per the manufacturer's protocol. This process converts non-methylated cytosine nucleotide residues to uracil, whereas methylated cytosine nucleotides remain unchanged.

Methylation-specific PCR (MSP) was performed on sodium bisulfate-treated DNA of CRC and normal tissues to detect the epigenetic modification of TNKS1. The promoter methylation of TNKS1 was measured in 50% of our samples. PCR products after MSP of converted CRC samples and paired normal match had unmethylated promoters (Fig. 3(B)), and no epigenetic alteration was detected in TNKS1.

Methylation-Specific Polymerase Chain Reaction (MSP): DNA methylation pattern of TNKS1 promoter was examined by methylation-specific PCR on 50% of our CRC samples with different mRNA expression. Forward and reverse primers of the TNKS1 promoter (Macrogen, Korea) were designed using MethPrimer software program shown in (Tab. 2). For the reaction, 2 µl sodium bisulfite-treated DNA was added to 10.5 µl reaction buffer [0.2 mM dNTP, 180 mM $(NH4)_2SO_4$, 600 mM Tris buffer, pH 8.9, 50 mM $MgCl_2$, and

TABLE 1

Sequence of the gene expression primers used during this study

Gene	Sequence for the gene expression primers
TNKS1	F - GTC CCT GAC AGC CTA GAA ATA AG
	R - GGG AAC AGT AGC AGT TGA GTA TG
GAPDH	F - GGT ATC GTG GAA GGA CTC ATG AC
	R - ATG CCA GTG AGC TTC CGT TCA GC

F: Forward primer; R: Reverse primer

TABLE 2

Primers used in the methylation-specific polymerase chain reaction

Gene Template	Primer Sequence (5'→3')	Product Size (bp)	Ta (°C)
Methylated TNKS1	F - TTAGGGGTTTTAGCGTCGTC R - AAATCGAAACCTATCGAACGAA	177	58
Unmethylated TNKS1	F - AGGGGTTTTAGTGTTGTTGT R - AATCAAAACCTATCAAACAAA	174	54

F: Forward primer; R: Reverse primer

1.25 U·µl^{-1} Platinum® Taq DNA Polymerase High Fidelity (Invitrogen, USA)] containing 1 µl of 10 pmol·µl^{-1} of forward and reverse primers, each, specific to the unmethylated and methylated DNA sequences. The reaction mixture was denatured for 2 min at 94°C and incubated for 35 cycles (denaturing for 30 s at 94°C, annealing for 30 s at variable °C depending on primers, and extending for 30 s at 68°C). Final extension was continued for 5 min at 68°C by a Veriti® thermocycler (Applied Biosystems, USA). EpiTect® control DNA, methylated and unmethylated (Qiagen, Germany) was used as a positive control for methylated and unmethylated reactions, respectively. The PCR products were resolved by electrophoresis in a 2% agarose gel, and the ethidium bromide-stained PCR products were visualized under UV illumination (Biometra, DE).

Sequencing of Ankyrin Domain of Tankyrase-1

Sequencing of exon 10 of TNKS1 was performed by Sanger sequencing method using BigDye® Cycle Sequencing Kit (Applied Biosystems, USA). For the reaction, a 0.25 dilution of the BigDye terminator mix was used, and the total reaction volume was 20 µl, which contained 2 µl of 2.5X Ready Reaction Premix, 3 µl of 5X BigDye Sequencing Buffer, 2 µl of 3.2 pmol·µl^{-1} forward primer, and 13 ng of purified PCR product. Cycle sequencing was performed using Veriti® thermocycler (Applied Biosystems, USA) for 25 cycles of 96°C for 2 min, 96°C for 10 s, 50°C for 5 s, and 60°C for 4 min; the rapid thermal ramp of all steps was 1°C·s^{-1}.

Statistical analysis

Statistical analysis was performed using the GraphPad Prism version 7.03 (GraphPad Software, USA) and Microsoft Excel®. The relative gene expression was calculated by using the 2^(-ΔΔCT) method, and the statistical association between the variables were assessed by Independent t-test analysis. The differences were considered statistically significant if p-value ≤ 0.05 was obtained. For Sanger sequence analysis, DNA Baser Sequence Assembler V4.3 was used to sequence data assembled and mutation detection.

Results

TNKS1 expression level in CRC

Tankyrase-1 mRNA was expressed in 100% of tumor tissues and adjacent normal tissues, but the expression level was variable between samples. The mean relative TNKS1 gene expression was (0.19 ± 0.27), which represents the up-regulation of TNKS1 gene in CRC samples compared with normal samples but the difference was not statistically significant (Fig. 1).

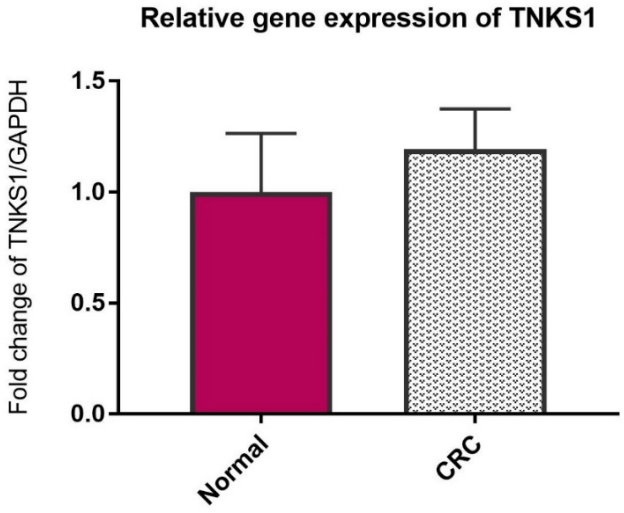

FIGURE 1. Relative gene expression of TNKS1 gene.Promoter methylation of TNKS1.

PCR products after MSP of TNKS1 promoter in CRC samples and paired normal match had unmethylated promoters with no epigenetic alteration as clearly seen in Fig. 2.

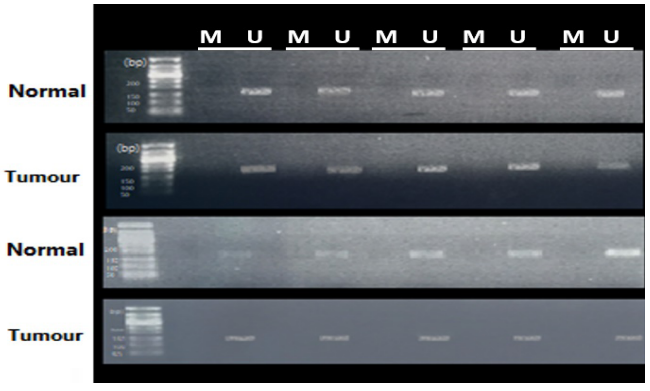

FIGURE 2. PCR product of converted CRC samples paired with normal match with different methylated (M) and unmethylated (U) primers.

Sequencing of Ankyrin Domain of TNKS1

The PCR fragments of the tumor and normal genomic DNA were sequenced and no mutation, such as frameshift mutation, was detected in the mononucleotides repeat of the exon 10, and the sequence in CRC tissues was the same as in the wild type sequence (Fig. 3).

Correlation Between TNKS1 Expression and Its Regulatory Factors

The expressional levels of TNKS1 in CRC tissues and their adjacent normal tissues were diverse between samples. Somatic mutations and/or epigenetic modification play a role in the expressional level change of genes. Tumor tissues and normal match tissues with down-regulation of TNKS1 gene expression have unmethylated promoters and intact exon 10 sequences similar to that in the wild type sequence (Fig. 4).

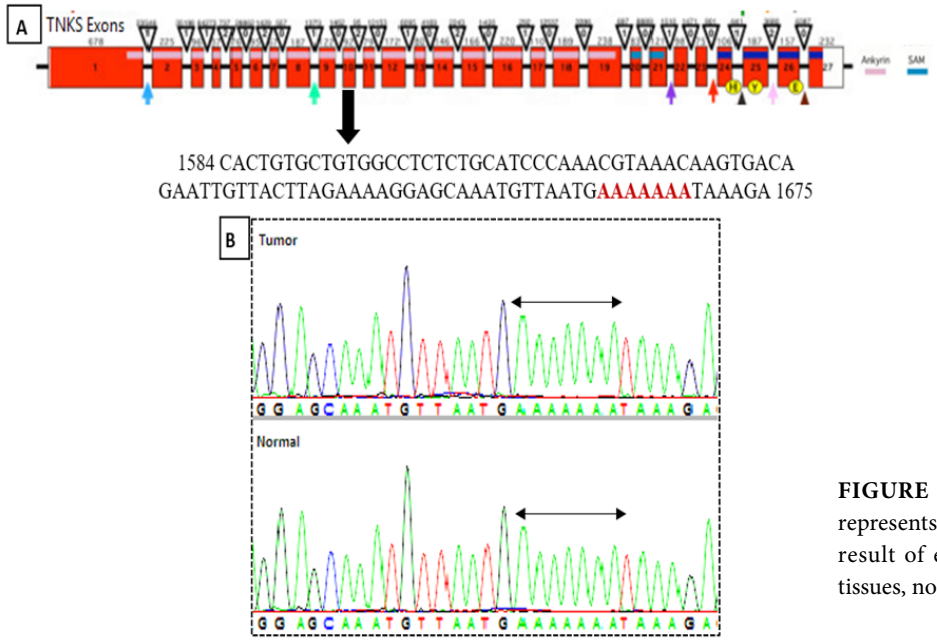

FIGURE 3. A) Exons of TNKS1 gene, exon 1-19 represents the ankyrin domain [85]. B) Sequencing result of exon 10 of TNKS1 in CRC and normal tissues, no mutation was detected.

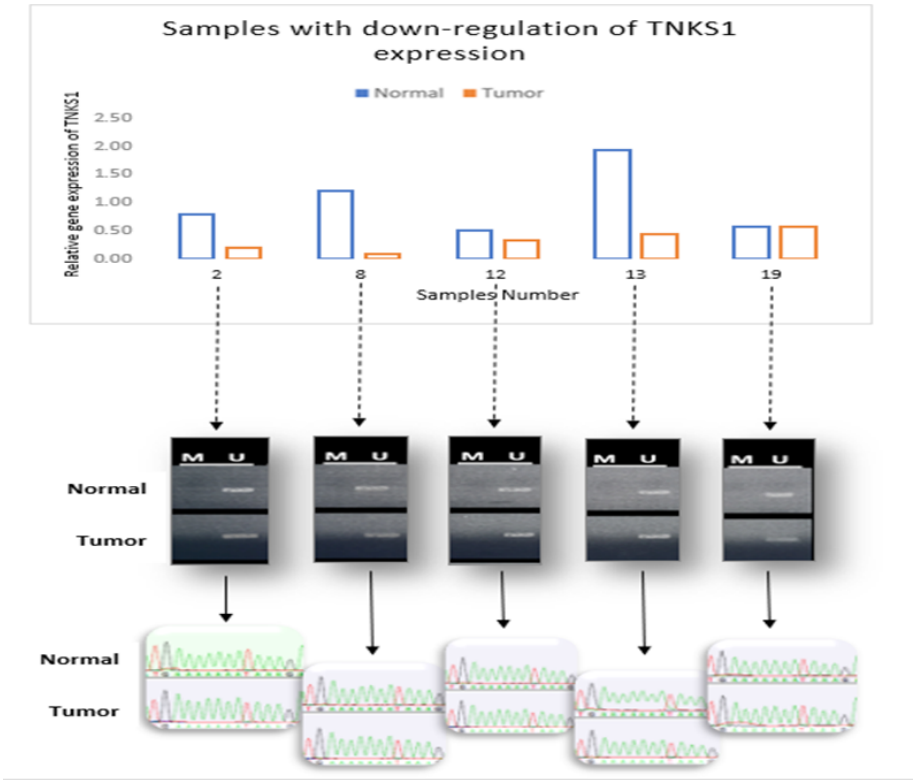

FIGURE 4. Correlation between the decrease in TNKS1 expression in tumor versus control, and the promoter methylation as well as exon 10 mutations in TNKS1.

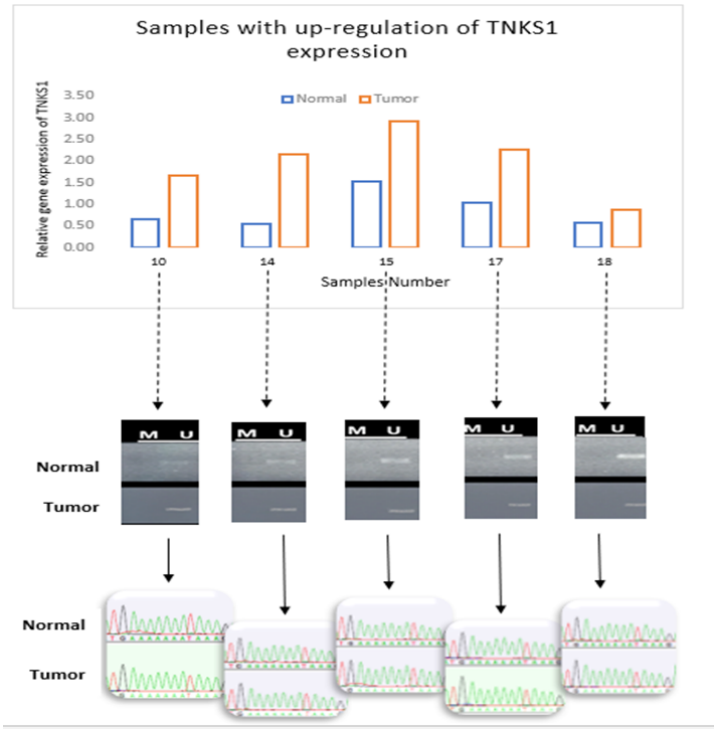

FIGURE 5. Correlation between the increase in TNKS1 expression in tumor versus normal samples, and the promoter methylation as well as exon 10 mutations in TNKS1.

Furthermore, TNKS1 up-regulation that was represented in some of the tumor and normal match tissues in the early stage of CRC also have unmethylated promoters and intact exon 10 sequences same as in the wild type sequence such as seen in a sample with down-regulation (Fig. 5). Both down and up-regulation of TNKS1 expression in CRC and normal match tissues have no correlation with the somatic mutation of TNKS1 gene as well epigenetic modification (promoter methylation).

Discussion

The present study explores TNKS1 gene expression level and its regulation in colorectal cancer. Our results showed that all cancerous and normal tissues express TNKS1, but the expression level was variable between samples. In an earlier study by Gelmini *et al.* (2006), high TNKS1 expression levels were reported in colon cancer. On the contrary, another study reported overexpression of TNKS2 and under expression of TNKS1 in colon cancer tissues (Shebzukhov *et al.*, 2008). Interestingly, Bhardwaj *et al.* (2017), explained the contribution of TNKS1 and TNKS2 in telomere length regulation and they found that either TNKS1 or TNKS2 is adequate for telomere maintenance. Our results showed that the up-regulation of TNKS1 in CRC tissues was significantly associated with the tumor stage though none of the other parameters, such as age, gender, and tumor location, showed any correlation. The expression of TNKS1 was significantly higher in early stages (I, II) than late stages (III, IV) of CRC development. These results agree with Gelmini *et al.* (2006), who found that TNKS1 level was inversely related to colon cancer stages. TNKS1 level was directly proportional to tumor stage in other types of cancers such as bladder cancer, astrocytoma neoplasm, gastric cancer, and astroglial brain tumor (Gelmini *et al.*, 2007; Tang *et al.*, 2012; Gao *et al.*, 2011; La Torre *et al.*, 2013).

Overexpression of TNKS1 may be involved in tumor development and progression in the early stages. According to previous studies, in these stages, cell division is increased and accelerates shortening of the telomeres, and Tankyrase-1 assists telomeres in achieving their maximum stability through length maintenance by decreasing the affinity of TRF1 from the telomeric complex and promoting telomerase access to telomeres in the CRC tissue. A strong relationship between Tankyrase-1 and telomerase has been reported in gastric cancer (Gao *et al.*, 2011), astroglial brain tumor (La Torre *et al.*, 2013), multiple myeloma, and plasma cell leukemia (Xu *et al.*, 2001). Some publishers have shown a high level of telomerase activity in CRC and others have reported the relation between tumor progression and telomerase activity (Valls-Bautista *et al.*, 2009; Kojima *et al.*, 2011; Safont *et al.*, 2011; Bertorelle *et al.*, 2013). However, in this study, down-regulation of TNKS1 level in the late stages (III, IV) of CRC development did not affect the progression of cancer, implying that CRC cells can reach late stages with a low level of TNKS1. Consistent with this, a recent study has also shown the survival of the colon adenocarcinoma cell line even with the inhibition of Tankyrases (Bhardwaj *et al.*, 2017).

Our results showed that the promoter of TNKS1 was unmethylated in CRC and normal tissues; no epigenetic modification was detected in TNKS1, and the promoter methylation did not participate in the regulation of TNKS1 expression. Our findings agree with the report on this aspect in the Catalogue of Somatic Mutations in Cancer (COSMIC) database, which showed that the promoter of TNKS1 is normally hypomethylated in most of the cancers including CRC (Forbes *et al.*, 2017).

During this investigation, we studied the somatic mutation of TNKS1, through the sequencing of exon 10 of the gene, which is a part of the ankyrin repeat domain (ANK) (Otto *et al.*, 2005). ANK repeat is an important domain of

Tankyrase1 and mediates the protein-protein interaction between Tankyrase1 and telomeric TRF1 or other binding partners (Guettler *et al.*, 2011). No mutation was detected in exon 10 of TNKS1 gene in CRC and normal match tissues, and the gene sequence in the CRC tissues was the same as in the wild type sequence. Thus, no correlation was demonstrated between the exon 10 mutation and TNKS1 expression level. In a similar manner, a study on a Korean population observed a frameshift mutation in exon 9 of Tankyrases 1 in patients with gastric cancer and in exon 10 in gastric and CRC patients (Kim *et al.*, 2011). It can thus be suggested that other somatic mutations in the gene might participate in the regulation of TNKS1 expression in CRC.

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