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Exploring genetic and epigenetic alterations in *hTERT* gene in colorectal cancer

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Abstract

Introduction: Colorectal cancer (CRC) is a heterogeneous and multifactorial malignancy driven by a series of genetic and epigenetic alterations. In this field, telomere/telomerase dysfunction contributes to CRC carcinogenesis by impairing genomic stability and cellular replication.

Objective: This study aimed to evaluate genetic and epigenetic alterations in CRC by examining mutation rates in the human telomerase reverse transcriptase (*hTERT*) promoter region, relative telomere length (RTL), *hTERT* gene expression, and DNA methylation in the *TERT* hypermethylated oncological region (THOR).

Methods: A total of 45 CRC and 34 adjacent normal tissue samples from Moroccan patients were analyzed using molecular approaches, such as Sanger sequencing, quantitative PCR (qPCR), reverse transcription qPCR (RT-qPCR), and methylation-specific PCR (MSP).

Results: No mutations in the *hTERT* promoter region were identified. However, hypermethylation in the THOR region was reported in 82.2% of CRC samples and 79.4% of adjacent normal tissues. High *hTERT* expression was detected in 50% of CRC patients. In addition, telomere length was significantly shorter (p=0.002) in cancerous tissues (1.41 [1.36 – 1.43]) compared to normal mucosa (1.559 [1.46 – 1.63]), with an RTL ratio less than 1 (0.90 [0.86 – 0.95]). No significant differences were found between clinicopathological features and *hTERT* expression, THOR methylation, or RTL, except for a significant correlation between THOR hypermethylation and smaller tumor size (p=0.017) and between THOR methylation and RTL in CRC tissues (p=0.034).

Conclusion: These results suggest that telomere lengthening is crucial for CRC initiation and progression, and cancer cells tend to shorten telomeres to maintain the chromosomal instability (CIN) required for tumor progression. Further research

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is needed to elucidate the mechanisms underlying telomere shortening in CRC and understand the role of telomerase/telomere complex in CRC initiation and progression, which could provide new diagnostic, prognostic, and therapeutic targets.

Keywords: Colorectal cancer; THOR methylation; *hTERT* promoter mutation; Gene expression; Telomere length

1. Introduction

Colorectal cancer (CRC) is the third most common cancer and the second leading cause of cancer-related death worldwide. According to GLOBOCAN 2022, 1,926,425 new cases were diagnosed and 904,019 deaths were registered, representing 9.6% of all diagnosed cancers and 9.3% of all cancer-related deaths.[1] In Morocco, the overall incidence of CRC has noticeably increased during the past decades. This increasing burden is globally linked to changes in lifestyle, including dietary patterns, urbanization, and increasing life expectancy.[2] Indeed, the incidence of CRC is currently recognized as one of the leading markers of nutritional and epidemiological transitions in societies undergoing socioeconomic development and a shift to an industrialized lifestyle.[3] According to the Great Casablanca Cancer Register, one of the largest in North Africa and encompassing both rural and urban populations, CRC ranks third in both men and women, with overall incidence rates of 10.6 and 10.4/100,000 persons/year, respectively.[4]

CRC is a heterogeneous and multifaceted malignancy characterized by a series of genetic and epigenetic alterations in oncogenes and tumor suppressor genes responsible for the initiation and progression of the adenoma-carcinoma sequence.[5] The development of CRC is associated with three major events: chromosomal instability (CIN), the CpG island methylator phenotype, and microsatellite instability.[6] Approximately 65 – 70% of sporadic CRCs are related to CIN, mainly associated with chromosome segregation defects, DNA damage repair, and excessive telomere breakage.[6,7] In recent years, assessment of such genetic alterations has made an outstanding contribution to our understanding of CRC development and progression. In this field, telomere dysfunction is reported as a key driving mechanism in the CIN.[8] Telomere shortening can play two opposing roles: (1) inducing cell death to suppress tumor formation or (2) promoting tumors by inducing genetic instability – one of the key events in CRC initiation.[8] In most human tumors, telomere maintenance is achieved through telomerase activation.[9]

The human telomerase reverse transcriptase (*hTERT*) is the catalytic subunit of the telomerase and is responsible for elongating telomeres, eventually leading to cellular immortality. The *hTERT* gene is located on chromosome 5p15.33, comprises 16 exons, and encodes a protein of 1132 amino acids with a molecular mass of 127 kDa.[10] The *hTERT* gene is highly expressed in embryonic stem and germ cells, downregulated during differentiation and development, and silenced in fully differentiated somatic cells.[8,11] *hTERT* is expressed in many types of cancer, including CRC, and plays a pivotal role in oncogenesis by providing proliferation, survival, and anti-apoptosis signals required for tumor progression.[12]

hTERT is regulated by both genetic and epigenetic mechanisms. Several regulatory mechanisms have been identified, including point mutations in the promoter region, promoter methylation, and *hTERT*-targeting miRNAs.[13,14] Specifically, a mutated and hypermethylated region within the *hTERT* promoter, named *TERT* hypermethylated oncological region (THOR), has been associated with *hTERT* upregulation in cancer. *hTERT* promoter mutations occur frequently in different tumor types and are commonly characterized by two hotspot mutations located at positions -124 (C228T) and -146 (C250T) upstream of the start codon, both of which drive a significant upregulation of expression.[15,16] In CRC, however, mutations in the core region of the *hTERT* promoter are rare and often undetected, suggesting that other regulatory mechanisms may play a key role in *hTERT* expression.[17-19] DNA methylation of the *hTERT* promoter is one of the most common and stable regulators of gene expression. While promoter methylation typically silences gene expression, in the case of *hTERT*, hypermethylation of the promoter core region has been associated with increased expression. Conversely, demethylation of the catalytic site leads to a notable reduction in telomerase activity and thus telomere shortening.[18,19]

In CRC, telomerase activity and expression of *hTERT* have been extensively studied, highlighting their crucial roles in cancer progression. Moreover, these studies underscore the clinical relevance of assessing telomerase status in the management of many cancers, including CRC. Overall, telomerase activity has been detected in up to 95% of CRC patients,[20] although this activity is not necessarily

associated with telomere lengthening. Several studies have shown that telomeres are shortened in colorectal tumors compared to adjacent healthy tissues.[8,21-24]

Given the pivotal role of telomeres and telomerase in colorectal carcinogenesis, this prospective study aims to explore *hTERT* promoter mutations, *hTERT* expression, DNA methylation status, and relative telomere length (RTL) in CRC patients from Morocco. By focusing on these factors, the study seeks to uncover potential regional or ethnic variations in telomere-related mechanisms driving CRC development.

2. Materials and methods

2.1. Patients and sampling

This prospective study included 45 fresh frozen CRC biopsies and 34 adjacent normal tissues from patients undergoing curative surgery at the Digestive Surgery Department of Mohammed V Military Teaching Hospital and the Surgery Department of Ibn Sina University Hospital in Rabat, Morocco. Only histopathologically confirmed CRC cases scheduled for curative resection of the primary tumor were included. Patients who had received neoadjuvant therapy were excluded. Tumor staging was assessed by pathologists using the pathological Tumor-Node-Metastasis (pTNM) classification system.

All clinicopathological data, including tumor site, histological differentiation, vascular embolization, and perineural invasion (PNI), were retrieved from patients' medical records. Moreover, face-to-face interviews were conducted with all patients to collect information on age, gender, and smoking history. From each patient, a fresh tumor biopsy and adjacent normal mucosa were collected, immediately preserved in the RNAlater (Invitrogen, USA), and stored at −80°C until use.

The study protocol was approved by the Ethics Committee for Biomedical Research at the Faculty of Medicine and Pharmacy of Rabat, Morocco (Ref 73/23). Written informed consents were obtained from all patients before surgery, ensuring full ethical compliance.

2.2. DNA and RNA extraction

Genomic DNA was extracted from CRC fresh frozen tissues and adjacent normal mucosa using the phenol/chloroform method.[25] Total RNA was isolated from CRC tissues stored in RNAlater using TRIzol™ Reagent (Labbox, France), according to the manufacturer's instructions. The yields and quality of extracted DNA and RNA were assessed using the NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, USA).

2.3. Evaluation of the mutational status of the *hTERT* core region

The mutational status of the *hTERT* core region was assessed by Sanger sequencing. First, a 163 bp region of the *hTERT* core promoter was amplified using the primers listed in Table 1. The PCR reaction mixture (25 µL) contained 5 µL of 5X MyTaq reaction buffer, 10 µM of each primer, 1 U of MyTaq DNA polymerase (Bioline, UK), and 2 µL of genomic DNA (50 – 100 ng/µL). Amplification was carried out with an initial denaturation at 95°C for 1 min, followed

Table 1. Primer sequences used for the assessment of *hTERT* promoter mutations, methylation, gene expression, and telomere lengths

Analysis target	Primers (5'–3')	Sequences (5'–3')	Tm (°C)
hTERT mutation	F	CACCCGTCCTGCCCCTTCACCTT	60
	R	GGCTTCCCACGTGCGCAGCAGGA	
hTERT expression	F	CCGCCTGAGCTGTACTTTGT	60
	R	CAGGTGAGCCACGAACTGT	
Telomere length	F	ACACTAAGGTTTGGGTTTGGGTTTGGGTTTGGGTTAGTGT	60
	R	TGTTAGGTATCCCTATCCCTATCCCTATCCCTATCCCTAACA	
hTERT methylation	M		
	F	GAGGTATTTCGGGAGGTTTCGC	58
	R	ACTCCGAACACCACGAATACCG	
	U		
	F	GGGAGGTATTTTGGGAGGTTTTGT	58
	R	CAAACTCCAAACACCACAAATACCA	
B2M expression	F	GAGGCTATCCAGCGTACTCCA	60
	R	CGGCAGGCATACTCATCTTTT	

Abbreviations: *hTERT*: Human telomerase reverse transcriptase; *B2M*: β2 microglobulin; F: Forward; R: Reverse; M: Methylated; U: Unmethylated.

by 35 cycles of 95°C for 30 s, 60°C for 30 s, and 72°C for 30 s, with a final extension at 72°C for 5 min. A negative control was added in each reaction. PCR products were then purified using the ExS-Pure™ Enzymatic PCR Cleanup Kit (NimaGen, The Netherlands), following the manufacturer's instructions.

All samples were subsequently prepared for Sanger sequencing using the Big Dye Terminator v3.1 cycle sequencing kit (Applied Biosystems, USA). Sequencing reactions were carried out in a final volume of 10 µL containing 10 µM of forward or reverse primer (Table 1), 1 µL of 2.5X BigDye Ready reaction mix v3.1, and 2 µL of purified PCR product. The reaction mixture was then incubated at 96°C for 1 min, followed by 25 cycles of denaturation at 96°C for 10 s, primer annealing at 50°C for 5 s, and extension at 60°C for 4 min. The sequencing reaction products were purified using the ethanol-ethylenediaminetetraacetic acid method. Finally, the purified sequencing products were sequenced on an ABI 3130 XL DNA analyzer (Applied Biosystems/Thermo Fisher Scientific, USA). The resulting sequences were aligned with the reference gene sequences from the GenBank database (NCBI Gene ID: 7015) using Sequencing Analysis Software v5.4 (Applied Biosystems/Thermo Fisher Scientific, USA).

2.4. Telomere length measurement

The RTL was determined using the quantitative PCR (qPCR) method established by Cawthon *et al.*,[26] offering a precise and standardized approach. The amplification reaction was performed in a total volume of 10 µL containing 2X Power-Up™ SYBR™ Green Master Mix (Thermo Fisher, USA), 10 µM of each primer (Table 1), and 20 ng of genomic DNA. Amplification was carried out on the StepOne qPCR system (Applied Biosystems, USA) using the following conditions: 95°C for 2 min, followed by 40 cycles of 95°C for 3 s and 60°C for 30 s. To minimize inter-sample variation, each sample was analyzed in triplicate, and final RTL values were calculated as the mean of three replicates. The single-copy reference gene used in this study was β2-microglobulin (*B2M*), and the corresponding primers are also reported in Table 1.

A standard curve was generated using five concentrations of the reference human genomic DNA sample – prepared by 10-fold serial dilutions (100 ng, 50 ng, 5 ng, 0.5 ng, etc. per well). RTL values were expressed as the ratio of the threshold cycle (Ct) value of telomere repeat copy number (T) to that of the single-copy gene (S), referred to as the T/S ratio. The RTL ratio was further calculated as the RTL in CRC tissue divided by the RTL in adjacent mucosa. A ratio greater than 1 indicated longer telomeres in CRC tissue compared to the adjacent mucosa.

2.5. Epigenetic profiling

2.5.1. Sodium bisulfite modification

Genomic DNA extracted from tumors and their adjacent normal tissues was treated with sodium bisulfite to convert unmethylated cytosines to uracil while leaving methylated cytosines unchanged. Sodium bisulfite modification and DNA purification were performed using the EZ DNA Methylation™ kit (Zymo Research, USA), following the manufacturer's instructions.

2.5.2. Methylation-specific PCR (MSP)

DNA methylation patterns in the THOR region of the *hTERT* gene were assessed using MSP. Two primer pairs of the *TERT* promoter (THOR region) were used to discriminate between the methylated (M) and unmethylated (U) DNA (Table 1). The PCR reaction was carried out in a final volume of 25 µL containing 2 µL of bisulfite-modified DNA, 2.5 µL of 10X PCR buffer, 10 µM of dNTPs, 10 µM of each primer, and 1 U of HotStarTaq DNA Polymerase (Qiagen, Germany). Amplification was performed using the GeneAmp PCR System 9700 (Applied Biosystems, USA). The cycling conditions included an initial denaturation at 95°C for 15 min, followed by 40 cycles comprising a denaturation at 95°C for 30 s, annealing at 60°C for 30 s, and extension at 72°C for 45 s. A final extension was performed at 72°C for 10 min. In each PCR reaction, a negative control was included. The PCR products were then analyzed on a 2% agarose gel, stained with ethidium bromide (10 mg/mL; Sigma-Aldrich, USA) and visualized under UV illumination.

2.6. Gene expression study

In the present study, real-time qPCR (RT-qPCR) was used to assess *hTERT* expression levels. First, cDNA was synthesized from 1 µg of purified RNA by using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, USA), following the manufacturer's protocol. Expression levels of the targeted gene were measured by qPCR. Specifically, 2 µL of cDNA was added to a reaction mixture containing 2X PowerUp SYBR Green master mix, 10 µM of each primer (Table 1), in a final volume of 10 µL. QPCR was conducted under the following conditions: an initial cycle at 95°C for 10 min, followed by 40 cycles of denaturation at 95°C for 15 s and annealing at the gene-specific temperature for 1 min. Melting curve analysis was carried out to confirm the absence of primer-dimer formation, using the following conditions: 95°C for 15 s, 60°C for 1 min, and a final incubation at 95°C for 15 s. Each sample was run in triplicate, and a template-free control was included for each primer pair to detect any potential contamination or non-specific amplification. To ensure

accuracy, *B2M* was selected as the housekeeping gene for normalization due to its stable expression across CRC cells.

The Ct values were determined for both *hTERT* and *B2M*, and the relative expression of *hTERT* was calculated using the formula: $2^{-\Delta Ct}$, where ΔCt is the difference between the average Ct values of *hTERT* and *β2M*. The resulting data were statistically analyzed to compare gene expression levels across different experimental groups.

2.7. Statistical analysis

Statistical analyses were performed using Jamovi biostatistical software (Version 2.3.28, The jamovi project, Australia)[27] and GraphPad Prism software (Version 8, GraphPad Software, Inc., USA). For descriptive analysis, qualitative variables were displayed as counts and percentages. Quantitative variables with a symmetrical distribution were presented as means ± standard deviations (SDs), while those with an asymmetrical distribution were expressed as medians ± quartiles (25^{th} and 75^{th} percentiles). Normality of quantitative variables was evaluated using the Shapiro–Wilk test. A *p*-value more than 0.05 indicated normal distribution, while a $p \leq 0.05$ suggested non-normality. Correlations between two quantitative variables were assessed using Pearson's or Spearman's correlation coefficient. McNemar's test was used to evaluate the concordance of the *THOR* methylation status between CRC tissues and their corresponding adjacent normal tissue. Associations between RTL and clinicopathological parameters were analyzed using the Student's t-test or Mann–Whitney U test for comparisons between two groups, and ANOVA or Kruskal–Wallis test for comparisons involving more than two groups. Comparisons between qualitative variables were performed using the Chi-square (χ^2) test or Fisher's exact test, depending on expected cell counts. A $p \leq 0.05$ was considered statistically significant.

3. Results

3.1. Patient characteristics

The distributions of the demographic, clinical, and pathological characteristics of the 45 CRC patients are summarized in Table 2. Among the CRC patients, 55.6% were women (25/45) and 44.4% were men (20/45), with a sex ratio of 0.8. The average age of CRC patients was 66 years, ranging from 29 to 89 years old, and most CRC patients were older than 60 years, accounting for about 66.7% (30/45). The smokers in our cohort represented 4.44% (2/45). Among CRC cases, 62.2% (28/45) of tumors measured 5 cm or less, and 55.6% (25/45) were located in the colon. Furthermore, a double location of CRC was observed, with almost 15.6% (7/45) and 4.4% (2/45) of

Table 2. Demographic, clinical, and pathological characteristics of CRC patients

Characteristics	Cancer tissue		Normal mucosa	
	n	%	*n*	%
Total number of cases	45	100	34	100
Age				
≤60	15	33.3	10	29.4
>60	30	66.7	24	70.6
Sex				
Male	20	44.4	17	50.0
Female	25	55.6	17	50.0
Smoking status				
Smokers	2	4.44	1	2.9
Non-smokers	43	95.5	33	97.1
Alcohol consumption				
Yes	0	0.0	0	0.0
No	45	100.0	45	100.0
Tumor location				
Colon	25	55.6	18	52.9
Rectum	6	13.3	3	8.8
Sigmoid	5	11.1	4	11.8
Rectum-sigmoid	7	15.6	7	20.6
Colon-sigmoid	2	4.4	2	5.9
Tumor size (cm)				
≤5	28	62.2	-	-
>5	17	37.8	-	-
Tumor stage				
I	4	8.9	-	-
II	24	53.3	-	-
III	13	28.9	-	-
IV	4	8.9	-	-
Grade				
1	19	42.2	-	-
2	26	57.8	-	-
Histological differentiation				
High	19	42.2	-	-
Intermediate	26	57.8	-	-
Vascular embolization				
Absent	31	68.9	-	-
Present	14	31.1	-	-
PNI				
Absent	39	86.7	-	-
Present	6	18.3	-	-

(Cont'd...)

Table 2. ***(Continued)***

Characteristics	Cancer tissue		Normal mucosa	
	n	%	*n*	%
Red meat consumption (per week)*				
<2 times	10	35.7	-	-
≥2 times	18	64.3	-	-
Fruit consumption (per week)*				
<2 times	3	10.7	-	-
≥2 times	25	89.3	-	-

Notes: *Data were missing for 17 patients.
Abbreviations: CRC: Colorectal cancer; PNI: Perineural invasion.

patients having tumors in the rectum-sigmoid and colon-sigmoid, respectively.

According to the pTNM classification, most patients were diagnosed at stage II (53.3%; 24/45), followed by stage III (28.9%; 13/45), while only 8.9% of patients were diagnosed at stage IV. The distribution of patients based on their histological differentiation showed that 57.8% of cases had intermediate cell differentiation (26/45), and 42.2% exhibited high differentiation (19/45). Vascular embolization and PNI were present in only 31.1% (14/45) and 18.3% (6/45) of tumor biopsies, respectively. In our cohort, red meat (64.3%; 18/28) and fruit (89.3%; 25/28) were frequently consumed twice or more per week.

3.2. Evaluation of the mutational status of the *hTERT* promoter region

In the current study, sequencing of PCR products of the promoter region of the *hTERT* gene clearly showed the absence of any point mutation in the core region in both cancer tissues and matched normal mucosa samples.

3.3. Telomeres are significantly shorter in CRC tissues than their adjacent normal mucosa

The RTL assessment was performed using q-PCR, and for each sample, the relative ratio between the telomere (T) and β2M (S), used as the single-copy gene, was calculated (T/S ratio). The distribution of CRC cases and their normal mucosa according to RTL values and the clinicopathological characteristics are summarized in Table 3. Overall, a significant difference was observed between RTL in CRC tissues (1.41 [1.36 – 1.43]) and in adjacent normal mucosa (1.559 [1.46 – 1.63]), with an RTL ratio of 0.90 (0.86 – 0.95), clearly indicating that tumor tissues had shorter telomeres (p=0.002).

In CRC tissues, no significant association was observed between RTL and clinicopathological features, namely age (p=0.673), sex (p=0.191), smoking status (p=0.349), tumor location (p=0.850), tumor size (p=0.826), tumor stage (p=0.141), histological differentiation (p=0.986), vascular embolization (p=0.410), PNI (p=0.07), red meat intake (p=0.344), and fruit consumption (p=0.366) (Table 3).

Patient-by-patient comparisons between the RTL values of tumor tissues and adjacent normal mucosa, as well as their correlation with age, are reported in Figure 1. The analysis revealed the presence of longer telomeres in non-cancerous mucosa in 97% (33/34) of cases, while only 1 case (3%) had longer telomeres in the cancer tissue, with a p=0.002 (Figure 1A). A negative correlation was found between age and RTL in adjacent normal mucosa, although the association was not statistically significant (r = –0.286; p=0.101) (Figure 1B). No significant correlation was detected between age and RTL in tumor tissue (r = 0.013, p=0.931) (Figure 1C). Of note, a significant difference was observed between RTL ratio and sex (p=0.012), due to the difference in telomere length in normal mucosa between males and females. In fact, telomeres were significantly longer in females (1.62 [1.55 – 1.67]) than in males (1.46 [1.42 – 1.56]), with a p=0.02 (Table 3).

3.4. Assessment of the promoter methylation status of the THOR region

The methylation status of the *THOR* region in the *hTERT* gene was successfully analyzed in all samples, and the associations between the methylation profile in CRC tissues and their adjacent normal mucosa with clinicopathological features are summarized in Table 4. Overall, hypermethylation of the promoter region of the *hTERT* was detected in 82.2% of CRC cases (37/45) and 79.4% of their matched normal mucosa samples (27/34).

No significant association was observed between the methylation status and sociodemographic characteristics, including age, sex, smoking status, and alcohol consumption (p>0.05) for both tumor tissues and matched normal mucosa.

Regarding clinicopathological features, a significant association was found between DNA hypermethylation of the THOR region and tumor size (p=0.017). Specifically, 92.8% of patients with tumors less than or equal to 5 cm showed hypermethylation (26/28), whereas hypermethylation was reported in only 64.7% of patients with tumor sizes larger than 5 cm (11/17), indicating that smaller tumors are more likely to exhibit hypermethylation. No significant associations were found for tumor location, stage, histological differentiation, vascular embolization, or PNI (p>0.05). Likewise, dietary factors such as red meat intake and fruit consumption showed no impact on the methylation status of the *hTERT* gene (p>0.05).

Table 3. Distribution of RTL in CRC tissues and their adjacent normal mucosa according to the clinicopathological characteristics of patients

Clinicopathological characteristics	*n* (%)	RTL ratio		*p*	*n* (%)	RTL of tumor		*p*	*n* (%)	RTL of normal mucosa		*p*
		Mean±SD	Median (IQR)			Mean±SD	Median (IQR)			Mean±SD	Median (IQR)	
Total	34 (100.0)	0.91±0.06	0.90 (0.86 – 0.95)		45 (100.0)	1.40±0.05	1.41 (1.36 – 1.43)		34 (100.0)	1.55±0.13	1.56 (1.46 – 1.63)	
Age (years)												
≤60	10 (29.4)	0.88±0.07	0.88 (0.80 – 0.91)	0.086	15 (33.3)	1.39±0.07	1.42 (1.34 – 1.44)	0.637	10 (29.4)	1.60±0.16	1.62 (1.49 – 1.69)	0.205
>60	24 (70.6)	0.92±0.06	0.93 (0.87 – 0.96)		30 (66.7)	1.40±0.05	1.41 (1.38 – 1.43)		24 (0.70)	1.53±0.11	1.52 (1.45 – 1.60)	
Sex												
Male	17 (50.0)	0.93±0.06	0.95 (0.87 – 0.96)	**0.012***	20 (44.4)	1.39±0.06	1.38 (1.36 – 1.43)	0.191	17 (50.0)	1.49±0.11	1.46 (1.42 – 1.56)	**0.02***
Female	17 (50.0)	0.88±0.06	0.88 (0.83 – 0.90)		25 (55.6)	1.41±0.05	1.42 (1.41 – 1.44)		17 (50.0)	1.62±0.11	1.62 (1.55 – 1.67)	
Smoking status												
Smokers	1 (2.9)	0.96±0.00	0.96 (0.96 – 0.96)	0.436	2 (4.4)	1.47±0.07	1.47 (1.45 – 1.50)	0.349	1 (2.9)	1.49±0.00	1.49 (1.49 – 1.49)	0.642
Non-smokers	33 (97.1)	0.96±0.07	0.89 (0.86 – 0.95)		43 (95.6)	1.40±0.05	1.41 (1.36 – 1.43)		33 (97.1)	1.55±0.13	1.56 (1.45 – 1.63)	
Alcohol consumption												
Yes	0 (0.0)	–	–	NA	0 (0.0)	–	–	NA	0 (0.0)	–	–	NA
No	34 (100.0)	0.91±0.06	0.90 (0.86 – 0.95)		34 (100.0)	1.40±0.05	1.41 (1.36 – 1.43)		34 (100.0)	1.55±0.13	1.56 (1.46 – 1.63)	
Tumor location												
Colon	18 (52.9)	0.90±0.05	0.89 (0.86 – 0.96)	0.993	25 (55.6)	1.40±0.06	1.42 (1.36 – 1.43)	0.850	18 (52.9)	1.55±0.11	1.53 (1.46 – 1.61)	0.163
Rectum	3 (8.8)	0.91±0.03	0.89 (0.89 – 0.92)		6 (13.3)	1.41±0.04	1.42 (1.39 – 1.43)		3 (8.8)	1.58±0.04	1.58 (1.56 – 1.60)	
Sigmoid	4 (11.8)	0.92±0.08	0.95 (0.91 – 0.95)		5 (11.1)	1.40±0.09	1.43 (1.38 – 1.44)		4 (11.8)	1.53±0.2	1.50 (1.39 – 1.64)	
Rectum-sigmoid	7 (20.6)	0.89±0.07	0.90 (0.83 – 0.96)		7 (15.6)	1.38±1.25	1.39 (1.35 – 1.41)		7 (20.6)	1.55±0.12	1.59 (1.44 – 1.65)	
Colon-sigmoid	2 (5.9)	0.91±0.19	0.93 (0.86 – 0.99)		2 (4.4)	1.41±0.07	1.41 (1.38 – 1.43)		2 (5.9)	1.56±0.38	1.56 (1.42 – 1.69)	
Tumor size (cm)												
≤5	19 (55.9)	0.91±0.07	0.89 (0.86 – 0.95)	0.928	28 (62.2)	1.40±0.06	1.42 (1.36 – 1.43)	0.826	-	-	-	
>5	15 (44.1)	0.90±0.07	0.91 (0.86 – 0.95)		17 (37.8)	1.40±0.05	1.41 (1.38 – 1.43)		-	-	-	
Tumor stage												
I	2 (5.8)	0.93±0.19	0.93 (0.86 – 0.99)	0.553	4 (8.9)	1.41±0.04	1.41 (1.38 – 1.44)	0.147	-	-	-	
II	19 (55.9)	0.92±0.06	0.94 (0.89 – 0.95)		24 (53.3)	1.39±0.06	1.41 (1.36 – 1.43)		-	-	-	
III	11 (32.3)	0.89±0.06	0.88 (0.85 – 0.93)		13 (28.9)	1.40±0.06	1.41 (1.36 – 1.44)		-	-	-	
IV	2 (5.9)	0.87±0.05	0.87 (0.85 – 0.88)		4 (8.9)	1.43±0.01	1.43 (1.42 – 1.43)		-	-	-	
Histological differentiation												
High	12 (35.3)	0.91±0.07	0.85 (0.87 – 0.95)	0.961	19 (42.2)	1.40±0.06	1.41 (1.36 – 1.44)	0.986	-	-	-	
Moderate	22 (64.7)	0.90±0.06	0.91 (0.86 – 0.95)		26 (57.8)	1.40±0.05	1.42 (1.38 – 1.43)		-	-	-	

(Cont'd...)

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Table 3. ***(Continued)***

Clinicopathological characteristics	*n* (%)	RTL ratio		*p*	*n* (%)	RTL of tumor		*p*	*n* (%)	RTL of normal mucosa	
		Mean±SD	Median (IQR)			Mean±SD	Median (IQR)			Mean±SD	Median (IQR)
Vascular embolization											
Absent	25 (73.5)	0.92±0.06	0.91 (0.88 – 0.96)	0.119	31 (68.9)	1.41±0.06	1.42 (1.38 – 1.44)	0.410	-	-	-
Present	9 (26.5)	0.88±0.06	0.86 (0.83 – 0.90)		14 (31.1)	1.39±0.05	1.41 (1.36 – 1.43)		-	-	-
PNI											
Absent	30 (88.2)	0.91±0.07	0.91 (0.87 – 0.96)	0.418	39 (86.7)	1.41±0.05	1.42 (1.38 – 1.44)	0.07	-	-	-
Present	4 (11.8)	0.88±0.06	0.88 (0.85 – 0.91)		6 (13.3)	1.35±0.06	1.36 (1.33 – 1.38)		-	-	-
Red meat consumption**											
<2 times	10 (38.5)	0.89±0.08	0.87 (0.81 – 0.96)	0.394	10 (35.7)	1.41±0.05	1.43 (1.39 – 1.43)	0.344	-	-	-
≥2 times	16 (61.5)	0.91±0.06	0.90 (0.88 – 0.95)		18 (64.3)	1.39±0.06	1.42 (1.36 – 1.43)		-	-	-
Fruit consumption**											
<2 times	2 (7.7)	0.92±0.04	0.92 (0.91 – 0.94)	0.675	3 (10.7)	1.36±0.05	1.34 (1.33 – 1.38)	0.366	-	-	-
≥2 times	24 (92.3)	0.90±0.07	0.89 (0.85 – 0.95)		25 (89.3)	1.40±0.06	1.43 (1.36 – 1.43)		-	-	-

Notes: RTL ratio is computed by dividing RTL of tumor by RTL of the corresponding normal mucosa. *$p \leq 0.05$. **The total number of patients for CRC tissues and the adjacent normal mucosa is 28 and 26, respectively, and the consumptions were assessed per week.

Abbreviations: CRC: Colorectal cancer; RTL: Relative telomere Length; SD: Standard deviation; IQR: Interquartile range; PNI: Perineural invasion; NA: Not applicable.

3.5. Evaluation of *hTERT* gene expression

hTERT relative expression analysis was successfully explored in 26 fresh colorectal biopsies using RT-qPCR. Overall, the median *hTERT* relative expression among CRC patients in our study was 0.346 (0.112 – 2.39), suggesting a large variability in gene expression across the cohort. The median value of 0.346 was used as a cut-off to subdivide the studied cases into high (*hTERT* relative expression >0.346) and low (*hTERT* relative expression <0.346) expression groups. Accordingly, 50% of the CRC cases (13/26) showed high *hTERT* expression, while the other 13 cases had low expression.

The distribution of *hTERT* gene expression according to the clinicopathological parameters is reported in Table 5. Despite higher expression detected in some subgroups, such as tumors in the sigmoid (4.63 [0.287 – 10.1]), Stage III (1.46 [0.334 – 3.45]), and patients with PNI (1.27 [0.848 – 1.96]), statistical analysis showed no significance. Moreover, no significant association was found between *hTERT* relative expression and sociodemographic or other clinicopathological characteristics, including age (p=0.959), sex (p=1.000), smoking status (p=0.615), tumor site (p=0.939), tumor size (0.107), stage (p=0.783), histological differentiation (p=0.844), vascular embolization (p=0.567), PNI (p=0.471), and fruit consumption (p=0.837) (Table 5).

3.6. Correlation between *hTERT* relative expression, methylation profile of THOR region, and RTL

In the current study, we also explored the relationship between *hTERT* relative expression levels, THOR region methylation status, and RTL in CRC patients (Figure 2). Notably, patients with hypermethylation of the THOR region exhibited higher *hTERT* expression, though the association was not statistically significant (p=0.308; Figure 2A). Furthermore, a negative but non-significant correlation was observed between *hTERT* expression and RTL in CRC tissues (r = −0.220; p=0.279; Figure 2B).

Intriguingly, when examining the link between *hTERT* promoter methylation and RTL of both CRC tissues and adjacent normal mucosa, a significant correlation emerged (p=0.034). Specifically, CRC cases with hypermethylation in the *hTERT* promoter region displayed shorter telomeres than their hypomethylated counterparts. Alternatively, hypermethylated cases presented a longer RTL in adjacent normal mucosa compared to CRC tissues Figure 2C.

4. Discussion

Telomerase plays a pivotal role in cancer development by controlling telomere length in cancerous cells, preserving their ability to proliferate, and promoting tumor progression in 90% of human cancers.[28] Thus, research on

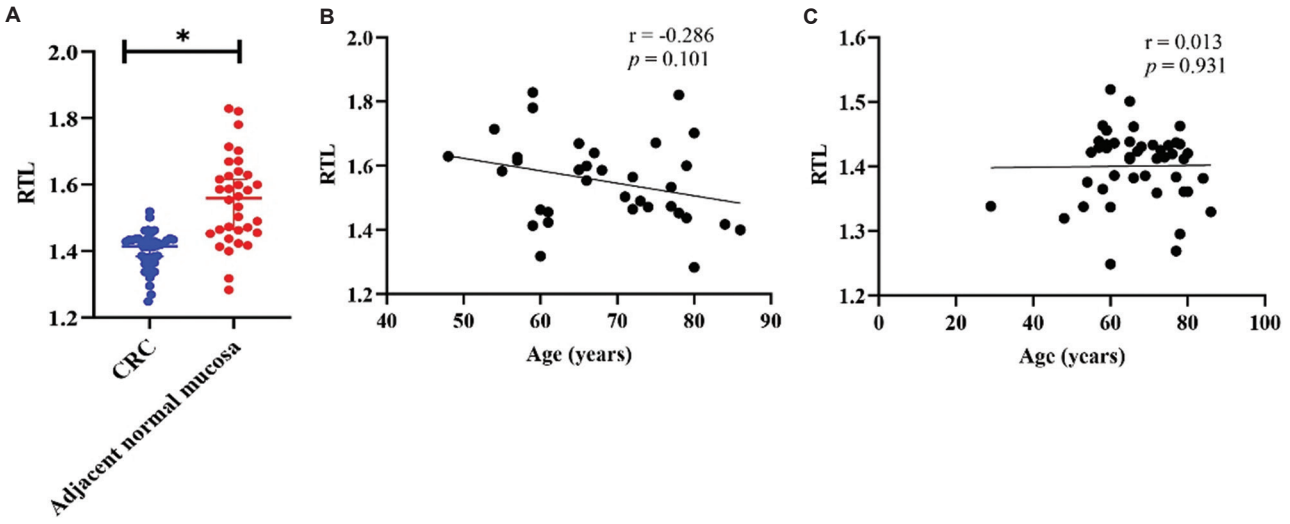

Figure 1. Relative telomere length (RTL) analysis in colorectal cancer (CRC). (A) Comparison of RTL between CRC tissues and their adjacent normal mucosa. Correlation between RTL and age in normal mucosa (B) and cancer tissues (C).
Note: Statistical significance in (A) was determined using t-test; *p<0.05. Pearson correlation was used for (B) and (C), with corresponding r and p-values indicated

Table 4. Association between methylation profile of THOR region in CRC and their adjacent normal mucosa and clinicopathological features

Clinicopathological characteristics	Total	CRC				Total	Adjacent normal mucosa			
		Hypermethylated		Hypomethylated			Hypermethylated		Hypomethylated	
		n	%	*n*	%		*n*	%	*n*	%
Total	45	37	82.2	8	17.8	34	27	79.4	7	20.6
Age (years)										
≤60	15	14	93.3	1	6.7	10	9	90.0	1	10.0
>60	30	23	76.7	7	23.3	24	18	75.0	6	25.0
p			0.168					0.324		
Sex										
Male	20	17	85.0	3	15.0	17	14	82.4	3	17.6
Female	25	20	80.0	5	20.0	17	13	76.5	4	23.5
p			0.663					0.671		
Smoking status										
Smokers	2	1	50.0	1	50.0	1	0	0.0	1	100.0
Non-smokers	43	36	83.7	7	16.3	33	27	81.8	6	18.2
p			0.327					0.206		
Alcohol consumption										
Yes	0	0	0.0	0	0.0	0	0	0.0	0	0.0
No	45	37	82.2	8	17.8	34	27	79.4	7	20.6
p			NA					NA		
Tumor location										
Colon	25	20	80.0	5	20.0	18	14	77.8	4	22.2
Rectum	6	5	83.3	1	16.7	3	2	66.7	1	33.3
Sigmoid	5	4	80.0	1	20.0	4	3	75.0	1	25.0

(Cont'd...)

Table 4. ***(Continued)***

Clinicopathological characteristics	Total	CRC				Total	Adjacent normal mucosa			
		Hypermethylated		Hypomethylated			Hypermethylated		Hypomethylated	
		n	%	*n*	%		*n*	%	*n*	%
Rectum-sigmoid	7	6	85.7	1	16.7	7	6	85.7	1	14.3
Colon-sigmoid	2	2	100.0	0	0.0	2	2	100.0	0	0.0
p			0.963					0.900		
Tumor size (cm)										
≤5	28	26	92.8	2	7.1			-		
>5	17	11	64.7	6	35.3					
p			0.017*							
Tumor stage										
I	4	4	100.0	0	0.0			-		
II	24	19	79.2	5	20.8					
III	13	10	76.9	3	23.1					
IV	4	4	100.0	0	0.0					
p			0.545							
Histological differentiation										
High	19	18	94.7	1	5.3			-		
Intermediate	26	19	73.1	7	26.9					
p			0.061							
Vascular embolization										
Absent	31	25	80.6	6	19.3			-		
Present	14	12	85.7	2	14.3					
p			0.681							
PNI										
Absent	39	31	79.5	8	20.5			-		
Present	6	6	100.0	0	0.0					
p			0.221							
Red meat consumption (per week**)										
<2 times	10	8	80.0	2	20.0			-		
≥2 times	18	13	72.2	5	27.8					
p			0.649							
Fruit consumption (per week**)										
<2 times	3	3	100.0	0	0.0			-		
≥2 times	25	18	72.0	7	28.0					
p			0.290							

Notes: *$p<0.05$; **Data were missing for 17 patients.
Abbreviations: CRC: Colorectal cancer; NA: Not applicable; PNI: Perineural invasion.

telomerase holds significant promise for its potential in cancer diagnosis, prognosis, and therapeutic applications in various cancers, including CRC.

The interaction between telomere length and telomerase status is crucial for preserving chromosomal stability and the replicative potential of cells. Abnormal functioning of this interaction has been identified as one of the main drivers of carcinogenesis, including CRC.[29] In this context, the present study was conducted to evaluate the mutational and methylation status of the *hTERT* promoter region and their impacts on the gene expression and telomere lengths in a Moroccan population, involving

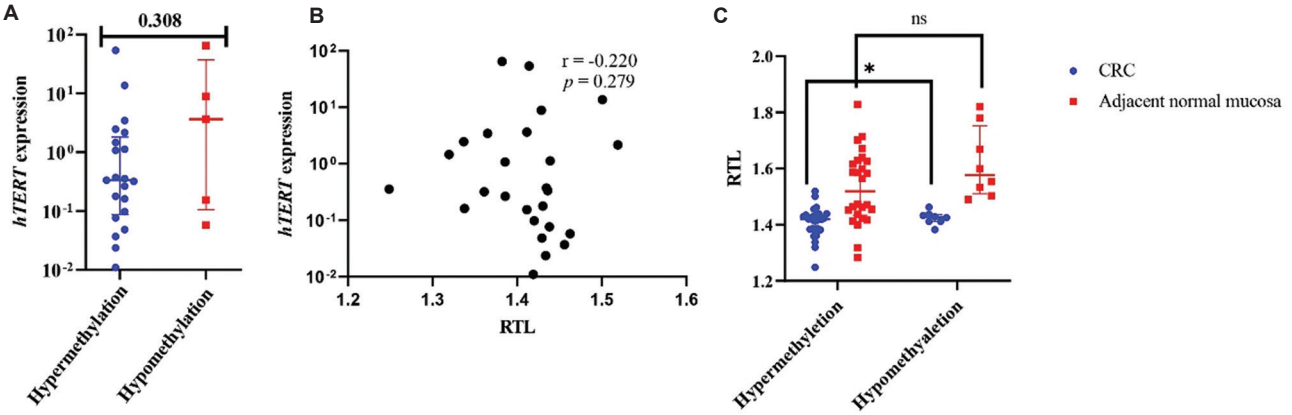

Figure 2. Relationship between *hTERT* expression, relative telomere length (RTL), and THOR methylation profile in colorectal cancer (CRC) patients. (A) Distribution of *hTERT* relative expression (log_{10}-transformed) according to methylation profile of the THOR region. (B) Correlation between hTERT expression and RTL in CRC tissues. (C) Association between RTL and THOR methylation profile in CRC tissues and adjacent normal mucosa. *Denotes significant association ($p<0.05$); ns denotes no significant association ($p\geq0.05$)

Table 5. Distribution of *hTERT* relative expression and clinicopathological characteristics of CRC patients

Clinicopathological characteristics	*n*	*hTERT* relative expression		*p*
		Median	IQR	
Total	26	0.346	0.112 – 2.39	NA
Age (years)				
≤60	11	1.13	0.105 – 2.31	0.959
>60	15	0.320	0.127 – 2.36	
Sex				
Male	7	0.357	0.293 – 1.62	1.000
Female	19	0.334	0.087 – 3.55	
Smoking status				
Smokers	1	2.17	2.17 – 2.17	0.615
Non-smokers	25	0.334	0.098 – 2.46	
Alcohol consumption				
Yes	0	-	-	NA
No	26	0.346	0.112 – 2.39	
Tumor site				
Colon	14	0.353	0.067 – 3.20	0.844
Rectum	2	1.10	1.09 – 1.11	
Sigmoid	4	4.63	0.287 – 10.1	
Rectum-sigmoid	4	0.222	0.172 – 0.566	
Colon-sigmoid	2	0.179	0.108 – 0.250	
Tumor size (cm)				
≤5	19	0.320	0.087 – 1.30	0.107
>5	7	3.65	0.256 – 31.3	
Tumor stage				
I	3	0.320	0.179 – 0.346	0.473
II	11	0.357	0.105 – 5.54	
III	9	1.46	0.334 – 3.45	
IV	3	0.098	0.087 – 0.138	
Histological differentiation				
High	13	1.13	0.334 – 2.46	0.072
Intermediate	13	0.162	0.057 – 0.357	
Vascular embolization				
Absent	18	0.365	0.157 – 2.39	0.567
Present	8	0.256	0.088 – 1.96	
PNI				
Absent	22	0.327	0.082 – 2.39	0.471
Present	4	1.27	0.848 – 1.96	
Red meat consumption (per week)*				
<2 times	7	0.266	0.067 – 1.30	0.837
≥2 times	9	0.178	0.057 – 0.334	
Fruit consumption (per week)*				
<2 times	0	-	-	NA
≥2 times	16	0.222	0.055 – 0.550	

Note: *Data were missing for 10 patients.
Abbreviations: CRC: Colorectal cancer; *hTERT*: Human telomerase reverse transcriptase; IQR: Interquartile range; PNI: Perineural invasion.

a cohort of 45 CRC patients and 34 matched adjacent normal tissue samples.

Consistent with previous studies, mutation assessment of the *hTERT* promoter revealed that all samples, including

45 CRC tissues and their 34 adjacent normal tissues, were wild-type. The hotspot mutations (C228T and C250T), as well as rare mutations (G245A, C243T, C242T, C230T, and C229T), were not detected in our cohort. These findings corroborate several studies reporting the absence of *hTERT* promoter mutations in CRC patients from Brazil,[17] America,[30] and Canada.[31] Interestingly, other studies have reported the occurrence of these mutations at low frequencies in CRC patients from the Middle East (1%) and the USA (3.9%), with notably higher rates in Black American patients (6%).[19,32] Therefore, the rare occurrence or absence of mutations in the CORE region of the *hTERT* gene in CRC suggests the involvement of other mechanisms, such as epigenetic regulation, in modulating the telomere/telomerase complex.[18] Consistently, THOR hypermethylation within the *hTERT* promoter has been reported as an epigenetic mechanism associated with *hTERT* upregulation in different types of cancer.[33,34]

In the present study, hypermethylation of the THOR region in the *hTERT* promoter was observed in 82.2% of CRC cases. These results are consistent with previous studies highlighting that hypermethylation of the *hTERT* promoter is a common mechanism required for transcriptional activation of *hTERT* during malignant transformation.[31,35-37]

Of particular interest, hypermethylation of the THOR region was also detected in 79.4% of adjacent normal mucosa. In Spain, using methylation-sensitive single-strand conformation analysis (MS-SSCA), the average methylation rates in tumoral, transitional, and normal CRC mucosa were 51%, 35%, and 20%, respectively.[22] However, Lee *et al*. reported aberrant THOR methylation in 94% of cancer tissues while hypermethylation was absent in normal samples.[31] These findings underscore the complex nature of adjacent normal mucosa, which may be phenotypically non-cancerous but already undergoing oncogenic changes. The presence of stem cells – or more broadly niche cells – in the colorectal crypts may partially explain THOR methylation, which is generally associated with hypermethylation of this region.[22]

Scientific evidence has shown that hypermethylation of the *hTERT* promoter region is associated with gene activation, and high expression of *hTERT* is common across several types of cancers, including CRC. This upregulation plays a pivotal role in tumorigenesis by providing the proliferative, survival, and anti-apoptotic signals required for tumor progression.[12] In our investigation, the median *hTERT* relative expression among CRC patients was 0.346 (0.112 – 2.39), suggesting variability in expression levels. Using the median value as a cut-off, 50% of CRC cases showed high *hTERT* expression. These results are consistent with previous studies showing overall high expression in 21 – 57% of CRC patients.[38-40]

To gain a better understanding of the mechanisms underlying telomerase activation, RTL was measured in both CRC tissues and adjacent normal mucosa. Overall, the RTL ratio was less than 1 (0.90 [0.86 – 0.95]), and the RLT was significantly longer in adjacent normal mucosa (1.559 [1.46 – 1.63]) compared to cancer tissues (1.41 [1.36 – 1.43]), with a significance difference (p=0.002). These results align with numerous studies showing shorter RTL in CRC tissues compared to their corresponding normal mucosa,[24,41-45] confirming that telomere shortening is a consistent hallmark of CRC. It is well-established that telomere stabilization is necessary for the unlimited proliferation of tumor cells, making telomere length a valuable diagnostic biomarker.[46] In addition, RTL has been proposed as a predictive biomarker in metastatic CRC patients treated with anti-epidermal growth factor receptor (anti-EGFR) therapy[47] and has been widely used for survival prediction, as patients with longer telomeres tend to have improved survival outcomes.[24,41]

Analysis of clinicopathological parameters showed that hypermethylation of the THOR region and the *hTERT* overexpression were detected at all tumor stages, including both early (Stage I) and advanced (Stage IV) stages of CRC. This confirms that telomere shortening is an early event in colorectal tumorigenesis.[42]

Exploring *hTERT* status alongside clinicopathological features unveiled a significant association between THOR hypermethylation and tumor size. Remarkably, hypermethylation was significantly higher in smaller tumor size (p=0.017), with 92.8% of these tumors showing hypermethylation, compared to 64.7% of larger tumors. This compelling association hints that THOR hypermethylation could represent an early event in tumorigenesis, potentially driving *hTERT* activation and telomere maintenance in smaller tumors.

No significant correlation was identified between THOR hypermethylation or *hTERT* overexpression and other clinicopathological features, including age, sex, smoking status, tumor location, clinical stage, or histological differentiation. This lack of correlation is consistent with several studies[21,24,41] and further supports the notion that telomere shortening is a universal feature of CRC, independent of tumor characteristics.

Interestingly, our study observed higher *hTERT* expression in tumors located in the sigmoid colon (4.63 [0.287 – 10.1]) compared to other tumor locations, in line with previous findings by Niiyama *et al*., although statistical significance was not reached.[48] hTERT expression was also higher

among patients under 60 years of age, those with stage III disease, and those with well-differentiated tumors, again without statistical significance. These observations are consistent with the findings of Saleh *et al.*,[38] who reported no statistically significant associations between hTERT expression and clinicopathological features. However, other studies have reported significant correlations with age (p=0.05), sex (p=0.02), and tumor grade (p=0.04).[39,40]

In the present study, hypermethylation and overexpression of hTERT did not promote telomere lengthening. In fact, tumor tissue showed shorter telomeres than adjacent normal mucosa (p=0.002). These results are in agreement with previous reports, showing the role of THOR methylation in regulating gene expression which is not linearly correlated with telomerase activity and TL, and highlighting the complex but central effect of the hTERT gene in CRC development and progression.[6,7,41] In the present study, a comparison between RTL and clinicopathological characteristics clearly showed the absence of any significant association, consistent with other studies.[9,41,43] Valls-Bautista *et al.*[22] reported that hTERT overexpression correlates more closely with telomerase activity – measured through fluorescent telomeric repeat amplification protocol (TRAP-F) – than with telomere length. This suggests that telomerase may influence other molecular pathways involved in CRC beyond simply lengthening telomeres. Indeed, CRC cells may sustain proliferative capacity and CIN without extensive telomere elongation, which may support tumor progression, therapy resistance, and metastasis

The present study confirms that telomere shortening is a universal feature of CRC, occurring early in all oncogenic processes and independently of clinicopathological characteristics. Moreover, telomere shortening is not directly associated with *hTERT* methylation and expression levels. However, several limitations must be acknowledged. The small sample size may have limited the statistical power to detect significant associations. Moreover, the use of adjacent normal mucosa as a control is suboptimal, as these tissues may already be undergoing pre-neoplastic changes. Future studies should include healthy tissue controls for more accurate comparisons. Finally, although q-PCR was employed to measure RTL, it may not be the optimal technique for assessing telomerase activity. More advanced assays, such as TRAP-F, are better suited to offer a more accurate and detailed evaluation of telomerase enzymatic activity.

6. Conclusion

The current study reinforces the notion that mutations in the *hTERT* gene promoter are rare events and they support telomere shortening, which is crucial for CRC initiation and progression. Despite the frequent hypermethylation and overexpression of the hTERT gene in CRC tissues, these alterations do not lead to telomere elongation. Notably, the persistence of short telomeres alongside hTERT upregulation implies that CRC cells may maintain CIN as a strategy to support tumor evolution and adaptability. These findings underscore the complexity of telomerase/telomere regulation in CRC and a better understanding of this interplay may pave the way for the development of novel diagnostic, prognostic, and therapeutic approaches for CRC.

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Conflict of interest

The authors declare that they have no competing interests.

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Writing – review & editing: Meryem Jafari, Boutaina Addoum, Abdelilah Laraqui, Imane Chaoui, Mohammed Attaleb

Ethics approval and consent to participate

The study protocol was approved by the Ethics Committee for Biomedical Research of the Faculty of Medicine and

Pharmacy of Mohammed V University in Rabat (Ref 73/23). All participants provided written informed consent before surgery, obtained through face-to-face interviews, in accordance with the guidelines approved by the institutional ethics committee.

Consent for publication

All patients provided written informed consent for the use and publication of their data included in this study. Consent was obtained through face-to-face interviews before surgery, and all identifying information has been anonymized to ensure privacy.

Availability of data

The data supporting our findings are available from the corresponding author upon reasonable request.

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