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Original article

Telomere 1 (*POT1*) gene expression and its association with telomerase activity in colorectal tumor samples with different pathological features

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ABSTRACT

The ends of chromosoms, telomeres are bound with a number of proteins which protect and stabilize telomeres against degredation, end to end fusion and aberrant recombinations. Telomeric DNA is bound of two groups of proteins, which are double-stranded telomeric DNA bindings proteins, and single stranded telomeric binding proteins. Among telomere binding proteins, protections of telomere 1 protein is a single stranded telomere binding proteins and suggested to be a significant player for telomere elongation and has an association with an enzyme called as telomerase which is an intrinsic reverse transcriptase. Telomerase synthesizes hexameric telomeric repeats onto the chromosomes thereby compansating telomere loss in immortal cells, such as tumor cells, whereas telomeres are shorthened with each division in normal cells. PCR-based TRAP (telomeric repeat amplification protocol) assay is a very sensitive assay for the detection of enzymatic activity of telomerase even if a few numbers of cancerous cells are available. The association between telomerase activity and hPOT1 expression in colorectal cancer is still unclear. Protein extraction was performed from specimens of matched normal and colorectal cancer specimens. Protein concentrations were determined by Bradford assay. Optimized protein concentrations were used for TRAP Assay. TRAP products were seperated by vertical gel electrophoresis on 12.5% polyacrylamide gels and visualized by silver staining. Gene expression of hPOT1 was determined by qPCR analysis. The results demonstrated that all tumor tissues were telomerase positive whereas all corresponding normal tissue was telomerase negative. Among clinicopathological findings, telomerase activity was found to be associated with stage, histology, localization, distant metastasis and lymph node metastasis of tumor in the current study. Although all of the clinicopathological findings differed in the expression of hPOT1 compared to normal tissues, they did not differ from each other significantly, except side of tumor and lymph node metastasis. Telomerase activity and hPOT1 gene expression may serve as a promising tumor marker for colorectal cancer and there is a close association between the enzymatic activty of telomerase and the expression of human protection of telomere 1 gene.

1. Introduction

Colorectal cancer is the third most common and second leading cause of cancer death worldwide among malignancies both in men and women [1–3]. Turkey is ranked fourth on the incidence of colorectal cancer in males and fifth in females and it is estimated that around 6000 new cases are seen each year [4]. A genetic model was proposed for colorectal cancer showing stepweise accumulation of mutations and genetic alterations [5]. One of the six hallmarks of cancer is the limitless replicative potential through either non-homologous recombination or aquisition of telomerase activity, which is an intrinsic reverse transcriptase stabilizing the ends of chromosomes, called as telomeres [6,7]. Telomerase is a holoenzyme composed of two essential subunits: hTERT (human telomerase reverse transcriptase) and hTR (human telomerase RNA) [7]. Telomeric DNA is composed of both double-stranded tandemly repeated sequences and single stranded G-rich overhang bound by six specialized proteins called altogether as "shelterin complex" which protects and shapes the ends of chromosomes. These proteins can be categorized in two groups: double-stranded

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telomeric DNA bindings proteins and single stranded telomeric DNA binding proteins [8,9]. POT1 (in human), cdc 13 (in budding yeast), TEBP (telomere end binding protein in ciliate) specifically bind to single stranded G-overhang of telomere whereas TRF1, TRF2, TIN2, RAP1 bind to double-stranded telomeric DNA [10]. Protection of telomere 1 protein (POT1) is a single stranded telomeric DNA binding protein and binds to G-overhang of telomeres through the NH2 terminal oligonucleotide binding folds [11]. In human, only one *POT1* gene is found whereas mice have two *POT1* genes, which encodes two different proteins (POT1a and POT1b) [12]. Studies revealed that hPOT1 has distinct functions effecting telomere lenght and the activity of telomerase. The functions of hPOT1 are thought to prevent telomeric DNA loss by nucleases, which provide the stability of cap structure of telomeres, and to recruit the telomerase to the ends of chromosomes [13,14]. hPOT1 recruitment to chromosomal ends is mediated by TPP1 protein. These complexes have been shown in a study to enhance the processivity of telomerase enzyme in vitro [15]. Additionally, in vivo studies showed the involvement of hPOT1 in the regulation of telomerase function positively and negatively [16].

In this study, telomerase activity in both tumor and adjacent normal tissues was determined by a quantitative telomeric repeat amplification protocol (TRAP)-silver staining assay. The level of hPOT1 mRNA expression was analysed by quantitative real time-polymerase chain reaction (qRT-PCR).

2. Materials and methods

2.1. Patients and tissue samples

Twenty matched tumor and adjacent normal tissues were obtained from colorectal cancer patients at Gülhane Millitary Medical Academy (GATA) in Turkey. Ethical approval was obtained from the committee for the collection of tissue specimens and further studies. Both tumor and normal tissues stored in RNAlater® solution (Sigma–Aldrich, USA) were incubated overnight at 4 °C, stored at –80 °C. Additionally, main tissue specimens were fixed in 10% phosphate-buffered neutral formalin. Samples were embedded in paraffin according to macroscopy guidelines. After 4 μm sections were taken, slides were stained with hematoxylin and eosin for histopathological analysis. Tumors were categorized in accordance with TNM (tumor-node-metastasis) system endorsed by American Joint Committee on Cancer (AJCC, Version7).

2.2. TRAP (telomeric repeat amplification protocol)–silver staining assay

Telomerase activity was determined according to manufacturer's instructions provided by the TRAPeze® telomerase detection kit (Millipore, Germany). Each tumor and normal tissue specimens were assayed in triplicate. Each experimental setup included a heat inactivated control, CHAPS lysis buffer only and a positive control. After homogenization of fresh frozen tissue specimens with liquid nitrogen, the fine powder was transferred with a sterile surgical blade to a RNAse free 1.5 mL microcentrifuge tube containing 40 unit/uL of RNAse inhibitor and 250 μL ice-cold CHAPS lysis buffer (10 mM Tris–HCl, pH 7.5, 1 mM $MgCl_2$, 1 mM EDTA, 0.1 mM benzamidine, 5 mM β-mercaptoethanol, 0.5% CHAPS, 10% glycerol). The homogenate was incubated on ice for 30 min and then spun in a benchtop microcentrifuge at 12,000 × *g* for 20 min at 4 °C. About 160 μL of supernatant was collected in a sterile RNAse free plastic tube, then stored at –80 °C for further use. Protein concentration of each sample was determined by Bradford assay. An aliquot of extract containing 0.6 μg of protein was used for each TRAP assay. PCR conditions performed were 30 °C for 30 min for the elongation of TS (telomere substrate) primer by telomerase and followed by 33 cycles of PCR amplification (94 °C for 30 seconds, 59 °C for 30 seconds and 72 °C for 60 seconds). The PCR products were analyzed by electrophoresis in a 12.5% polyacrylamide (PAGE) non-denaturating gel for 45 min at 300 V. PAGE gel was stained with modified silver staining method. Specimens were considered telomerase positive which showed six base pair incremental bands starting from 50 bp band according to manufacturers instructions. The gel images were analysed with Image J software.

2.3. Expression analyses of hPOT1 gene

2.3.1. RNA isolation

Total RNA was extracted from tissue samples by using TRIzol Reagent according to manufacturer's instructions (Sigma–Aldrich, USA). The concentration and purity of isolated RNA samples were determined by measuring optical densities at 260 nm and 280 nm using NanoDrop 2000C spectrophotometer (Thermo Fischer Scientific, USA). The intactness of isolated RNA and DNA contamination were checked by agarose gel electrophoresis.

2.3.2. cDNA synthesis

cDNA synthesis was performed by using QuantiTect Reverse Transcription Kit (Qiagen, Germany) according to manufacturer's instructions. Then, 1 μg total RNA and 25 pmol of gene specific primers, either of GAPDH, hPOT1 were used for each reaction. cDNA was stored at –20 °C until further studies.

2.3.3. Real time quantitative polymerase chain reaction (RT-qPCR)

hPOT1 mRNA levels were determined by quantitative real time PCR (qRT-PCR) performed in Rotor-Gene 6000 (Corbett Research, Australia). Light-Cycler-FastStart SYBR Green I DNA master mix kit was used. Briefly, total volume of reaction mix was 20 μl containing 10 μl SYBR Green 2X master mix, 2.8 μL of cDNA, 0.15 μM of reverse and forward primers (Table 1) and appropriate amount of nuclease free water. All samples were run as triplicates in each run including a non-template control to check background signal. Relative quantification of qPCR products was perfomed by using $2^{-\Delta\Delta Ct}$ method.

2.4. Statistical analysis

All data are representative of three independent experiments and expressed as mean ± standard error of the means (SEM). Relative telomerase activity (RTA) results of tumor and normal tissues were analyzed by two-tailed *t*-tests to assess the association. The relationship between clinical variables and RTA values was analyzed by Mann–Whitney *U* test. The difference in the expression levels of *hPOT1* mRNA between tumor and normal tissue samples was determined using *t*-test. One-way ANOVA test and post-hoc Tukey analyses were carried out to find groups whose mean differences were significant. The results were significant at the 0.05 level.

Table 1
Primers used in quantitative real time-polymerase chain reaction (qPCR).

Primers	Sequence	Amplicon size
hPOT1 sense	5′GAAGTGGACGGAGCATCATT3′	127 bp
hPOT1 antisense	5′TTTGTAGCCGATGGATGTGA3′	
GAPDH sense	5′ATGGGTGTGAACCATGAGAA3′	79 bp
GAPDH antisense	5′GTGCTAAGCAGTTGGTGGTG3′	

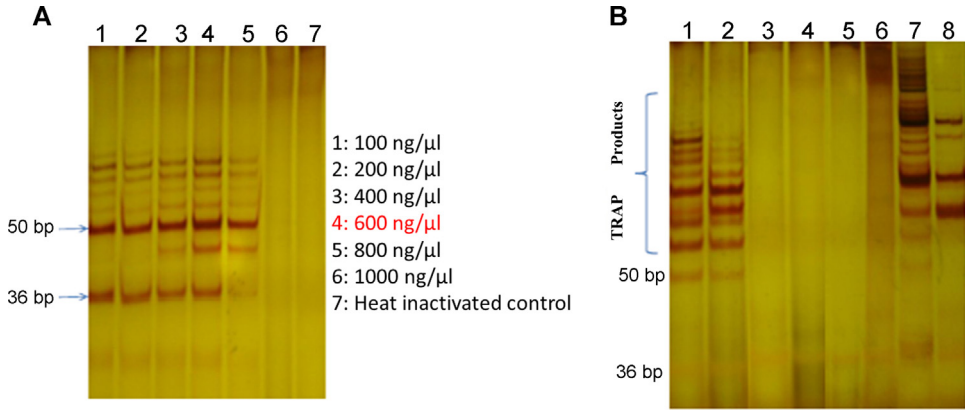

Fig. 1. Optimization of protein content for TRAP assay (A). Representative results of TRAP–silver staning assay. LaneS 1 and 2: tumor specimens, laneS 3 and 4: normal specimens, lane 5: CHAPS only, lane 6: heat inactivated control, lanes 7 and 8: positive controls at different concentrations (B).

3. Results

Twenty matched samples from colorectal cancer patients were obtained and studied for the current study. Thirteen out of 20 tumors were localized in left colon and 65% of left-sided tumors were found in rectum and remaining were found in sigmoid colon. Tumors in right colon (7/20) were localized in hepatic flexure, ascending colon, cecum. Tumor size ranged from 1.8 cm to 10 cm. According to TNM classification, 70% of patients were evaluated as late stage (stage III and IV) whereas 30% of patients were at early stage (stage I and stage II). Only one patient was diagnosed at early onset with stage I which is a rare event in Turkish population. About 65% of patients showed lymph node metastasis. Although the number lymph of node metastasis was variable, metastasis in 4 or more regional lymph node was common among patients evaluated in the current study. Distant metastasis (M) status was also evaluated and 10% of cases showed metastasis to liver and peritoneum. Patients with rectal tumors took chemoradiotheraphy as neoadjuvant therapy, however, other patients did not take any therapy before surgery.

3.1. TRAP–silver staining assay

Protein extracts of tissue specimens from colorectal cancer patients were diluted several folds to find out the optimal protein concentration for the TRAP–silver staning assay. Fig. 1A demonstrated telomerase activity at different protein concentrations.

In the current study, all colorectal cancer cases showed telomerase activity at different levels, whereas all of normal tissues were telomerase negative (Fig. 1B). TRAP assay is the best analysis method of enzymatic activity. In this assay, specimens were considered as positive regarding telomerase activity showing six base pair incremental bands which starts from 50 bp (i.e. 50, 56, 62, 68, 74, etc.) which are called also regular TRAP products (Fig. 1, lanes 1 and 2). Beside regular TRAP products, 36 bp internal control (IC) band also is seen. In contrast, telomerase negative specimens show only 36 bp IC band (Fig. 1, lanes 5 and 6). Some lanes may exhibit weak IC band due to high telomerase activity. Representative results of both tumor and normal samples were illustrated in Fig. 1, since there is a competition for amplification between internal control and TRAP products. A heat-treated sample extract was used as a negative control for each assay because telomerase is a heat sensitive enzyme. To check contamination or primer dimer artifacts, CHAPS lysis buffer was used instead of sample extract in each experimental setup. Besides negative controls, a positive control was also included in the TRAP assay.

The association between telomerase activity and the clinicopathological features of patients with colorectal cancer were shown in Figs. 2–6.

As shown in Fig. 2, telomerase activity has a tendency to be higher in young ages compared to old ages, however, this is not statistically significant ($P > 0.05$). This could be an indicator of poor prognosis. Likewise, the difference between man and woman regarding telomerase activity was not significant ($P > 0.05$). In the current study, a noteworty difference was observed between tumor site and telomerase activity. Tumors located at left colon had significantly higher telomerase activity compared to right colon as shown in Fig. 3. In this study, 6 out of 20 patients in stage I and II were compared with 14 patients out of 20 in stage III and IV regarding telomerase activity. Telomerase activity increased significantly with advanced stages (stage III and stage IV) compared to early stages (stage I and stage II) (Fig. 4). This could be related to the accumulation of genetic alterations and mutations during carcinogenesis. A comparison was made between histopathological types of the colorectal cancer tumors and their relative telomerase activities (Fig. 5). Although the major and usual histopathological type, colorectal adenocarcinomas were also positive with respect to telomerase activity, the unusual histological type, mucinous adenocarcinomas, showed significantly higher telomerase activity compared to usual colorectal adenocarcinomas ($P < 0.05$). Generally, tumors with grade 3 had slightly higher relative telomerase activity than tumors with grade 2, however, the difference was not found to be statistically significant as shown in Fig. 5. Tumors with distant metastasis (M1) and lymph

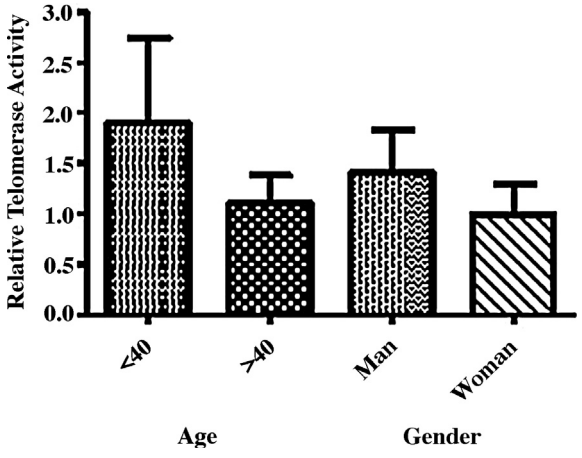

Fig. 2. Relation between telomerase activity and age and gender parameter.

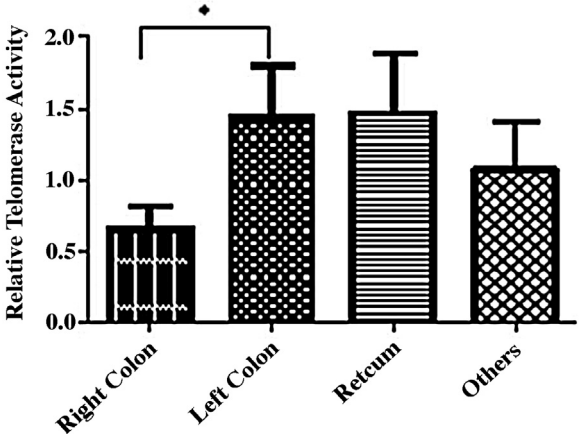

Fig. 3. Relation between tumor site and telomerase activity. Others include tumors located at hepatic flexure, ascending colon, cecum, sigmoid colon. (*Results were signifcant with a $P < 0.05$).

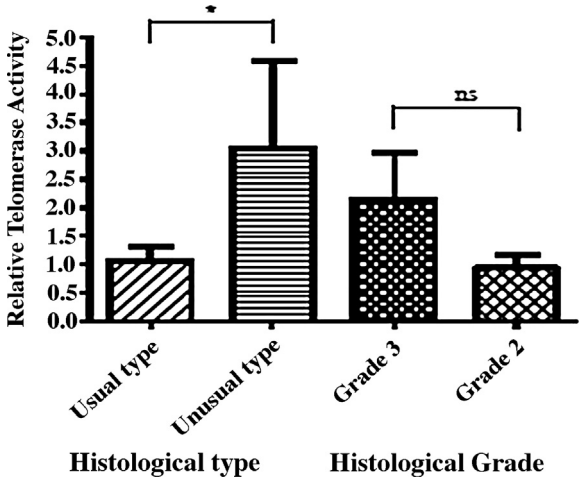

Fig. 5. The association among relative telomerase activity, histopathological features, and grade of tumors (*Results were signifcant with a $P < 0.05$).

node metastasis revealed high telomerase activity when compared to tumors without distant metastasis (M0) and lymph node metastasis (N0), respectively (Fig. 6).

3.2. *Expression analysis of* hPOT1 *gene in colorectal cancer samples*

According to RT-qPCR results, all of the tumor and adjacent normal tissues were found to express *hPOT1*, however, tumor tissues showed 2.6-fold more *hPOT1* expression compared to their normal adjacent tissues (Fig. 7A). The relative fold change of *hPOT1* gene expression was compared among different tumor parameters. Early stage tumors were found to express slightly higher level of *hPOT1* compared to normal tissues but statistically non-significant. However, tumors at late stage expressed 2.55-fold more *hPOT1* compared to normal tissues (Fig. 7B). The difference in the expression level of *hPOT1* was not statistically significant between late stage tumors and early stage tumors. Therefore, it can be concluded that gene expression of *hPOT1* was found to upregulate as the cancer progressed. In addition, the tumor site showed significant difference wih respect to expression of *hPOT1*. Tumors located at left colon showed notably high level of *hPOT1* expression compared to tumors at right colon and normal tissues (Fig. 7C).

Moreover, tumors with grade II and both usual and unsual type tumors showed significant difference regarding the expression level of *hPOT1* compared to normal tissues in the current study. However, the difference among histological type and grade of tumors in the level of *hPOT1* expression was not statistically significant as shown in Fig. 7C. Among TNM parameters, tumor with lymph node metastasis, tumors with and without distant metastasis demonstated significantly high level of *hPOT1* expression compared to normal tissues. However, the difference regarding *hPOT1* expression between tumors with distant metastasis and tumors without distant metastasis was not found to be statistically significant as shown in Fig. 7D.

Considering that all tumors in the current study were telomerase positive together with elevated expression of *hPOT1* may indicate an association between telomerase activity and the expression of *hPOT1*. Telomerase activity is seemed to be correlated with the expression of *hPOT1*.

Table 2 summarizes fold changes in the expression of *hPOT1* and relative telomerase activity with respect to the clinicopathological features.

Fold changes were represented as "mean ± SEM". SEM values were determined from three independent experiments, each run in dublicates.

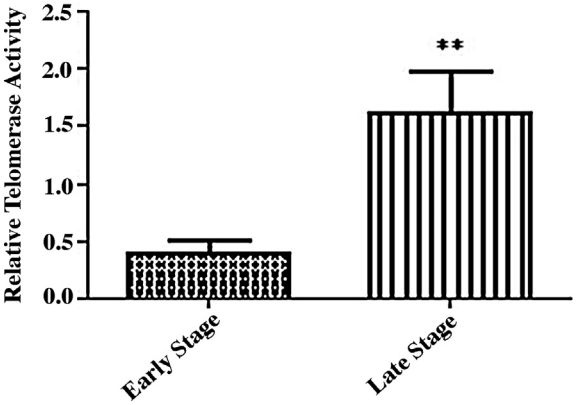

Fig. 4. Association between stage of colorectal tumors and relative telomerase activity (**Results with a $P < 0.01$).

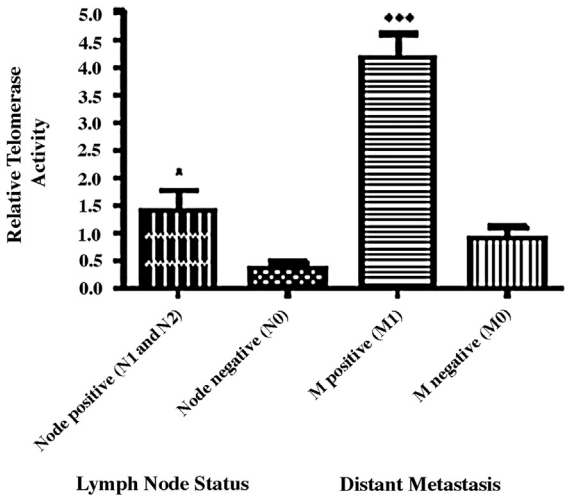

Fig. 6. Evaluation of RTA in different TNM classification parameters (*Results were signifcant with a $P < 0.05$ and ***results with a $P < 0.0001$).

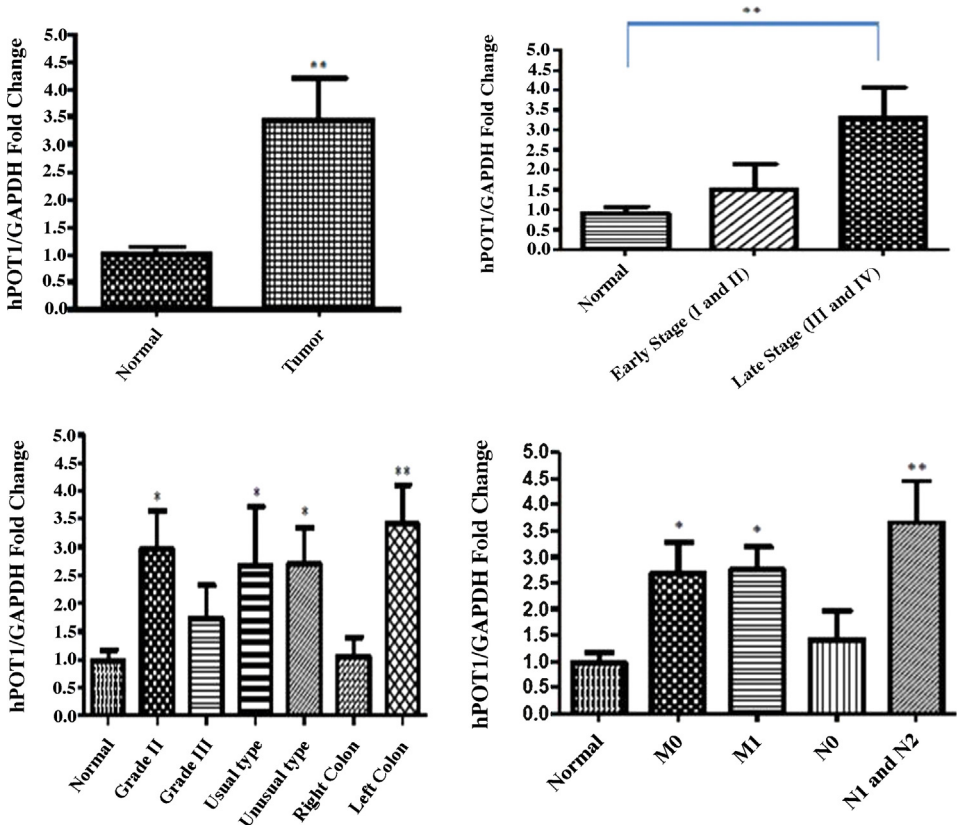

Fig. 7. *hPOT1* expression and its association with tumor parameters (*Results with a $P < 0.05$, **results with a $P < 0.01$).

4. Discussion

Among the regulators of telomerase gene activity, single stranded telomere binding protein, *hPOT1*, may play a significant role [17]. To date, there is no study conducted to elucidate the relationship between telomerase activity and *hPOT1* expression in colorectal cancer, together with clinicopathological findings of patients. In the current study, all tumor specimens were both telomerase positive and expressed significantly high *hPOT1* mRNA compared to normal tissues. Moreover, *hPOT1* expression was found to be associated with stage of tumor, site of tumor, lymph node metastasis significantly in the current study. Previous studies regarding the expression of *hPOT1* and its relation with telomerase acitivity and telomere elongation were controversial. In the current study, a significant correlation was found between relative telomerase activity and the expression of *hPOT1*. When considered, all tumors in the current study were telomerase positive together with elevated level of expression of *hPOT1*, this may indicate the recruitment of telomerase to the end of the telomeres by *hPOT1*.

Table 2
Fold changes in expression of *hPOT1* and relative telomerase activity (RTA).

Groups	Fold Change (hPOTI)	RTA
Normal	0.99 ± 0.14	0.000
Tumor	3.43 ± 0.75	2.987 ± 0.015
Early stage	1.49 ± 0.64	0.387 ± 0.113
Late stage	3.32 ± 0.73	1.609 ± 0.358
Right colon	1.40 ± 0.34	0.653 ± 0.159
Left colon	3.40 ± 0.71	1.432 ± 0.362
Grade 2	2.95 ± 0.67	0.941 ± 0.228
Grade 3	1.71 ± 0.59	2.156 ± 0.814
Usual type	2.67 ± 0.96	1.078 ± 0.232
Unusual type	2.68 ± 0.58	3.038 ± 1.551
Lymph node metastasis negative (N0)	1.42 ± 0.43	0.382 ± 0.100
Lymph node metastasis positive (N1 and N2)	3.65 ± 0.78	1.419 ± 0.353
Distant metastasis negative (M0)	2.67 ± 0.60	0.922 ± 0.191
Distant metastasis positive (M1)	2.74 ± 0.44	4.169 ± 0.420

Previously, *hPOT1* was shown to act on telomere extention positively and negatively by telomerase depending on its position at telomeres. It was suggested that telomerase activity was positively regulated when hPOT1 protein binds to more internally on the telomeric DNA, thereby leaving more open and accessible 3′ terminal [18]. On the other hand, the binding of *hPOT1* near the 3′ terminal leads to unaccessible structure for telomerase to bind to the ends of chromosomes [19]. Previously, another study also confirmed these findings and stated that both overexpression and knockdown of *hPOT1* gene led to telomere elongation proposing that there could be a homeostatic regulation for the control of telomere length in telomerase positive cells for which a certain level of *hPOT1* expression is responsible. Overexpression or downregulation of *hPOT1* gene may result in telomere elongation depending on the activity of telomerase. Colgin et al. suggested that *hPOT1* acts as telomerase dependent, positive regulator of telomere lenght. Because they found that cells with overexpression of *hPOT1* gene were also telomerase positive and had longer telomeres [19].

The activity of telomerase is regulated through a number of levels, including transcriptional and post-transcriptional regulation of TERT, phosphorylation of TERT protein, telomeric binding proteins, cellular transport and association of each components required for the holoenzyme assembly [20]. We previously reported that gene expression of *hTERT* was determined by qPCR analyses. The expression of hTERT was observed both in tumor and normal tissues, however; tumor tissues expressed 4.33-fold more hTERT than normal tissues [21]. Gene expression analysis

demonstrated that both *hTERT* and *hPOT1* were overexpressed in tumor tissues compared to normal tissues. The expression levels of *hTERT* and *hPOT1* were found to increase with the stage of tumor. Late stage tumors expressed significantly high level of *hTERT* and *hPOT1* compared to early stage tumors, which is paralel with telomerase activity.

Eskiocak et al. investigated the effect of doxorubicin on telomerase activity and apoptotic gene expression in doxorubicin resistant and sensitive MCF-7 cells. They found that 72 hours doxorubicin incubation caused a decrease in telomerase activity in parallel with a small decrease in hTERT level in both sensitive and resistant MCF-7 cells [22]. Ning et al. demonstrated that the loss of hPOT1 by RNAi in BGC823 cells leads to an increase in multi-nucleated giant cells, a decrease in cell proliferation and colony formation, induction of senescence and apoptosis, shortened telomere length, upregulation of *hTERT* gene [23]. Wan et al. showed that transfection of *hPOT1* siRNA into SGC-7901 cells led to a decrease in cell proliferation, colony formation and invasion, and also an increase of apoptosis. A downregulation of hTERT were found in gastric cancer cells with hPOT1 siRNA [24].

5. Conclusion

The results in the current work were consistent with previous studies, which have found a positive correlation with telomerase activity and *hPOT1* gene expression in colorectal cancer. These results may indicate that telomerase activity and expression of human protection of telomere1 (*POT1*) gene could be a promosing marker for colorectal cancer in the future. In this study, some relations or and associations found were statistically insignificant. However, this could be related with a limited number of patients categorized in different clinicopathological parameters. It may be useful to screen these associations in a larger group of patients.

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