mRNA expression of nuclear factor of activated T-cells, cytoplasmic 2 (NFATc2) and peroxisome proliferator-activated receptor gamma (PPARG) transcription factors in colorectal carcinoma

Venus Zafari[1,2], Shahryar Hashemzadeh[2,3], Mohammadali Hosseinpour Feizi[4], Nasser Pouladi[5], Leila Rostami Zadeh[1], Ebrahim Sakhinia[1, 2,6*]

[1]Department of Biochemistry and Clinical Laboratories, Division of Medical Genetics, Faculty of Medicine, Tabriz University of Medical Sciences, Tabriz, Iran, [2]Tuberculosis and Lung Disease Research Center of Tabriz University of Medical Sciences, Tabriz, Iran, [3]General and Vascular Surgery Department of Tabriz University of Medical Sciences, Tabriz, Iran, [4]Department of Animal Biology, University of Tabriz, Tabriz, Iran, [5]Department of Biology, Azarbaijan Shahid Madani University, Tabriz, Iran, [6]Tabriz Genetic Analysis Center (TGAC), Tabriz University of Medical Sciences, Tabriz, Iran

ABSTRACT

Transcription factors are involved in cell cycle and apoptosis regulation and thus have a key role in the carcinogenesis of different tumors. Nuclear factor of activated T-cells, cytoplasmic 2 (NFATc2) and peroxisome proliferator-activated receptor gamma (PPARG) transcription factors are important in the carcinogenesis of colorectal cancer (CRC). In this study, we examined whether the expression of *NFATc2* and *PPARG* genes is significantly altered during the carcinogenesis of CRC. A total of 47 tumor samples and matched normal tissue margins were collected during surgery from patients with CRC. In addition, three CRC cell lines (HCT119, SW480, and HT29) and healthy cell line were used. After total RNA extraction and cDNA synthesis, mRNA expression levels of *NFATc2* and *PPARG* were examined by real-time polymerase chain reaction. The results showed that *NFATc2* is overexpressed in the tumor tissues compared with normal tissue margins ($p \leq 0.05$). However, the mRNA expression levels of *PPARG* were not significantly different between the tumor tissues and tissue margins. Our results indicate that *NFATc2* may be used as an early diagnostic or predictive biomarker for CRC as well as a therapeutic target, providing that upcoming studies confirm these results.S

KEY WORDS: NFATc2; PPARG; colorectal cancer; gene expression; biomarker; CRC; targeted therapy
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INTRODUCTION

Cancer is one of the leading causes of death and colorectal cancer (CRC) is the third most prevalent cancer worldwide [1]. As in other cancers, different factors such as obesity, physical inactivity, smoking cigarettes, inflammation, and genetic factors play important roles in the development of CRC. CRC initiation is associated with genetic variation; for example, in nearly 10% of CRC cases, hereditary impairments were detected as the underlying cause [2]. Alterations of different genes in cancer cells contribute to the changes in the related molecular pathways. Among the important molecular pathways in cancer development are the Wnt signaling pathways. Thus, the genes associated with the Wnt signaling are potential therapeutic targets, especially in CRC [3-10].

Nuclear factor of activated T-cells, cytoplasmic 2 (NFATc2) is a transcription factor involved in the last steps of a non-canonical Wnt signaling pathway [11]. Generally, transcription factors regulate the expression of other genes and thus have an important role in cell proliferation and apoptosis. Previously, the association of NFAT family members with breast and pancreatic cancers has been reported [12-17]. Among the five members of NFAT family, Nfat1 (Nfatc2) is the most commonly overexpressed in human colorectal cells [18].

Dephosphorylation of NFATc2, mediated by calcineurin, leads to NFATc2 activation. Recent studies have showed

*Corresponding author: Ebrahim Sakhinia, Department of Biochemistry and Clinical Laboratories, Division of Medical Genetics, Faculty of Medicine, Tabriz Genetic Analysis Center (TGAC), Tabriz University of Medical Sciences, Golgasht Street, postal code 5166/15731, Tabriz, Iran. E-mail: ebrahim666saxi@gmail.com.

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that an aberrant activation and subsequent overexpression of *NFATc2* gene can lead to cancer and metastasis [19-24]. Peroxisome proliferator-activated receptors (PPARs) are a group of nuclear receptor proteins. Among the three types of PPARs (alpha, gamma, and delta [beta]) PPAR-γ or PPARG is the most commonly expressed in colon tissue [25-30]. In general, PPARs are involved in cellular lipid and whole-body glucose homeostasis. Moreover, PPARs have a key role in inflammatory pathways by controlling prostaglandin and leukotriene production. More specifically, it was demonstrated that PPARG negatively regulates inflammatory responses in the large intestine [31]. Due to its important role in cell metabolism, inflammation and energy homeostasis, PPARG is a potential therapeutic target in different cancers, especially in CRC [26,31-33].

Early diagnosis of cancer is very important for effective therapy. In all types of cancers, molecular alterations occur at early stages, and they are usually evident before morphological changes. Therefore, by establishing new and effective biomarkers for an initial cancer screening at the molecular level, more accurate diagnosis and better treatment strategies can be achieved [1-3]. Considering the important role of *PPARG* and *NFATc2* genes in cancer-related pathways, the main purpose of this study was to investigate the potential of *PPARG* and *NFATc2* as biomarkers for CRC diagnosis. The mRNA expression levels of *PPARG* and *NFATc2* genes, both in clinical samples and colorectal cancer cell lines, were assessed.

MATERIALS AND METHODS

Clinical samples

Forty-seven CRC tissue samples (all adenocarcinoma) and their matched tumor-free margins were collected during surgeries, from 2011 to 2013, from patients referred to Imam Reza Hospital, Tabriz University of Medical Sciences. The pathological diagnosis was performed before the surgery by an expert pathologist. All tumors were staged according to the American Joint Committee on Cancer (AJCC) classification. The collected samples were then immediately placed into RNAlater RNA stabilization solution (Qiagen, Germany) to stabilize and protect the cellular RNA. Written informed consent was obtained from all participants and the study was approved by the Ethical Committee of Tabriz University of Medical Sciences.

Cell lines and cell culture

Human CRC cell lines HCT116, SW480, and HT29 as well as the immortal colorectal healthy cell line CRL1831 were purchased from Pasture Institute of Iran. All cell lines were cultured in RPMI-1640 medium (Gibco, UK) supplemented with %10 fetal bovine serum (FBS; Gibco, UK), containing 10 U/ml streptomycin-penicillin (Sigma-Aldrich, USA). The cells were incubated at 37°C in a water-saturated atmosphere with 5% CO_2.

Quantitative reverse transcription polymerase chain reaction (RT-qPCR)

RNA extraction and complementary DNA (cDNA) synthesis

RNeasy™ mini kit (Qiagen, Germany) was used to extract RNA from the tissue samples and cell lines. The concentration of the extracted RNA was measured by NanoDrop spectrophotometer at 260/280 nm (NanoDrop ND-2000C Spectrophotometer, Thermo Fisher Scientific, USA) and the RNA quality was determined via electrophoresis. First-strand cDNA was synthesized using first-strand cDNA synthesis kit (TaKaRa, China) according to the manufacturer's protocol. Briefly, 4 µl of isolated RNA (30 µg) was first mixed with 1 µl of random hexamer primer and 7 µl of RNAse-free H_2O and then incubated at 65°C for 5 minutes. Afterward, the micro tubes were cooled on ice followed by addition of 4 µl of reaction buffer, 1 µl of RNase inhibitor, 2 µl of dNTP mix, and 1 µl of reverse transcriptase to each sample. The samples were immediately incubated at 25°C for 5 minutes and then at 42°C for 60 minutes. Finally, the reaction was terminated by heating the samples at 70°C for 5 minutes. The reverse transcription reaction was performed with the final volume of 20 µl per tube.

Real-time PCR

The primers used for real-time PCR (Table 1) were designed using Oligo 7 software (Molecular Biology Insights, Inc., Cascade, CO, USA) and the Basic Local Alignment Search Tool (BLAST) at National Center for Biotechnology Information (NCBI) site was used to determine the specificity and accuracy of the primer sequences. The real-time PCR was performed using *SYBR* green EX taq™ master mix (TaKaRa, China). To calibrate the PCR reaction, a dilution series of human genomic standards was constructed. After homogenizing human genomic DNA (hgDNA), hgDNA was serially diluted (from 1/10 to 1/10000) in Tris EDTA (TE) buffer.

The quantitative analysis was carried out using StepOnePlus Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). Each reaction mixture contained a total volume of 20 µl (10 µl of master mix, 2 µl of cDNA [5 ng/ml], 1 µl of assay mix, and 7 µl of H_2O). The real-time PCR conditions were as follows: 50°C for 2 minutes, 95°C for 10 minutes, then 40 cycles at 95°C for 15 seconds, and 60°C for 1 minute. The mRNA expression levels of *NFATc2* and *PPARG* were calculated using the Pfaffl method [34] after normalization with *GAPDH* gene expression as an internal control.

TABLE 1. Primer sequences used in real-time polymerase chain reaction

Target gene	Primer sequence	Amplicon size (bp)	Temperature (°C)
NFATc2-F	5′-TGTTGTTTCCATTAGAGCAGG-3′	117	62
NFATc2-R	5′-GGTGAGTGAGGTTCTTTGACA-3′		
PPARG-F	5′-AGCTGAACCACCCTGAGTCC-3′	156	58
PPARG-R	5′-TCATGTCTGTCTCCGTCTTCTTG-3′		
GAPDH-F	5′-CATGGCCTCCAAGGAGTAAG-3′	219	58
GAPDH-R	5′-GCTTGAGCACAGGGTACTTTA-3′		

NFATc2: Nuclear factor of activated T-cells, cytoplasmic 2; *PPARG*: Peroxisome proliferator-activated receptor gamma; *GAPDH*: Glyceraldehyde-3-phosphate dehydrogenase; bp: base pair

Statistical analysis

For statistical analysis of real-time PCR results, the REST 2009 and SPSS for Windows Version 16.0. (SPSS Inc., Chicago) software packages were used. The independent *t*-test was used for comparing the average expression of target genes between tumor tissues and normal tissue margins, Pearson's correlation test was used for evaluating the correlation between the expression of target genes and patient clinical profiles, Kolmogorov-Smirnov normality test was used to determine the normality of data, and Levene's test was used for assessing the equality of variances. All results were expressed as mean ± standard deviation (SD) with statistical significance level at 5%.

RESULTS

Gene specific PCR

After the extraction of RNA and construction of cDNA library, gene specific PCR was performed to confirm that the poly(A) cDNA can be used to detect the expression of *NFATc2* and *PPARG*. In all samples, the amplicons were within the recommended size range, indicating that the poly(A) cDNA contained the target transcripts (cDNA library confirmation).

Calibration of real-time PCR

Standard curve is essential for calibration of real-time PCR. We used a dilution series of hgDNA as a standard, applying the same DNA quantity and PCR program setting as for the target genes. Using the threshold cycle (Ct) values of the serial dilutions, standard curves were plotted for all genes. Calculated from the slope of the standard curves, the efficiency for *NFATc2*, *PPARG*, and *GAPDH* was 1.02, 0.98 and 1, respectively.

RT-qPCR results

RT-qPCR analysis of the *NFATc2* and *PPARG* genes was carried out in 47 pairs of CRC tissues and matched normal tissue margins. The mRNA expression level of *NFATc2* gene was significantly increased in CRC tissues compared with the normal tissue margins (Figure 1; fold change = +2.58; $p = 0.021$). However, there was no significant difference in the expression level of *PPARG* gene between the tumor tissues and normal tissue margins (Figure 2).

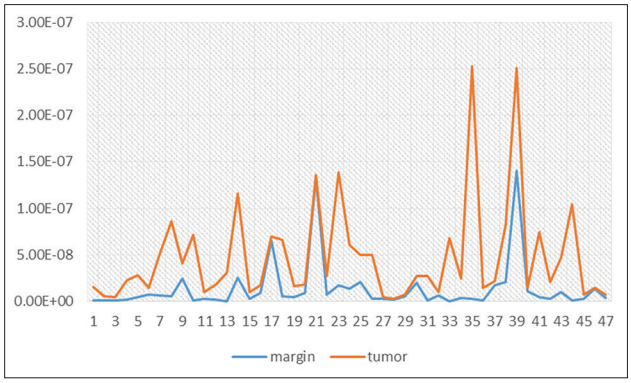

FIGURE 1. Comparison of the expression level of *NFATc2* in colorectal cancer (CRC) tissues and normal tissue margins. The mRNA expression level of *NFATc2* gene was significantly increased in the CRC tissues compared with normal tissue margins (fold change = +2.58; $p = 0.021$).

The relative mRNA expression of *NFATc2* was significantly upregulated in HCT119 and HT29 cell lines compared with the healthy cell line (fold change = +2.3 and +3.1; $p = 0.019$ and 0.007, respectively). However, no significant difference was observed in *NFATc2* mRNA expression between SW480 and healthy cell line. Furthermore, there were no significant differences in the mRNA expression of *PPARG* gene between HCT119, SW480, and HT29 and healthy cell line (Figure 2).

Specificity and sensitivity of *NFATc2* and *PPARG* genes in predicting CRC

Receiver operating characteristic (ROC) curves were plotted for *NFATc2* and *PPARG* genes. Next, the area under the curve (AUC) was calculated to assess the specificity and sensitivity of *NFATc2* and *PPARG* in predicting CRC. *NFATc2* mRNA had a ROC area of 0.653 (Figure 3; $p < 0.05$; CI: 0.543-0.764). The ROC area for *PPARG* gene was not statistically significant ($p > 0.05$) and the plot showed the sensitivity and specificity at different cut-off points. To determine the optimal cut-off value, we carried out a post-test from pretest probability of 0.5 and cost ratio of 1.00. The optimal cut-off point for *NFATc2* was ≤6.57, with 0.85 sensitivity and 0.38 specificity.

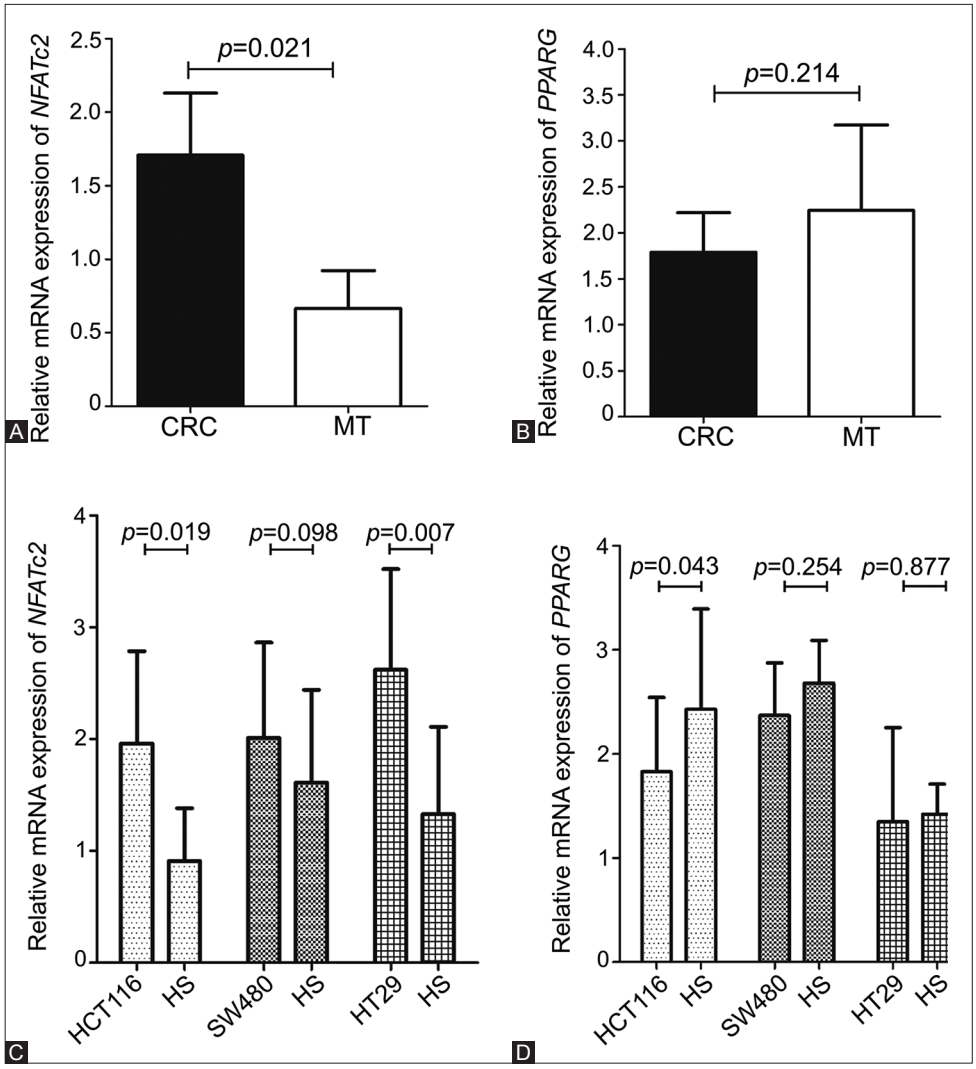

FIGURE 2. Relative mRNA expression level of *NFATc2* (A) and *PPARG* (B) genes in CRC tissues and normal tissue margins; relative mRNA expression level of *NFATc2* (C) and *PPARG* (D) in three CRC cell lines (HCT119, SW480, and HT29) and healthy cell line. CRC: Colorectal cancer; MT: Margin tissue; HS: Healthy subjects.

Correlation between *NFATc2* and *PPARG* expression levels and clinicopathological characteristics

There was no significant relationship between the mRNA expression levels of *NFATc2* and *PPARG* and clinicopathological variables, including age, gender, tumor grade and depth, lymph node metastasis, venous invasion, liver metastasis, and CRC stages according to the AJCC classification (Table 2).

DISCUSSION

The diagnosis and treatment of CRC have been improved over the past decades; however, due to the late detection of CRC, at the time of diagnosis, most patients are in an advanced or metastatic stage, resulting in a poor prognosis [35]. To improve the treatment rate of CRC patients, early detection of tumor is important. So far, the conventional methods and markers have not been effective in early detection of CRC [36]; thus, it is necessary to investigate new biomarkers that could be used for the initial screening.

Transcription factors play a key role in the regulation of cell cycle, and can potentially be used as biomarkers. In the present study, we analyzed the expression level of two transcription factors, NFATc2 and PPARG, which are involved in CRC development. Our results showed that NFATc2 was markedly upregulated in CRC tissues compared with normal tissue margins, suggesting that the high expression of NFATc2 might be associated with colorectal carcinogenesis. According to previous studies, a pro-tumorigenic role of NFATc2 in CRC is the result of its involvement in cytokine production, cell-cycle and apoptosis regulation, and activation of calcium signaling [37]. Moreover, NFATc2 promotes angiogenesis by inducing vascular endothelial growth factor (*VEGF*) gene expression [38], which contributes to tumor migration induced by COX2 [13], also suggesting a tumor-promoting function for NFATc2.

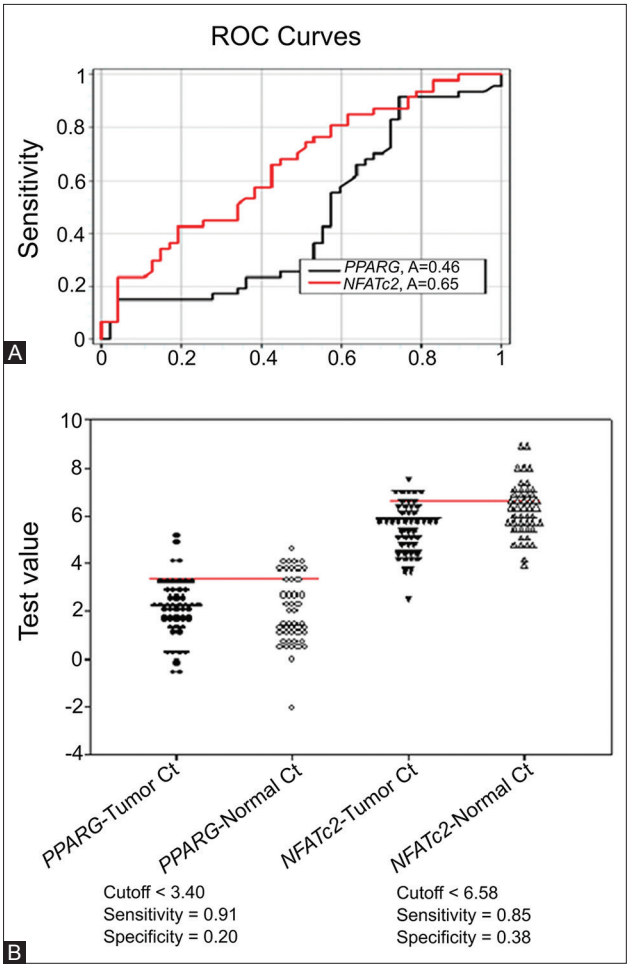

FIGURE 3. The receiver operating characteristic (ROC) curve was automatically generated from 36 points of cut-off values set by Sigma Plot software. The area under the curve (AUC) was 0.65 and 0.46 out of 1 for *NFATc2* and *PPARG* genes, respectively (A). The dot histogram shows the optimum cut-off points for each gene (B).

Similar to our results in CRC, the mRNA expression level of NFATc2 was also upregulated in pancreatic [39] and colitis-associated colorectal cancer [24], compared with normal tissues. Conversely, in another study, biopsies from patients with bronchial adenocarcinoma revealed that the expression level of NFATc2 was significantly lower compared with normal tissues [40]. In our study, the sensitivity and specificity of NFATc2 at the optimal cut-off point were 0.85 and 0.38, respectively. These results indicate the potential of NFATc2 as a diagnostic and prognostic marker in CRC.

Previous studies on *PPARG* expression in CRC showed disparate results. Some studies demonstrated overexpression of *PPARG* in CRC tissues [41] while other studies showed decreased *PPARG* mRNA expression [42-44]. In addition, discrepant results have been obtained regarding the expression of *PPARG* in other cancer types. For example, upregulation of *PPARG* mRNA expression in HER2-overexpressing breast cancer was reported [45] while it was down-regulated in patients with neuroblastoma [26]. These data suggest that *PPARG* can act as a tumor suppressor or oncogene depending on the tissue type, cellular environment, and genetic background of a patient [46]. Our results showed no significant difference in the mRNA expression level of *PPARG* between tumor tissues and normal tissue margins from CRC patients. Considering the controversial role of *PPARG* in tumor initiation and development, and according to our results, it is still not possible to consider this gene as a potential therapeutic target.

In this study, we also analyzed the possible relation between the mRNA expression level of *NFATc2* and *PPARG* and clinicopathological features of patients with CRC. Our results showed no significant relationships between the expression level of both genes and clinicopathological characteristics of CRC patients, including age, gender, AJCC stage, tumor grade and depth, lymph node metastasis, venous invasion, and liver metastasis. These results possibly indicate that the expression quantity of *NFATc2* and *PPARG* does not affect the clinical manifestations of CRC patients and *vice versa*.

In this study, normal tissue margins were considered as the control group, which eliminates confounding factors such as race, and geographic and individual differences. Nevertheless, with regard to the *PPARG* gene, our study did not demonstrate any significant difference in the *PPARG* expression level between the tumor tissue and normal tissue margins and thus has no prognostic value.

CONCLUSION

Alterations in the expression of molecular markers during the initiation and progression of carcinogenesis can be the basis for designing more effective drugs, and may prevent cancer development in early diagnosed patients. These molecular markers can then be used as a target for new therapeutic drugs. In addition, except for the end-stage cases of CRC, the expression analysis of selected genes can be helpful in reducing surgical errors during tumor removal and tumor clearance and can improve the surgery outcomes. Due to a 25-year interval between the initiation of colon adenoma and appearance of symptoms, a panel of molecular markers can be used in screening and early detection of CRC. Our results indicate that the *NFATc2* gene may be used in these analysis. However, because a small sample size was used in this study, further studies are required to confirm the application of *NFATc2* in screening and diagnosing CRC.

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TABLE 2. Relationships between *NFATc2* and *PPARG* expression levels in CRC tissue samples and clinicopathological features of CRC patients

Manifestation	*NFATc2* (ΔCt)	*p* value	*PPARG* (ΔCt)	*p* value
Age				
<55	5.67+0.87	0.13	2.21+1.13	0.6
>55	5.31+1.05		2.11+1.28	
Gender				
Male	5.60+1.18	0.8	2.08+1.46	0.38
Female	5.47+0.93		2.17+0.97	
Tumor grade				
Well	5.44+0.97	0.84	2.30+1.43	0.76
Moderate	5.51+1.06		1.85+0.83	
Poor	5.71+0.87		2.33+0.49	
Tumor depth				
T2	5.31+0.60	0.99	1.83+0.86	0.55
T3	5.43+0.94		1.97+1.34	
T4	5.53+1.03		2.26+1.20	
Lymph node metastasis				
Yes	5.59+0.98	0.56	1.96+1.48	0.14
No	5.40+0.98		2.29+0.98	
Venous invasion				
Yes	5.43+0.95	0.93	1.99+1.55	0.36
No	5.51+0.99		2.22+1.06	
AJCC stage classification				
II, III	5.59+1.04	0.71	1.95+1.54	0.16
IV, V	5.44+0.95		2.27+0.98	
Liver metastasis				
Yes	5.47+1.01	0.55	2.12+1.26	0.36
No	5.60+0.70		2.42+0.61	

Ct: Cycle threshold; CRC: Colorectal cancer; *NFATc2*: Nuclear factor of activated T-cells, cytoplasmic 2; *PPARG*: Peroxisome proliferator-activated receptor gamma; AJCC: American Joint Committee on Cancer

DECLARATION OF INTERESTS

The authors declare no conflict of interests.

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