

Investigating the Expression Level of *CCAT2* and *BIM* Genes in Colorectal Cancer Patients

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ABSTRACT

Colon cancer ranks as the second most common cancer among women and the third among men globally. Research has demonstrated that reduced expression of the Bcl-2-like protein 11 (*BIM*) gene in various cancers, including colon cancer, lowers cancer cell apoptosis, thereby enhancing their survival. Another crucial molecular factor in cancer is non-coding RNAs, which influence protein-coding genes such as oncogenes and tumor suppressor genes. Colon cancer-associated transcript 2 (*CCAT2*), a long non-coding RNA, is known to play a regulatory role in several cancers, affecting gene expression. This study aimed to measure and compare the expression levels of *BIM* and *CCAT2* genes in colon cancer tissues and healthy tissues. Total RNA was extracted from paraffin-embedded tissue samples using the Trizol method. Specific primers for the *BIM* and *CCAT2* genes, along with the Glyceraldehyde-3-Phosphate Dehydrogenase (*GAPDH*) reference gene, were designed using Oligo 7 software. Following cDNA synthesis, RT-PCR was conducted to assess gene expression levels relative to the *GAPDH* control gene. Data analysis, performed using GraphPad Prism and Excel2020 with One Way ANOVA and one-sided T-test, revealed a 10-fold decrease in *BIM* gene expression in cancerous tissues compared to healthy tissues ($P < 0.01$). In contrast, *CCAT2* gene expression was 325 times higher in cancerous tissues ($P < 0.0001$). The present study revealed that the expression of the *CCAT2* gene was significantly elevated in colon cancer patients compared to healthy individuals. This increase may be mediated through various signaling pathways, including *BOPI*, *mTOR*, and *Wnt*. Additionally, interactions with other regulatory factors, such as miRNAs, can contribute to tumor cell proliferation, inhibition of apoptosis, and metastasis in patients. The reduced *BIM* expression could result from genetic and epigenetic mutations disrupting the *AKT* and *ERK* pathways, thereby promoting tumor growth.

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Introduction

Epidemiological studies have shown that cancer is the second leading cause of death in the world, behind cardiovascular diseases, with approximately 12 million new cases per year and approximately 1 million annual deaths (Aliperti *et al.*, 2021; Bahar *et al.*, 2023). Lung and breast cancer are the most common cancers before Colorectal cancer (CRC), making it the fourth most common cause of cancer death worldwide

(Chakraborty *et al.*, 2013; Delattre *et al.*, 2022). Despite improvements in CRC treatment that have led to better overall survival, its prevalence is still increasing, and the incidence of cancer in the Iranian population has also been rising (Faber *et al.*, 2012; Foßelteder *et al.*, 2018). Various environmental, genetic, and epigenetic factors influence the development of colon cancer. Environmental factors such as lifestyle and nutrition affect the prevalence of colon

cancer (Harada *et al.*, 2012; Huang *et al.*, 2020). Hereditary characteristics are significant risk factors for causing the disease. Familial adenomatous polyp and hereditary non-polyposis carcinoma are the genetic factors that cause this malignancy (Huang *et al.*, 2016; Javed *et al.*, 2020). Stages of colorectal cancer are typically determined by the TNM system, which stands for tumor, node, and metastasis. Predicting and treating colorectal cancer is influenced by age, TNM stage, distant metastasis, cancer grade, and tumor size (Kasagi *et al.*, 2017).

Genes significantly impact the development of colon cancer. Identifying people at risk, developing targeted treatments, and gaining a deeper understanding of this disease requires investigating genes in colorectal cancer (Ling *et al.*, 2013). A large number of RNA transcripts that do not code for any protein are known as non-coding RNAs (ncRNAs). LncRNAs (long non-coding RNAs) are a type of ncRNA that is longer than others and ranges from 200 to 1000 nucleotides (Liu *et al.*, 2020).

The function of these RNAs is divided into four main mechanisms: calling chromatin-modifying complexes to specific locations in the genome, creating molecular scaffolds, modulating the transcription process, and regulating expression (Aliperti *et al.*, 2021; Wang and Chang, 2011). Recent studies have shown that LncRNAs are increasingly involved in the pathogenesis of various diseases (Pirlog *et al.*, 2021).

The pathophysiology of cancer is influenced by certain LncRNAs, one of which is *Colon Cancer-Associated Transcript 2 (CCAT2)* that plays a significant role in carcinogenesis (Kasagi *et al.*, 2017). The lncRNA CCAT2 on chromosome 8q24.21 has been proven to be differentially expressed in various kinds of tumors, such as colorectal cancer (CRC). The *MYC* gene, an important oncogene, is also present in this region (Pirlog *et al.*, 2021).

The increased expression of the *CCAT2* gene has been demonstrated in various human cancers, including stomach, breast, lung, and esophagus cancer (Wang *et al.*, 2019; Xin *et al.*, 2017; Yao *et al.*, 2018; Zeng *et al.*, 2019). This increased expression is associated with tumor progression and a poorer prognosis for individuals who are affected (Huang *et al.*, 2020). In addition, studies have shown that overexpression of *CCAT2* is

associated with the dysregulation of key cellular processes, including cell proliferation, invasion of surrounding cells, and apoptosis, thereby contributing to the aggressive nature of cancer development (Zeng *et al.*, 2017; Zhao *et al.*, 2022).

MYC and *Wnt* signaling pathways play a significant role in *CCAT2*'s role in colorectal cancer, which is crucial for cellular regulation and tumorigenesis (Pirlog *et al.*, 2021). When *MYC* interacts with *CCAT2*, it enhances the expression of genes that are involved in tumor progression, increases the oncogenic potential of *MYC*, and regulates the aggressive behavior of cancer cells (Javed *et al.*, 2020; Swier *et al.*, 2019; Zhu and Li, 2023). *CCAT2* is responsible for uncontrolled cell growth and invasion by modulating the *Wnt* pathway. *CCAT2* plays a multifaceted role in colorectal cancer progression through its complex interaction with *MYC* and *Wnt* pathways (Pirlog *et al.*, 2021).

The expression of this gene is associated with overall survival, progression-free survival, and distant metastasis of human cancers. Functionally, the reduction of *CCAT2* expression can induce apoptosis in cancer cells and suppress cell proliferation, which indicates the potential of *CCAT2* as a therapeutic target (Moradi *et al.*, 2022).

The *BIM* gene is located at chromosomal position 2q13. This gene encodes a protein that belongs to the BCL-2 protein family (Warren *et al.*, 2019). BCL-2 family members can form hetero- or homodimers that regulate anti- or pro-apoptosis and are involved in a variety of cellular activities (Kim *et al.*, 2017). Dysregulation of *BIM* expression is a factor in the pathogenesis of different cancers. The activation of apoptotic pathways is facilitated by *BIM*'s interaction with anti-apoptotic BCL-2 family members (Harada and Grant, 2012; Sionov *et al.*, 2015; Warren *et al.*, 2019). Cancer cells can survive, proliferate uncontrollably, and escape apoptosis if *BIM* expression is decreased or dysfunctional (Shamas-Din *et al.*, 2013). Double main pathways control apoptosis in mammalian cells. A combination of apoptotic signals is completed through the cross-talk among several upstream signals and downstream effectors, influencing the decision between life and death. Changes in the apoptotic pathways

are intricate, with genetic alterations found mostly in the upstream supervisory proteins, delivering many potential aims for drug development. One of the major goals of cancer study is to develop safer and more effective therapies, which might capitalize on a better understanding of the cancer genome in addition to cancer-specific synthetic lethal interactions. A great challenge ahead is to understand in biochemical detail the integration modules and nodes of apoptotic pathways to help drug development and delineate therapeutic resistance mechanisms. Another big challenge is identifying biomarkers to help stratify patients for treatments and follow-up. Such efforts are expected to ultimately lead towards personalized medicine.

Furthermore, BIM expression changes are linked to chemotherapy resistance and poor prognosis in different cancers (Harada *et al.*, 2012). It has been proven that decreased *BIM* expression is connected to advanced tumor stage, metastasis, and poorer survival outcomes in colon cancer patients (Greenhoug *et al.*, 2010; Mhaidat *et al.*, 2019).

Investigating the expression level of the *BIM* gene and *CCAT2* gene in colorectal cancer is necessary due to its potential prognostic value, impact on treatment response, identification of therapeutic targets, and providing deeper insight into cancer progression. Understanding the expression level of these genes can provide important information regarding prognosis for patient management, predicting treatment response, choosing targeted treatments, and ultimately leading to improved outcomes for patients with colorectal cancer. Also, a better understanding of lncRNA function, especially the *CCAT2* gene, will help clarify the real impact of genomic pervasive transcription on cell biology and evolution.

Materials and Methods

Collection of samples

In this study, 30 tissue samples of colorectal cancer and 30 adjacent tissue samples from patients in Ilam City were collected through colonoscopic procedures and sent to Eram Pathobiology Laboratory Ilam. A pathologist tested the samples, and normal or cancerous

status was confirmed. According to the Helsinki Declaration, this study was approved by the ethics committee of the Ilam University of Medical Sciences. For molecular experiments, 10µm sections were prepared from paraffin blocks and placed in sterile 2cc microtubes. The samples were stored at -20°C until molecular experiments were performed.

RNA extraction

According to the kit method, first deparaffinization was performed, and then the other extraction steps were performed on the tissue without paraffin. RNA was extracted using TRIzol™ reagent (Sinaclon Co., Iran) according to the manufacturer's instructions. DNase I treatment (Sigma-Aldrich) was performed to remove DNA contamination. After extraction, the purity and concentration of RNA should be determined by measuring the absorbance at 260 nm (A260) in a spectrophotometer. The ratio of the readings at 260 nm and 280 nm (A260/A280) provides an estimate of the purity of RNA. The obtained RNAs were stored at -70°C until further steps. For RNA extraction, 500µl RNX Plus (Sinaclon Co., Iran) was added to the tube containing the sample. In the next step, 200 µl of chloroform (Merck, Germany) was added to the tube. Then, it was centrifuged for 15 minutes at 12,000 rpm. The aqueous portion was transferred to a new tube, and an equal volume of cold isopropanol was added. The tube was incubated for 1 hour at -20°C, then centrifuged for 15 minutes at 12,000 rpm, and the supernatant was discarded. In the next step, 1000 µl of cold 70% ethanol was added to the tube and then centrifuged for 8 minutes at 7,500 rpm. The supernatant was discarded and left at room temperature to dry. Then 30 ml of DEPC water was added to the tube and stored at -20°C.

cDNA synthesis

The cDNA was synthesized using a cDNA synthesis kit (Cinacloon, Iran) according to the manufacturer's instructions. To perform cDNA synthesis, first 1µl Random hexamer primer (Cinacloon, Iran) was mixed with 1 µl OligodT (Cinacloon, Iran) and 1 µl dNTP (Cinacloon, Iran) and then 10 µl of the additional RNA sample was mixed, and the resulting mixture was incubated for 5 minutes at 65°C and then placed

on ice. In the next step, 4.5µl water was mixed with 0.5µl M-MuLV (Cinacloon, Iran) and 2µl M-MuLV buffer (Cinacloon, Iran) and added to the previous mixture and placed at 42°C for 1 hour.

Real-time PCR

In this study, *BIM* and *CCAT2* were selected as target genes and *GAPDH* as a reference gene. The NCBI website was used to obtain the desired gene sequences, and Oligo7 software was used to design primers based on them. Then, primers were confirmed using the Primer-BLAST tool (<https://www.ncbi.nlm.nih.gov/tools/primer-blast/>). The primers were then sent to Cinacloon for

synthesis. Table 1 displays the specifics of the primers. Amplification was performed in a reaction mixture with a total volume of 20 microliters using Mastermix (2X Real-time PCR Master Mixb bimake (SYBR Green)), which is a ready-to-use solution for Real-Time PCR quantitative assay from Amplicon Company. A Stratagene mx3000p device was used to perform real-time reaction time PCR, and the melting temperature curve for each studied gene and the internal control gene (*GAPDH*) was calculated. The real-time PCR cycling conditions were: 50 °C 2 min, 95 °C 2 min, followed by 40 cycles of 95 °C 2 s and 60 °C 20 s.

Table 1. Specifications of primers used in quantitative real-time PCR.

Primer names	Oligomers (5'→3')	Target length	Target gene
<i>R-GAPDH</i>	5'-CGCTCAGCACAAAGAACCCTC	21	<i>GAPDH</i>
<i>F-GAPDH</i>	5'-TCACCAAGGACAAGCAGACAG	21	
<i>R-CCAT2</i>	5'-AAGTAGTTTCCATAGGTCTGA	21	<i>CCAT2</i>
<i>F-CCAT2</i>	5'-CTACCAGCAGCACCATTTCAG	21	
<i>R-BIM</i>	5'-AGGATCGAGACAGCAGGGAG	20	<i>BIM</i>
<i>F-BIM</i>	5'-GAAGGCAATCACGGAGGTG	19	

Statistical analysis

To compare gene expression in different groups, the normality of the data was checked using the Kolmogorov-Smirnov test. Considering that the expression of genes in different groups did have a normal distribution. Ct is a response cycle that occurs when the fluorescence signal crosses the threshold. The Ct difference between the desired gene and the reference gene is defined as ΔCt . The $\Delta\Delta Ct$ is the difference in ΔCt between cancerous and healthy samples. Change folding was calculated using the formula $2^{-\Delta\Delta Ct}$. Statistical analysis was performed using Excel 2020 software and GraphPad Prism 9 using the Fold Change method. An independent t-test was used to determine the significance level of the data with a $P < 0.05$.

Results

The melting curve of various genes can be seen in Figure 1. The melting curve of all three genes had one peak, suggesting that only one PCR product was present (Fig. 1). The single peak of the melting curve indicates the specificity of the PCR reaction products. The average relative expression of the *CCAT2* gene in colorectal

cancer tissue was 326, while in healthy tissue, it was 1.52. The difference in the average expression between the cancerous and healthy tissues was calculated as 324.48 ± 25.00 , indicating a significant increase in the expression of *CCAT2* in cancerous tissue compared to adjacent healthy tissue (Fig. 2A). Data analysis was done using paired t-test and the P value was less than 0.0001, which is statistically significant ($P \leq 0.05$). The average relative expression level of the *BIM* gene was 1.14 in the healthy tissue of the margin, 0.11 in the colorectal cancer tissue, and the difference in the average expression of the two genes was equal to 0.29 ± 1.04 , which indicates the decrease in the expression of the *BIM* gene in the cancerous tissue compared to the adjacent healthy tissue (Fig. 2B). The paired t-test was used to evaluate the data and the P value was 0.008, which indicates statistical significance ($P < 0.05$).

Discussion

Various genetic and environmental factors play a role in the complex disease of colorectal cancer (Ramzi *et al.*, 2014). In cancer biology, one of the first pieces of evidence that researchers seek

is gene expression differences between tumor and normal samples. The breadth of knowledge concerning CCAT2 and BIM expression profiles in tumor and normal samples is quite modest. We foresee potential uses of CCAT2 and lncRNAs in the clinical setting for oncology or for other fields. CCAT2 and lncRNAs may be useful as novel biomarkers for diagnosis, prognosis, and prediction of response to therapy. CCAT2 is located on chromosome 8q24, a region frequently amplified in various cancers, and has been implicated in promoting tumorigenesis

through multiple mechanisms (Grisanzio and Freedman, 2010). Studies suggest that CCAT2 enhances *Wnt* signaling, a pathway crucial for cell proliferation and survival, thereby contributing to colorectal carcinogenesis (Foßelteder *et al.*, 2018; Javed *et al.*, 2020). Increased levels of CCAT2 have been correlated with poor prognosis, higher tumor grade, and metastasis, indicating its potential as a biomarker for aggressive CRC phenotypes (Huang *et al.*, 2016; Wang *et al.*, 2019).

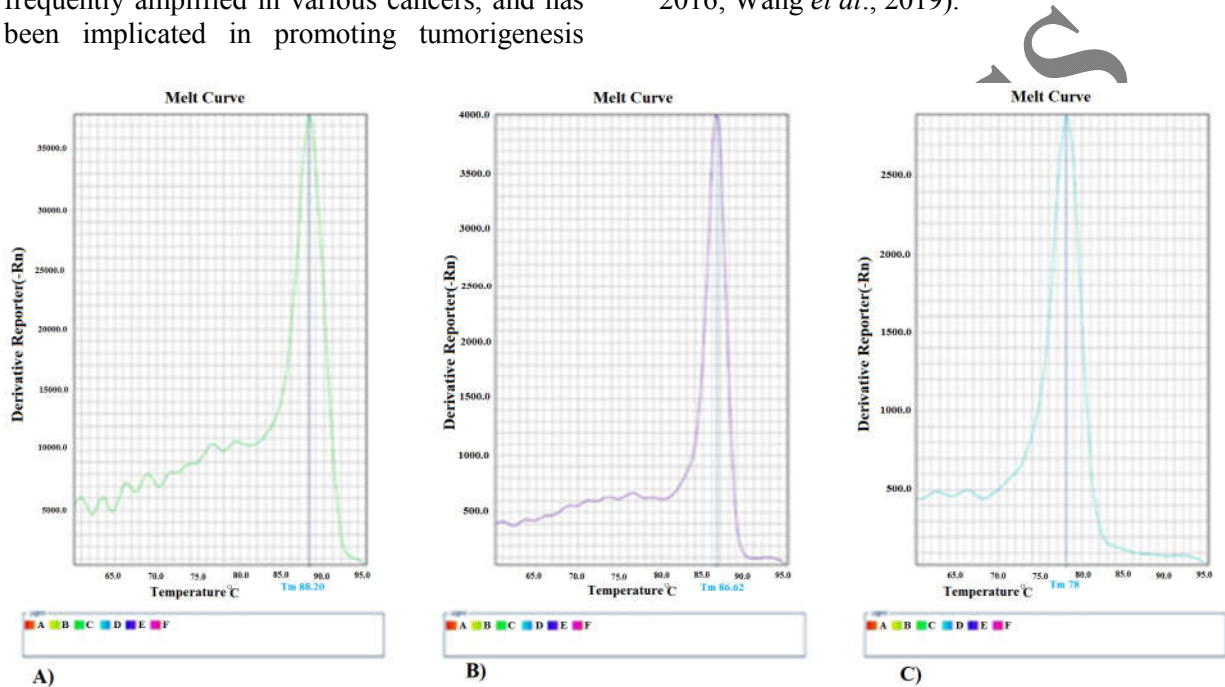


Fig. 1. Melting curve of various genes: A) Melting curve of CCAT2 gene. B) GAPDH gene melting curve. C) Melting curve of the BIM gene

In contrast, the BIM (BCL2L1) gene, which encodes a pro-apoptotic member of the BCL-2 protein family, is typically downregulated in CRC (Greenhough *et al.*, 2010; Tagscherer *et al.*, 2016). BIM is essential for the initiation of apoptosis, and its decreased expression can result in impaired apoptotic pathways, contributing to cancer cell survival and resistance to chemotherapy (Faber *et al.*, 2012). The suppression of BIM has been linked to various mechanisms, including epigenetic modifications and aberrant signaling pathways such as EGFR/MAPK and PI3K/AKT, which are commonly activated in CRC (Bahar *et al.*, 2023; Chakraborty *et al.*, 2013; Sanaei *et al.*, 2022; Steelman *et al.*, 2011). Decreased BIM expression correlates with resistance to apoptosis

and has been associated with poor clinical outcomes (Hata *et al.*, 2015). Understanding the differential expression of CCAT2 and BIM provides insights into the molecular underpinnings of CRC and highlights the potential for therapeutically targeting these pathways to improve patient outcomes.

Mhaidat *et al.* showed that tumor suppressor gene methylation is associated with cancer development and chemoresistance. One of such genes is Bim. Their study found a significant increase in Bim gene methylation among CRC patients compared to healthy controls. Their results showed that Bim gene methylation is associated with CRC development, metastasis, and chemosensitivity (Mhaidat *et al.* 2019). Their results showed that Bim gene methylation

is associated with CRC development, metastasis, and chemoresistance. The findings also indicated that Bim gene expression is reduced in CRC tissue, which could be due to Bim gene methylation. Therefore, evaluation of Bim gene methylation may help diagnose and treat CRC patients.

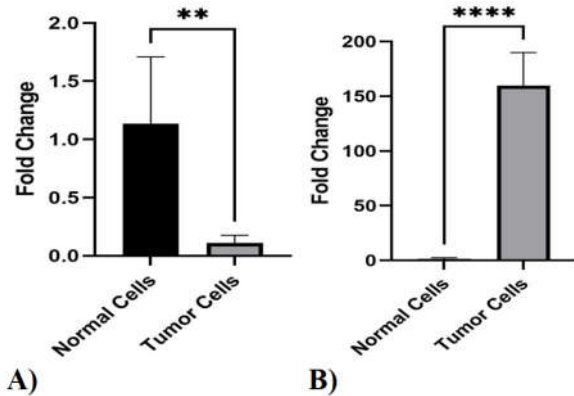


Fig. 2. Gene expression changes between the control and tumor cells: A) The average level of *BIM* gene expression changes in colorectal cancer tissue compared to healthy peripheral tissue B) The average level of *CCAT2* gene expression changes in colorectal cancer tissue compared to healthy peripheral tissue. The error bars show the standard deviation for all samples.

This study evaluated the expression of *CCAT2* in colorectal cancer tissue and adjacent normal tissue samples from 30 patients diagnosed with colon cancer. The results found that the expression level of *CCAT2* was significantly different between colorectal cancer and healthy tissue. The results are consistent with previous studies and emphasize the importance of *CCAT2* as a prognostic marker for cancer (Wu *et al.*, 2016).

In the study of Wang *et al.*, the *CCAT2* gene was found to be overexpressed in colon cancer cells. The activation of the *AKT/GSK3* signaling pathway resulted in an increase in cell growth and metastasis due to this increased expression (Wang *et al.*, 2019). Zhang *et al.* reported that increased *CCAT2* expression is associated with increased cell proliferation, decreased apoptosis, increased metastasis, and shorter patient survival (Zhang *et al.*, 2017). According to the results, *CCAT2* has the potential to be a promising target for colon cancer control and treatment. The level

of *BIM* gene expression in colon cancer tissue cells was decreased in comparison to the marginal healthy tissue in this study, which was statistically significant. This study's results were in line with the ones of Zaki *et al.*, who examined *BIM* gene expression in the serum of CRC patients under miR-92a's inhibitory mechanism (Zaki *et al.*, 2022).

The extremely high level of conservation among these lncRNAs, which are referred to as ultra-conserved genes or transcribed UCRs, is their most peculiar feature. Some lncRNAs bind to polycomb repressive complex 2, indicating that lncRNAs might regulate gene expression by directing the polycomb protein group to target DNA regions, inducing changes in histone marks and chromatin structure and ultimately suppressing transcription activity. Another class of lncRNAs that seems to regulate gene expression by changes in chromatin status includes antisense transcripts. Antisense ncRNA transcripts overlap PCG but are transcribed in the opposite direction. Although one would expect small interfering RNA (siRNA) machinery to degrade messenger RNA after the sense-antisense pairing, the mechanism in action instead seems to be the modifications of histone marks at the promoter region of the sense transcript (PCGs). Antisense lncRNAs drive (cytosine-5)-methyltransferase 3A (DNMT3A) to the DNA of the host PCG to methylate histones at lysine 9 and 27 or CpG islands and ultimately silence transcription (Liu *et al.*, 2020). Zaki *et al.* also investigated the expression level of this gene in the serum. Therefore, it is possible to investigate the expression of the *BIM* gene in future studies in the serum and plasma of patients (Zaki *et al.*, 2022). Increased miR-323 expression in colon cancer cells caused a decrease in *BIM* gene expression and increased cell proliferation through the *FOXO3a* pathway in another study (Cheng *et al.*, 2019). Another research showed that increasing the expression of a transcription factor called *SPZ1* in colon cancer cells decreases *BIM* gene expression, decreasing apoptosis and promoting cell growth (Liu *et al.*, 2020). Studies have demonstrated that boosting the level of *BIM* gene methylation in cancer cells decreases the expression of this gene (Cheng *et al.*, 2019). These findings are in accord with the ones from the present study and

could be one of the possible explanations for the decrease in *BIM* gene expression in the study.

Conclusion

The function of CCAT2 in either beginning or promoting the oncological phenotype of various cancer types is still an expanding niche in the area of tumor-associated non-coding RNAs. Presently, the major highlighted and reoccurring regulatory connections with tumor-promoting signaling pathways are Wnt/ β -catenin and MYC. Moreover, new insight concerning CCAT2's implication in stimulating cancer-associated genomic instability hints towards the multiple implications of these transcripts across the numerous types of cancers associated with chromosomal abnormalities.

In colorectal cancer, there is a significant increase in the expression level of the *CCAT2* gene, as shown by the present study. In the *BIM* gene, the expression pattern was reversed due to its higher expression level in normal tissue than in cancer cells. Furthermore, the *CCAT2* gene has an important role in pathology and the role of *BIM* gene functional defect in colorectal cancer. Therefore, CCAT2 could be considered a valuable pan-cancer biomarker that is associated with a more aggressive disease course, one with potential as a therapeutic target of new treatments or for use in strategies for overcoming chemo- and radio-resistance. Also, the assessment of *BIM* gene methylation might help identify and treat colorectal cancer patients. Consequently, these two genes can not only be considered as an important prognostic factor but also as a promising target candidate for new treatments.

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Conflicts of interest

The authors declare no conflict of interest.

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