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Investigation of the expression of LncRNA/CCAT2 gene in patients with colorectal cancer

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A B S T R A C T

Background and Aim: One of the most important topics in Colorectal Cancer (CRC) is identifying the genes that play a crucial role in cancer progression. Therefore, recognizing the important genes during the CRC progression is important as a biomarker for prognosis. Noncoding RNAs (ncRNAs), the major transcribed RNA molecules in animal cells, do not encode proteins and are mainly involved with the regulation of gene expression. The Colon Cancer Associated Transcript 2 (*CCAT2*) gene is transcribed to a LncRNA molecule and seems to be associated with several cancers. Methods: A total number of 20 colon cancer specimens and their corresponding healthy specimens from adjacent tissues were studied to investigate the expression of *CCAT2*. Total RNA was extracted, cDNA was synthesized, and finally, the expression of *CCAT2* was determined in CRC and healthy tissues using Real-time Polymerase Chain Reaction (RT-PCR) assay. Results: The expression of *CCAT2* in colorectal tumors and their adjacent healthy tissues was investigated. According to the results, the expression of *CCAT2* in colorectal tumors and healthy tissues was significantly different. In other words, the expression of *CCAT2* in cancer tissues was higher than the healthy tissues by 4.5 folds. Conclusion: The increased expression of LncRNA/*CCAT2* is a common characteristic of colorectal cancer, and could be a good prognostic marker for this cancer.

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Introduction

According to the epidemiological studies, cancer is a major leading cause of death in the world after cardiovascular diseases with an annual estimated 12,000,000 new cases (Haggar, et al., 2009). With almost 1 million annual death, colon cancer is considered the third cause of cancer-associated death worldwide (GLOBOCAN, 2020). The incidence of colorectal cancer has also been increasing in Iranian population (Pourhoseingholi, et al., 1392). Several environmental, genetic and epigenetic factors are associated with colon cancer development. Environmental factors such as lifestyle and nutrition influence the prevalence of colorectal cancer. Also, hereditary characteristics are important risk factors for the development of the

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disease. Genetic factors influencing the development of this malignancy include familial adenomatous polyps and hereditary non-polyposis carcinoma (Jasperson, et al., 2010). Epinephrine changes broadly cause changes in the methylation of cytosines, the structure of chromatins and histones, and expression of microRNAs (miRNAs) (Danese, et al., 2017). Susceptibility to cancers is mainly attributed to changes in the coding sequences of the genome. Researches have shown that a non-genetic imbalance in proteins causes uncontrolled cell growth (Xing, et al., 2021). Cancer cells tend to develop malignancy to nearby organs which is called metastasis (Gupta, et al., 2016). The malignancy is often initiated from a colonic ulcer in the distal part of the large intestine (left side of the colon). Colon cancer generally involves two different types, including familial and sporadic colon cancers. Approximately 80% of cases are related to the inherited type (Kory, et al., 2010). Risk factors for the disease include obesity, race, geography, lifestyle, the extent of tumor penetration into the intestinal wall, distant metastasis, lymph nodes metastasis, and the pathological stage (Fernandez, et al., 2010). In general, the survival rate of colorectal cancer is slightly higher in women than in men. Age, TNM (Metastases, Nude, tumor) stage, distant metastasis, cancer grade, and tumor size are important factors in predicting and treatment of colorectal cancer (Young, et al., 1999). Aspirins and other nonsteroidal anti-inflammatory drugs play a role in preventing colorectal cancer. Researches suggest that taking aspirin may cause 30 to 40 % reduction in the risk of adenoma and colorectal cancers (Badmaev, et al., 2009).

Non-encoding RNAs (ncRNAs) account for a large number of RNA transcripts that do not encode any protein. Some ncRNAs are longer than others, ranging from 200 to 1000 nucleotides that are called LncRNA (long non-coding RNAs) or lincRNA (long intervening non-coding RNAs). These molecules are found in the nucleus and cytoplasm and are transcribed by RNA polymerase II (Hauptman et al., 2013). LncRNA coding genes have epigenetic effects and are compatible with transcriptional genes and are transcribed through the RNA polymerase II enzyme. They are also regulated by transcription factors and are often expressed according to their tissue specificity. Copies of LncRNAs often undergo a polyadenylation process and are processed through their conventional processing station motifs (Cabili, et al., 2011). LncRNAs are divided into two groups of senses and antisense based on their location relative to protein-coding genes. Antisense LncRNAs are also divided into three separate groups (bilateral, intron, intergenerational) (Xing, et al., 2021).

LncRNAs have several functions, including chromatin organization, epigenetic regulation, transcriptional and post-transcriptional regulation (Hu, et al., 2018, Morlando, et al., 2018, Schmitz, et al., 2016). LncRNA molecules are usually controlled in tumors, but some LncRNAs play a role in the pathophysiology of cancer (Chiu, et al., 2018). Interruption of LncRNAs regulatory mechanism is significantly associated with the cellular promotion and colorectal cancer metastasis (Chiu, et al., 2018). The *CCAT2* is an oncogenic LncRNA that shows increased expression in cancer cells (Yu, et al., 2017). The LncRAN/*CCAT2* is highly conserved in the 8q24.27 region of the human genome. The expression of the *CCAT2* gene alters the metastatic potential of cancer cells and causes chromosomal instability (Ling, et al., 2013). Based on the reports, the *CCAT2* is overexpressed in several carcinomas, including colon cancer. So, the excessive expression of the *CCAT2* could be a prognostic marker for colorectal cancer. Also, reduction or silencing of the *CCAT2* may result in the suppression of tumor development and metastasis via reduction of cancer cell proliferation and migration, and also apoptosis induction (Yu, et al., 2017, Fobelteder, et al., 2018).

The LncRNA/*CCAT2* is expressed following the activation of the Wingless-related integration site (WNT) messaging pathway, the entry of B-catenin into the nucleus, and its association with the TCFL72 transcription factor. The presence of the rs6983267GG polymorphism in the LncRNA colon cancer-associated transcript *(CCAT2)* encoding gene increases the expression of the transcript relative to the rs6983267TT genotype and is associated with the increased susceptibility to colorectal cancer. Increased *CCAT2* expression is associated with the increased expression of downstream target genes, including the oncogenic transcription factor MYC, and has a positive

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feedback effect on WNT messaging activity. Elevated MYC levels increase the expression of its downstream targets, including miR17HG and miR20a, which are mediators of metastasis and play an important role in the development of metastatic phenotype (Ling, et al., 2013).

Considering the important role of *CCAT2* in the development of colorectal cancer, in this study we investigated the expression of *CCAT2* in colon cancer tissues in comparison with their adjacent healthy tissues.

Materials and Methods

Patient's samples

A total number of 20 biopsy samples from colorectal cancer tumors and their adjacent healthy tissues from the same individuals, received from the tumor bank of Imam Khomeini Hospital, Tehran, Iran during 2019 to 2020. Patients demographic is presented in table 1. This study was approved by the Biomedical Ethics Committee of the Islamic Azad University of Shahrekord and also complies with the Ethics Code of IR.IAU.SHK. REC.1398.044.

Table 1. Patients' demographic and clinical information (n = 20)

Variables	Number
Age	
<50	16
≥50	4
Sex	
Male	16
Female	4
TNM stage	
I	2
II	8
III	8
IV	2
Grade	
I	4
II	11
III	3
IV	2
Unknown	1
Tumor size (cm)	
<5	6
>5	14
Smoking	
Smoker	3
Non-smoker	17

RNA extraction and cDNA synthesis

Extraction of total RNA was performed using the SinaClone RNA extraction kit (Iran), according to the manufacturer's instruction. The quality and quantity of the extracted RNA samples were evaluated by measuring the 260/280 nm optical density ratio using a Nanodrop instrument (Thermo scientific-Nanodrop 2000), and also gel electrophoresis. Synthesis of cDNA was performed using the SinaClone cDNA synthesis kit (Iran), according to the manufacturer's protocol.

Real-time PCR

Real-time Polymerase Chain Reaction (RT-PCR) was used to determine the expression of *CCAT2* in colorectal and healthy tissues using a SYBR Green master mix (Takara) in a final volume of 20 µL containing 12 µL DEPC treated distilled water, 6 µL of SYBR Green master mix, 1 µL of cDNA (200 ng), and 0.5 µL of forward and reverse primers (10 pmol). The amplication program was as follows: initial denaturation (95 °C/5 min), and 40 cycles of denaturation at 95 °C/30 s, annealing at 60 °C for 30 s, and extension at 72 °C for 20 s. The Glyceraldehyde 3-phosphate dehydrogenase *(GAPDH)* gene was used as the reference gene. Finally, the expression of the *CCAT2* in cancer and healthy tissues was evaluated by measuring the cycle threshold (Ct) values. The primers used in this study were presented in Table 2.

Table 2. Primers used in this study

Name of primers	Sequence (5′-3′)	Amplicon size (bp)	Reference
CCAT2-Forward	CCCTGGTCAAATTGCTTAACCT	66	Lai et al., 2018
CCAT2-Reverse	TTATTCGTCCCTCTGTTTTATGGAT		
GAPDH-Forward	ACCCACTC CTCCACCTTTGAC	159	Lai et al., 2018
GAPDH-Reverse	TGTTGCTGTAGC CAAATTCGTT		

Statistical analysis

Data analysis was performed using the SPSS version 16.0 software (SPSS Inc., Chicago, IL). Statistically difference for the expression of

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CCAT2 was analyzed using *one-way* ANOVA and Tukey's posthoc tests and the *p-value* less than 0.05 was considered statistically significant. Also, the Kolmogorov-Smirnov test was used to check the normal distribution of data and in cases where the distribution was not normal, Wilcoxon nonparametric test, which is equivalent to nonparametric pair t test was used. The *p-value* less than 0.001 was considered statistically significant. Graphs were drawn in Excel 2016 and each experiment was repeated at least three times.

Results

The result of RNA extraction is presentated in figure 1. As shown in the figure. The presence of 18s and 28s rRNAs indicates the appropriate quality and quantity of extraction and no degradation of RNA.

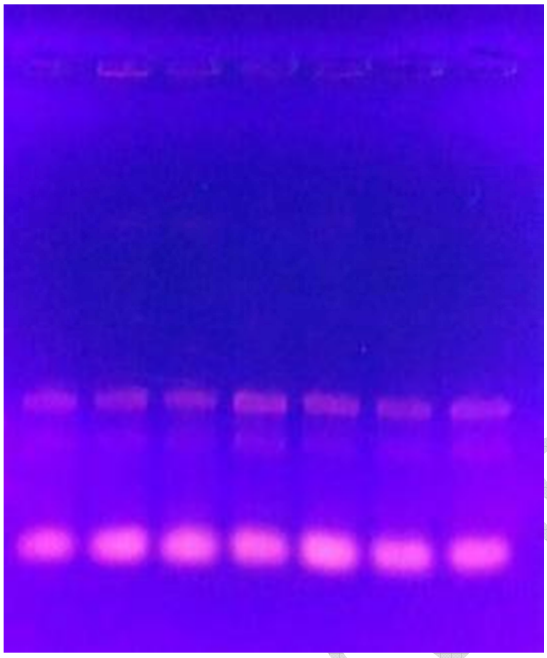


Fig. 1. Qualitative evaluation of the amount of RNAs extracted from the tissues.

The melting curve plotted for the target gene had single peaks, indicating amplification of only one specific product in the Real Time PCR reaction (figure 2).

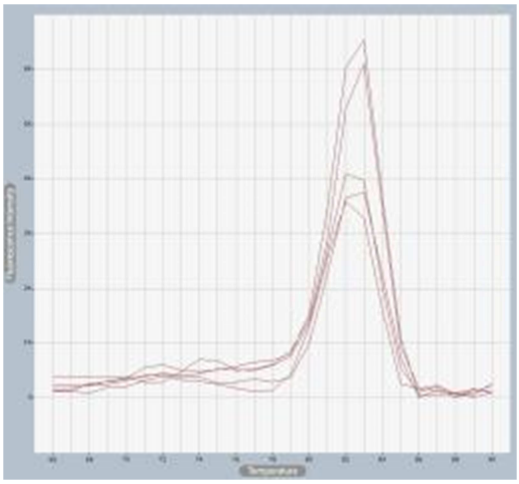


Fig. 2. The Melting curve diagram of *CCAT2* gene.

Examination of the normality of the data using Kolmogorov-Smirnov test showed that *CCAT2* expression was not normally distributed in case and control individuals, so Wilcoxon nonparametric test was used to compare gene expression in these two tissues. The results of gene expression in patients were 4.5 folds compared to healthy individuals, which was significant at the level of 0.001.

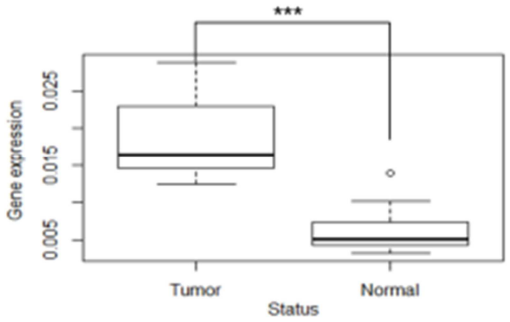


Fig. 3. Distribution of *CCAT2* gene expression in tumor and normal tissus (*p<0.001*)

The expression of *CCAT2* in colorectal tumors and their adjacent healthy tissues was investigated. According to the results, the expression of *CCAT2* in colorectal tumors and healthy tissues was significantly different. In other words, the expression of *CCAT2* in cancer tissues was higher than the healthy tissues by 4.5 folds. The relative expression of *CCAT2* in colorectal tumor and healthy tissues was presented in figure 4.

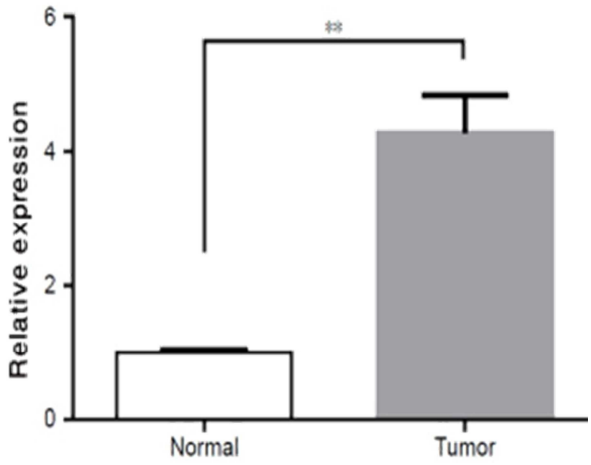


Fig. 4. The relative expression of the *CCAT2* in colorectal cancer and healthy tissues. **significantly different (*p<0.05*)

Discussion

Currently, the only comprehensive criteria employed in clinical practice to provide an indication of CRC prognosis or to guide adjuvant chemotherapy choices is the American Joint Committee on Cancer (AJCC) tumor node metastasis (TNM) staging system. Regrettably, the TNM system hasn't provided the clinicians with the optimal staging allocation, but has resulted in substantial under- or over-treatment of certain CRC patients over the past decades (Yang, et al., 2017).

Recent studies suggest that both oncosuppressive and oncogenic lncRNAs may have potential to be used as efficient biomarkers of CRC in clinical routine (Akbari, et al., 2020).

Some LncRNAs are functional components involved with the gene regulations at transcriptional and post-transcriptional levels. Among them, the LncRNA/*CCAT2* is considered as a functional molecule to be associated with several cancers (Yu et al., 2017). The increased expression of the *CCAT2* in several cancers has been reported previously (Yu, et al., 2017, Ling, et al., 2013). Also, this RNA molecule has been revealed to be overexpressed in colorectal cancer with a regulatory role in cancer development (Yu, et al., 2017, Ling, et al., 2013, Wang, et al., 2019). Therefore, in this work, we evaluated the expression of the *CCAT2* in colorectal cancer tissue and their adjacent normal tissue specimens from 20 patients with colon cancer. Our results showed a significant difference for the expression of *CCAT2* between colorectal cancer and healthy tissues. In the cancer tissues, the expression of *CCAT2* was higher than healthy tissues by 4.5 folds. Our results are in agreement with previous reports (Ling, et al., 2013, Shaker, et al., 2017).

Thus, the expression of *CCAT2* could a prognostic marker for colorectal cancer. Also, it has been reported that the increased expression of *CCAT2* is associated with tumor development and metastasis, and increased resistance to chemotherapy (Yu, et al., 2017, Yu, et al., 2015). Therefore, the *CCAT2* could also be a promising target for the control and treatment of colorectal cancers.

Several mechanisms have been introduced for the effect of *CCAT2* overexpression on cancer development. In a recent study, it was revealed that the oncogenic potential of *CCAT2* is involved with the inhibition of the miR-145, a tumor suppressor micro RNA. Recent studies showed that *CCAT2* selectively inhibits the maturation of the miR-145 molecules and thus enhances tumor development (Yu, et al., 2017, Ling, et al., 2013).

In addition, an association between some SNPs, including rs6983267 and rs7763881 at 8q24, and increased serum level of CCAT2 was reported. Thus, genetic polymorphisms are involved with the development of colorectal cancer through the regulatory mechanism of *CCAT2*. Previous works showed the strong association of the rs6983267 G allele with increased transcription of *CCAT2*, which is a risk factor for colorectal cancer development (Pourhoseingholi, et al., 1392). Also, it was shown that the increased expression of *CCAT2* results in the up-regulation of MYC, miR–17–5p, and miR–20a, which are involved with enhanced cell proliferation, tumor development, and metastasis (Ling, et al., 2013, Fobelteder, 2018). Also, the physical interaction of *CCAT2* and Transcription factor-7–like 2 (TCF7L2), an oncogenic transcription factor, enhances the WNT signaling activity, which may result in chromosome instability, cell transformation, and finally cancer development (Yu, et al., 2017, Ling, et al., 2013). Thus, *CCAT2* plays a critical role in colorectal cancer development and metastasis via several regulatory mechanisms.

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Conclusion

In this study, we found a significantly higher expression of *CCAT2* among colorectal cancer tissues in comparison to their adjacent healthy tissues. Owing to the vital role of the *CCAT2* in the development of colorectal cancers, this LncRNA not only could be regarded as an important prognostic factor but also could be considered as a promising target candidate for novel chemotherapy approaches.

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