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Investigation of *GNB1* derivative circular RNAs *hsa_circ_0009361* and *hsa_circ_0009362* expressions in colorectal cancer patients: potential new diagnostic factors

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ABSTRACT

Aim: We aim to investigate the relationship between *hsa_circ_0009361* plus *hsa_circ_0009362* expression levels and the clinicopathological features of colorectal cancer (CRC) patients.

Background: Circular RNAs (circRNAs) are implicated in the progression and development of CRC. CircRNAs have been recognized as diagnostic and prognostic biomarkers, opening up a new window to comprehend the molecular basis of CRC. Given the significance of circRNAs and the G protein subunit b1 (*GNB1*) gene in malignancies, the goal of the current investigation was to determine the expression levels of *GNB1* derivative circular RNAs circGNB1 (*hsa_circ_0009361* and *hsa_circ_0009362*) in CRC and adjacent control tissues.

Methods: The expression levels of the *GNB1* derivative circular RNAs (*hsa_circ_0009361* and *hsa_circ_0009362*) were evaluated using the quantitative real-time PCR (qRT-PCR) method in 45 CRC tissues and adjacent control tissues. Furthermore, we analyzed the diagnostic power of the mentioned circRNAs by plotting the receiver operating characteristic (ROC) curve. The association between the expression levels of *hsa_circ_0009361* and *hsa_circ_0009362* was evaluated using correlation analysis.

Results: Our results revealed that the expression levels of *hsa_circ_0009361* and *hsa_circ_0009362* were significantly down-regulated in CRC tissues compared to the adjacent control group. Analysis of patients' clinicopathological features indicated that expressions of *hsa_circ_0009361* and *hsa_circ_0009362* were differently related to lymph vascular invasion (P<0.001). ROC curve results showed that these circRNAs are good candidate diagnostic biomarkers in CRCs. Pearson's correlation test revealed a positive correlation between *hsa_circ_0009361* and *hsa_circ_0009362* expression levels (P<0.0001).

Conclusion: These results demonstrated that *hsa_circ_0009361* and *hsa_circ_0009362* expression levels may be used as possible diagnostic biomarkers for CRC.

Keywords: Circular RNA, *hsa_circ_0009361*, *hsa_circ_0009362*, Colorectal cancer

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Introduction

Colorectal cancer (CRC) is the third most common type of cancer worldwide and the second leading cause of cancer-related deaths (1). The majority of CRC cases are caused by genetic mutations and epigenetic changes that alter signaling pathways, leading to the transformation of normal tissue into carcinoma (2). A biological molecule that is present in blood, other bodily fluids, or tissues, serving as a marker for a normal or abnormal process, condition, or disease is referred to as a biomarker (3). Various epigenetic and genetic changes can be utilized as potential clinical indicators for individuals with CRC (4).

A class of endogenous RNAs with a closed loop structure are called circular RNAs (circRNAs) (5). CircRNAs are expressed more steadily and are less

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prone to degradation than standard linear RNAs (6). This particular subset of non-coding RNAs is crucial for anti-tumor activities and cancer metastasis (7). Furthermore, they are involved in exerting their biological functions, such as apoptosis, proliferation, and, invasion through different mechanisms (8). Indeed, circRNAs serve as potential biomarkers in cancer (9, 10). CircRNAs play pivotal roles in the initiation and promotion of diverse human malignancies, such as breast (11), gastric (12), hepatocellular (13), lung (14), and colorectal (6). A recent study has demonstrated a stark difference between the expression levels of circRNAs in different types of colorectal tissues and para-carcinoma control tissues (15). Based on previous studies, different expressions of circRNAs are involved in the pathogenicity and development of CRC (16-18). However, the different expression of many circular RNAs and their functions in biological processes in tumor cells are not yet fully understood (19).

G protein subunit b1 (*GNB1*) is a component of the G protein complex that participates in signal transduction and forms a heterotrimer complex with the G protein subunits Ga and Gc (20). *GNB1* gene is important for the initiation and development of malignancies (21). Some specific circRNAs have shown dysregulation in cancer, such as *hsa_circ_0009361* (15) and *hsa_circ_0009362* (22) derived from the *GNB1* gene. In studies, *Hsa_circ_0009362* in breast cancer was overexpressed, and *hsa_circ_0009361* in CRC showed reduced expression (15, 22). However, the expression levels of *hsa_circ_0009361* and *hsa_circ_0009362* in CRC as well as their regulatory mechanisms are still unknown. Given that circRNAs play a significant role in cancer, we hypothesized that *GNB1*-derivative circular RNAs may be dysregulated in colorectal tissues compared with adjacent control tissues. The aim of the current study is to assess the expression levels of *hsa_circ_0009361* and *hsa_circ_0009362* derived from the primary *GNB1* gene, and to identify whether the transcripts of the mentioned circRNAs are proper potential diagnostic biomarkers or not. Additionally, we aim to investigate the relationship between these two circular RNAs and the clinicopathological data of CRC patients.

Methods

Patients and samples

In this case-control study, 45 fresh CRC tissue samples and paired adjacent control tissues were collected from the Genomic Research Center, Tehran, Iran. The tumor tissue samples were verified by a board-certified pathologist to ensure their histological consistency with CRC. Patients who had not received any CRC-related therapy prior to the biopsy were included in the study, while those who had received such therapy, had hereditary CRC, or had any other malignancies were excluded. The patient's lifestyle demographic and histopathological information, including the clinical TNM staging, were also recorded.

RNA extraction and cDNA synthesis

RNA extraction was done according to the manufacturer's protocol (Cinna colon, Tehran, Iran). Briefly, 1 ml ice-cold RNXTM–PLUS solution was added to 2 ml tube containing homogenized sample. It was vortexed 5-10 sec and incubated at room temperature for 5 min. Next, 200 µl of chloroform was added, and was mixed well for 15 sec through shaking. The resulting compound was incubated on ice or 4 ºC for 5min. Then, centrifugation was done at 12000 rpm at 4 ºC for 15 min and the aqueous phase was transferred to a new RNase-free 1.5 ml tube, where equal volume of isopropanol was added. The mixture was centrifuged at 12000 rpm at 4 ºC for 15 min, the supernatant was discarded, and 1 ml of 75% ethanol was added. The supernatant was discarded, and the pellet was allowed to dry at room temperature for few minutes. Isolated RNA was eluted in 40 µl of RNase/free water. The total RNA content and purity were evaluated using a NanoDrop ND-1000 spectrophotometer. Thereafter, RNA was kept for further use at -80 °C. Reverse transcription reaction was carried out using a cDNA kit (Cinna Colon, Tehran, Iran). Specifically, 2 µg of total RNA was used to create cDNA using random hexamer and Oligo(dT) primers. The kit mix was run on a PCR thermocycler as follows: 10 min at 25 °C, 2 h at 37 °C, 5 min at 85 °C.

Quantitative Real-Time PCR (qRT-PCR)

Utilizing 2.0X Real qRT-PCR Master Mix with SYBR Green (Ampliqon, Denmark), quantitative real-time PCR (qRT-PCR) was carried out. The reaction of

Table 1. qRT-PCR primer sequences.

Genes	Primers	Sequences	Amplicon size (bp)
hsa_circ_0009361	Forward Primer	CAGATGCAACTCTCTCTCAGAC	159
	Reverse Primer	TTAAGTTGCTCGGCCTCCTG	
hsa_circ_0009362	Forward Primer	CAACTTAAGAACCAGATTCGAAC	130
	Reverse Primer	TCAAGCTCACTCATCTTCCGA	
Beta-2-microglogulin	Forward Primer	TGTCTTTCAGCAAGGACTGGT	143
	Reverse Primer	TGCTTACATGTCTCGATCCCAC	

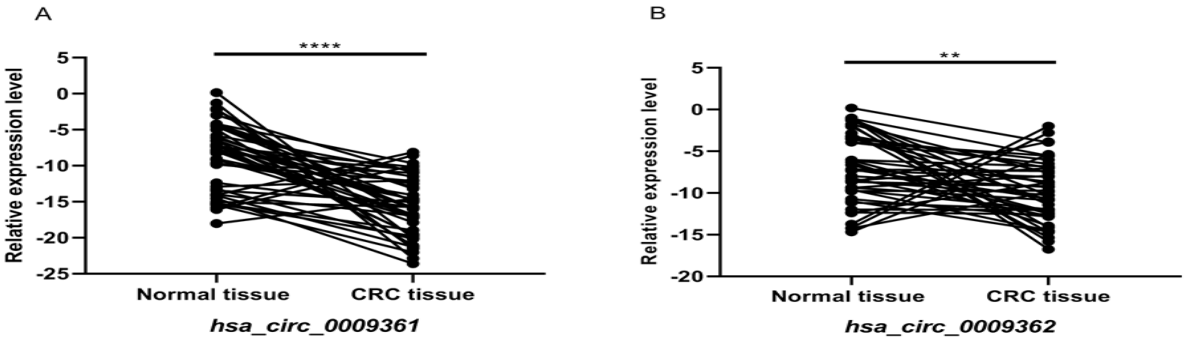

Figure 1. The relative expression levels (−ΔCt) of *hsa_circ_0009361* and *hsa_circ_0009362* in CRC samples and adjacent control tissues. As shown in the Figure 1 *hsa_circ_0009361* and *hsa_circ_0009362* were down-regulated in CRC tissues in comparison with adjacent control tissues. **P<0.01, ****P<0.0001.

each sample involved 10 µl 2X PCR Master Mix, 1 µl of each primer, 1 µl cDNA, and 7 µl of distilled water. The following thermal cycling settings were used to conduct the reactions using the StepOnePlus Real-time PCR System (Applied Biosystems, USA): Melt curve stage assessment after 15 minutes at 95 °C, 40 cycles at 95 °C for 5 s, and 60 °C for 30 s. Gene expression levels were normalized using the expression level of beta-2 macroglobulin (β2 M) as the housekeeping gene (23). The primer sets are reported in Table 1.

Statistical analysis

The gene expression ratio (Fold change $2^{-\Delta\Delta Ct}$) of the *hsa_circ_0009361* and *hsa_circ_0009362* was estimated using REST 2009 software. The expression ratio of the *hsa_circ_0009361* and *hsa_circ_0009362* was estimated using REST 2009 software. All other data were analyzed by GraphPad Prism software (La Jolla, CA). A two-tailed t-test was employed to determine the connection between the expression of the *hsa_circ_0009361* and *hsa_circ_0009362* as well as the clinicopathological factor of the CRC patients. The diagnostic values of the hsa_circ_0009361 and *hsa_circ_0009362* expression levels were estimated using the receiver operation characteristic (ROC) curve. The accuracy criterion for the analysis of the expression levels of *hsa_circ_0009361* and *hsa_circ_0009362* was the area under the ROC curve (AUC). The Pearson test was applied to determine the correlation between the expressions *hsa_circ_0009361* and *hsa_circ_0009362*. P value≤0.05 was considered as significant.

Results

Overall, we studied 45 CRC patients (22 females and 23 males) aged between 22 and 64 years (mean ± SD: 45.2 ± 12.4 years). In patients, tissue location of malignancy was as follows: 19 (42.2%) were colon and 26 (57.8%) were rectum. The patients clinical TNM stages were as follows: 16 (35.6%) were stage II, 18 (40%) were stage III, and 11 (24.4%) were stage IV. We measured the *hsa_circ_0009361* and *hsa_circ_0009362* expression levels in CRC tissue in comparison with adjacent control tissues. We observed *hsa_circ_0009361* and *hsa_circ_0009362* down-regulation in CRC tissues compared with controls (Figure 1).

Our results clarified that the expression levels of *hsa_circ_0009361* and *hsa_circ_0009362* were differently associated with the TNM stage (P<0.001). *Hsa_circ_0009361* and *hsa_circ_0009362* expressions

Table 2. The correlation of *hsa_circ_0009361* and *hsa_circ_0009362* levels (ΔCt) and clinicopathological features of the CRC patients.

Variable	Clinic pathological parameter	Number of samples (n = 45)	Mean ± SD *hsa_circ_0009361*	*P* value *hsa_circ_0009361*	Mean ± SD *hsa_circ_0009362*	*P* value *hsa_circ_0009361*
Age	≥60	20	14.75±3.837	*P*=0.2404	9.461 ±3.464	*P*=0.5786
	<60	25	16.20± 4.252		10.08 ±3.838	
Gender	Male	21	15.65± 3.34	*P*=0.8872	10.22± 2.82	*P*= 0.616
	Female	24	15.47 ±4.72		9.38 ±4.23	
TNM stage	II	15	6.52 ± 2.63	*P*<0. 0001	7.008 ± 3.51	*P*<0.0001
	III	18	10.79 ± 2.48		10.58 ± 2.75	
	IV	12	12.32 ± 3.36		12.77 ± 2.35	
Tumor size	<2	10	11.84 ± 3.46	*P*<0.0001	7.29 ± 3.78	*P*= 0.045
	2-3.5	12	15.03 ± 3.42		9.25 ± 3.90	
	3.5-5	13	16.17 ± 3.24		10.23± 2.77	
	>5	10	19.09 ± 3.38		12.30 ± 2.57	
Localization	Colon	19	15.37 ± 3.90	*P*=0.7959	9.64 ± 2.73	*P*= 0.95
	Rectum	26	15.69 ± 4.29		9.87 ± 4.21	
Lymphatic invasion	Positive	32	17.47± 3.06	*P*<0.0001	11.41± 2.72	*P<0.0001*
	Negative	13	10.82 ± 1.74		5.74 ± 2.10	

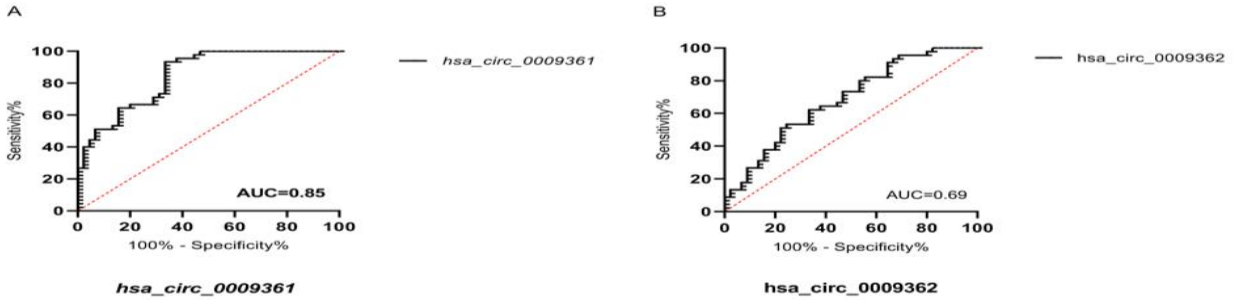

Figure 2. Potential diagnostic values of *hsa_circ_0009361* and *hsa_circ_0009362* in CRC. The sensitivity and specificity were 93.3 and 66.7 for *hsa_circ_0009361*, respectively (A). The sensitivity and specificity were 51.1 and 77.8 *for hsa_circ_0009362*, respectively (B).

in the stage IV group were down-regulated in comparison with the stage II and III groups. Clinical characteristics of the subjects are outlined in Table 2. The results revealed that expression of *hsa_circ_0009361* and hsa_*circ_0009362* were associated with lymph vascular invasion (LVI) (P<0.001). LVI+ was observed in 32 (71.1%) of 45 CRCs. The LVI+ group possessed a lower expression of *hsa_circ_0009361 and hsa_circ_0009362* compared with LVI- group (*hsa_circ_0009361*:17.48% vs. 10.83%, P<0.001, *hsa_circ_0009362* :11.42% vs. 5.74%, P < 0.001) (Table 2). ROC curve analysis demonstrated its effectiveness in assessing the performance of the *hsa_circ_0009361* and *hsa_circ_0009362* as good potential biomarkers for distinguishing CRC patients from control subjects (Figure 2). The areas under curve for *hsa_circ_0009361* and *hsa_circ_0009362* were 0.85 and 0.69, respectively. For *hsa_circ_0009361*, the sensitivity and specificity were 93.3 and 66.7, respectively, while for *hsa_circ_0009362*, they were 51.1 and 71.8, respectively.

Discussion

The abnormal expression of circRNAs in several malignancies has been proven by studies, indicating the significant role of circRNAs in the progression and development of various types of cancer (22, 24). CircRNAs alter signaling pathways and contribute to CRC pathogenesis (15, 25-27). So far, several circRNAs, such as *hsa_circ_0060927* (28), *hsa_circ_0005927* (29), and *hsa_circ_0001178* (30),

have been implicated in CRC development. By analyzing CRC-related circRNAs and confirming their role in cancer progression, we can introduce CRC-related circRNAs as viable therapeutic targets (31). Nevertheless, the expression level and function of circRNA in CRC are still unclear. Our results revealed that the expression levels of *hsa_circ_0009361* and *hsa_circ_0009362* were down-regulated in CRC tissues compared with control tissue samples. Our results are in agreement with a study conducted by Y Geng and colleagues. By analyzing CRC-related circRNAs, they identified *hsa_circ_0009361* down-regulation in CRC tissues and cells.

If we want to examine the mechanisms involved in the function of these circRNAs in CRC, we can refer to the epithelial-mesenchymal transition (EMT) process. EMT is an essential step in tumor metastasis induction. During EMT, adhesion and migration abilities of epithelial cells change, whereby the mesenchymal phenotype is obtained. E-cadherin and vimentin are two key markers of EMT. E-cadherin is a cell adhesion molecule, and its expression diminishes in epithelial cells during EMT. Thus, invasion is enhanced in tumor cells. In CRC cells, increased Wnt/β-catenin signaling inhibits E-cadherin expression and induces EMT. The overexpression of *hsa_circ_0009361* leads to a significant increase in E-cadherin expression and a decline in vimentin expression, and finally, EMT is inhibited in CRC cells. Silencing *hsa_circ_0009361* reverses these effects. So, in CRC, *hsa_circ_0009361* is known as a tumor suppressor. Adenomatous polyposis coli (APC) can inhibit the Wnt/β-catenin signaling pathway.

The structure of APC2 is similar to APC. Wnt signaling plays an important role in tumor progression. Upon inactivation of APC, β-catenin accumulates in the cytoplasm and is transported to the nucleus. In the nucleus, β-catenin can regulate the transcription of genes that control cell proliferation and apoptosis. miR-582 is able to bind to *hsa_circ_0009361*. *hsa_circ_0009361* relies on miR-582 to exert its tumor suppressive role, and the functions of *hsa_circ_0009361* are reversed by miR-582. APC2 is also known as a target gene of miR-582. On the other hand, in CRC cells, *hsa_circ_0009361* positively regulates APC2 and miR-582 negatively regulates APC2. APC2 is simultaneously regulated by *hsa_circ_0009361* and miR-582. Thus, in CRC, *hsa_circ_0009361* is known as a miR-582 sponge. Indeed, *hsa_circ_0009361* promotes APC2 overexpression via miR-582 sponge and represses CRC growth as well as metastasis by inhibiting Wnt/-catenin signaling. Therefore, they have hypothesized that the *hsa_circ_0009361*/miR-582/APC2 network may be used as a possible therapeutic target (15, 32).

Meanwhile, the mechanism of action of *hsa_circ_0009362* in CRC has not been investigated. Nevertheless, Liu P and colleagues showed increased expression of *hsa_circ_0009362* in triple-negative breast cancer (TNBC) tissues compared to normal breast tissues. *Hsa_circ_0009362* sponges miR-141-5p (22). To explain the reason for the reduction of circRNAs in CRC, we can point to these mechanisms: (a) the back-splice machinery is damaged; (b) deregulated miRNAs degrade circRNAs; (c) increased cell proliferation reducing the level of circRNAs (33). We analyzed the expression levels of *hsa_circ_0009361* and *hsa_circ_0009362* as well as clinicopathological features in different groups (Table 2). LVI is a particular pathogenic factor related to CRC metastases. LVI is defined by the presence of cancer cells within the lymphatic or vascular channels and is a common histological finding in CRC. Furthermore, LVI is a primary sign of lymph node metastasis and increases the risk of lymph node metastasis in patients with CRC. For cancer patients, understanding the oncological effect and prognostic relevance of LVI, which is connected with various types of malignancies (31-35), is of utmost importance. A few studies have shown that the circRNAs expression variations are considerably related to lymph vascular invasion (25, 34).

Our findings suggested that LVI may be a crucial marker for tumor behavior that is aggressive and is correlated with the expression level of *hsa_circ_0009361* and *hsa_circ_0009362* in CRC patients. The analysis revealed that the expression levels of circRNAs are significantly lower in the LVI+ group in comparison with the LVI- group ($P < 0.001$) (Table 2). The TNM staging system is the most significant tool for predicting the long-term survival of CRC patients. The TNM system is used to identify patients suitable for adjuvant treatment and provide an estimate of survival (35, 36). We found that the TNM stage is correlated with the expression of

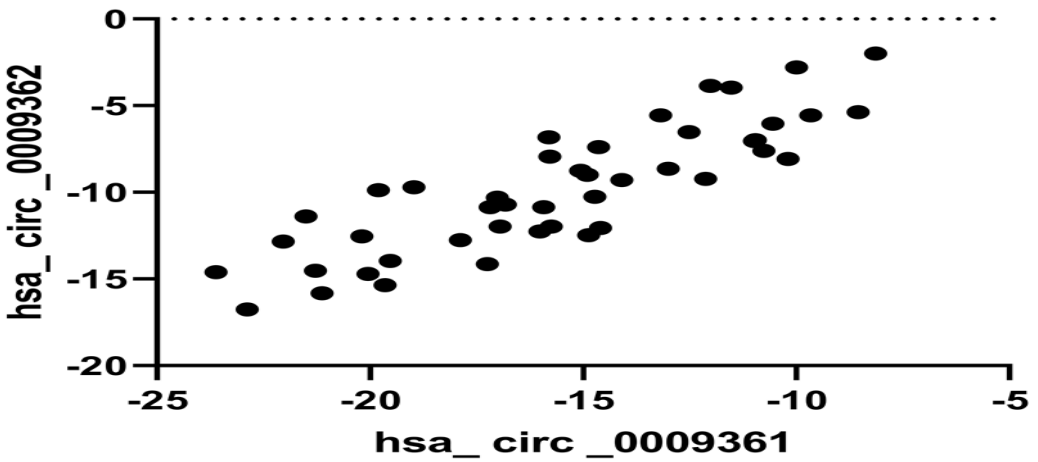

Figure 3. The statistically significant correlation between the expression of *hsa_circ_0009361* and *hsa_circ_0009362* (R2=0.730, N=45, P<0.0001)

hsa_circ_0009361 and *hsa_circ_0009362* in CRC patients, as in previous studies (37-39). As a result, our analysis revealed that *hsa_circ_0009361 and hsa_circ_0009362* expressions were significantly lower in advanced tumor stages (III and IV) than in lower tumor stages (II) (Table 2). ROC curve evaluates the predictive capability of *hsa_circ_0009361* and *hsa_circ_0009362* (i.e., sensitivity, specificity, and statistical significance) (Figures 1 and 2). The difference in the expression level of *hsa_circ_0009361* and *hsa_circ_0009362* between CRC tissues and adjacent control tissues indicated that these genes could serve as possible diagnostic biomarkers for CRC. The area under the ROC curve for *hsa_circ_0009361* and *hsa_circ_0009362* is shown in Figure 2. Additionally, in CRC samples, we demonstrated a positive correlation between the expression of *hsa_circ_0009361* and *hsa_circ_0009362* (Figure 3). We can also state that this is the first study to explore the potential relationship between has_circ_0009361 and *has_circ_0009362* in CRC. Therefore, it can be concluded that GNB1 derivative circular RNAs, *hsa_circ_0009361* and *hsa_circ_0009362*, may have a similar function in CRC pathogenesis. Further, these genes could serve as potential diagnostic biomarkers for CRC. In summary, we have indicated the expression levels of *has-circ-0009361* and *hsa_circ_0009362* are lower in CRC tissues compared with adjacent control tissues, and the expression levels of these circRNAs may potentially be used as diagnostic biomarkers for CRC. Also, some differences in the expression patterns of markers considering the patient's clinicopathological findings were observed. Since the relationship between circRNAs and CRC is multifaceted and dynamic, further investigations are required on the circRNA network in CRC.

The limitation of the study has been the small sample size; evaluating findings in larger numbers of patients allows for a better understanding of the potential impact in a clinical setting.

Conclusion

These results revealed that *hsa_circ_0009361* and *hsa_circ_0009362* expression levels may be potential new diagnostic factors to be used as possible diagnostic biomarkers for CRC.

Ethical statement

The study was compiled following the requirements confirmed by the Ethics Committee of the Shahid Beheshti University of Medical Sciences (Code No: IR.SBMU.RETECH.REC.1401.385). Additionally, informed written consent was taken from each individual.

Conflict of interests

The authors declare no conflict of interest.

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