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Platelet-Derived circRNAs hsa_circ_0004771 and hsa_circ_0019120 Differentially Expressed in Colorectal Cancer and Polyps

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Abstract

Background: Colorectal cancer (CRC) is the second most common cause of cancer-related deaths worldwide. Early detection is crucial for improving survival rates. Liquid biopsies, specifically analyzing circulating tumor-educated platelets (TEPs), have emerged as a promising tool for early CRC detection and monitoring treatment efficacy. This study investigated the expression levels of two specific circRNAs, hsa_circ_0004771 and hsa_circ_0019120, in the platelets of patients with CRC, advanced polyps, and healthy controls.

Methods: Blood samples were obtained from 25 individuals with CRC, 25 individuals with advanced polyps, and 25 healthy controls. Platelet-derived total RNA was extracted, and expression analysis was conducted using reverse transcription quantitative PCR (RT-qPCR). Differential expression and receiver operating characteristic (ROC) curve analysis were performed using GraphPad Prism.

Results: Both circRNAs were found to be upregulated in platelets from individuals with advanced polyps and CRC compared to healthy individuals. However, the upregulation was statistically significant only for hsa_circ_0004771 in CRC patients (p-value = 0.0036) and for hsa_circ_0019120 in both advanced polyp (p-value = 0.0175) and CRC patients (p-value = 0.0356). The combined analysis of both circRNAs achieved an area under the curve (AUC) of 0.8348 (95% CI: 0.7131 to 0.9565) with a sensitivity of 84% and specificity of 80% (p-value = 0.0002).

Conclusions: This study showed that hsa_circ_0004771 and hsa_circ_0019120 dysregulated in both CRC and polyps and have potential as a novel diagnostic biomarker of CRC.

Keywords: Biomarker, Blood Platelets, Circular RNA, Colorectal Cancer, Polyps.

Introduction

Despite improvements in detection and treatment approaches, colorectal cancer (CRC) remains the second most fatal cancer and the third most prevalent malignant tumor globally among both men and women (1, 2). Survival rates for CRC are greatly influenced by early identification, varying from a 90% five-year survival rate in stage I disease to just 10% in stage IV disease (3). At the advanced stage, surgical intervention is often impractical, leading to restricted treatment options and adversely affecting the prognosis of the patient (4, 5). Given the protracted transition from precancerous lesions (adenomas) to malignant formations (6), there is a window for early detection through screening (7). Timely identification of CRC facilitates effective treatment, and proactive

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screening efforts play a crucial role in reducing its occurrence.

In the current landscape of cancer screening, diagnosis, and treatment monitoring, liquid biopsy has emerged as a prominent area of interest (8-10). The utilization of circulating tumor-educated platelets (TEPs) in liquid biopsy has emerged as a valuable tool for early cancer detection and assessing treatment efficacy. TEPs offer distinct advantages over other blood-based resources due to their abundance, ease of isolation, and responsiveness to external signals through RNA processing (11). The significance of TEPs has been evaluated across various cancer types, including non-small cell lung cancer (NSCLC) (12, 13), colorectal cancer, breast cancer(14), liver cancer(15, 16), glioma (17), and gastroenteropancreatic neuroendocrine tumors (18). While previous studies on platelets of CRC patients have predominantly focused on mRNA expression analysis, it is notable that platelets also harbor a diverse array of non-coding RNA molecules, including long noncoding RNAs (lncRNA), circular RNAs (circRNA), microRNAs (miRNA), and mitochondrial DNA, all of which hold potential as valuable biomarkers (19).

Circular RNAs, characterized by their closed-loop structure lacking free 5′ and 3′ ends, exhibit exceptional stability compared to linear RNAs, even in the bloodstream (20).Their prolonged half-life and prevalence in human cells suggest that circRNAs could offer enhanced analytical validity as biomarkers (21-23). Despite the abundance of circRNAs in human platelets, there is a lack of studies exploring their potential as biomarkers in platelet samples from cancer patients. Previous research suggested hsa_circ_0004771(24-26) and hsa_circ_0019120 (27) as potential blood-based tumor biomarkers. In this study, we evaluate the expression levels of these circRNAs originating from platelets of patients with CRC and advanced polyps to explore their capability to distinguish between colorectal cancer, advanced polyps, and individuals without any specific gastrointestinal issues.

Materials and Methods

Sample collection and study population

This study received approval from the medical ethics committee at Baqiyatallah University of Medical Sciences, and all participants provided informed consent for the collection and analysis of their blood samples. The study included 25 patients with colorectal cancer (stage III and IV), 25 individuals with advanced polyps (defined as size ≥10 mm or the presence of more than 3 polyps) (28), and 25 healthy controls who were recruited from Baghiyyatollah Hospital. Blood samples were collected prior to any treatment or surgery. The diagnoses of cancer were confirmed through colonoscopy and pathology, while healthy individuals had negative colonoscopy reports. Participants with no familial history of cancer were included, and blood samples were obtained using EDTA-coated tubes for collection.

Sample Processing

The blood samples underwent centrifugation at 120× g for 20 minutes to separate platelet-rich plasma (PRP) from nucleated blood cells. The PRP was then centrifuged at 360× g for 20 minutes to gather the platelets. These centrifugation processes were conducted at room temperature. The resulting platelet pellets, approximately 250 µl in volume, were combined with 750 µl of YTzol Pure RNA reagent (YTA, Iran), vortexed, and allowed to incubate for 5 minutes at room temperature, before being stored at -80 °C.

RNA Isolation

Total RNA isolation was performed according to the YTzol Pure RNA reagent (YTA, Iran) protocol. Briefly, 200 µl of chloroform was added to lysed Platelets and incubated on ice for 10 minutes. Precipitation and washing were done with isopropanol and 75% ethanol, respectively. RNA pellets resuspended in 30 µl RNAse/DNAse free water. The concentrations

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were checked with a NanoDrop™ One/OneC Microvolume UV-Vis Spectrophotometer.

cDNA Library Construction

Purified RNA was converted to cDNA on the same day. cDNA synthesis was carried out in accordance with the manufacturer's instructions of the SMOBIO kit (Hsinchu, Taiwan). In short, a final volume of 20 µL of cDNA was obtained by reverse transcribing a 1500 ng of total RNA with random primers and then stored at -20 °C until the next use.

RT-qPCR

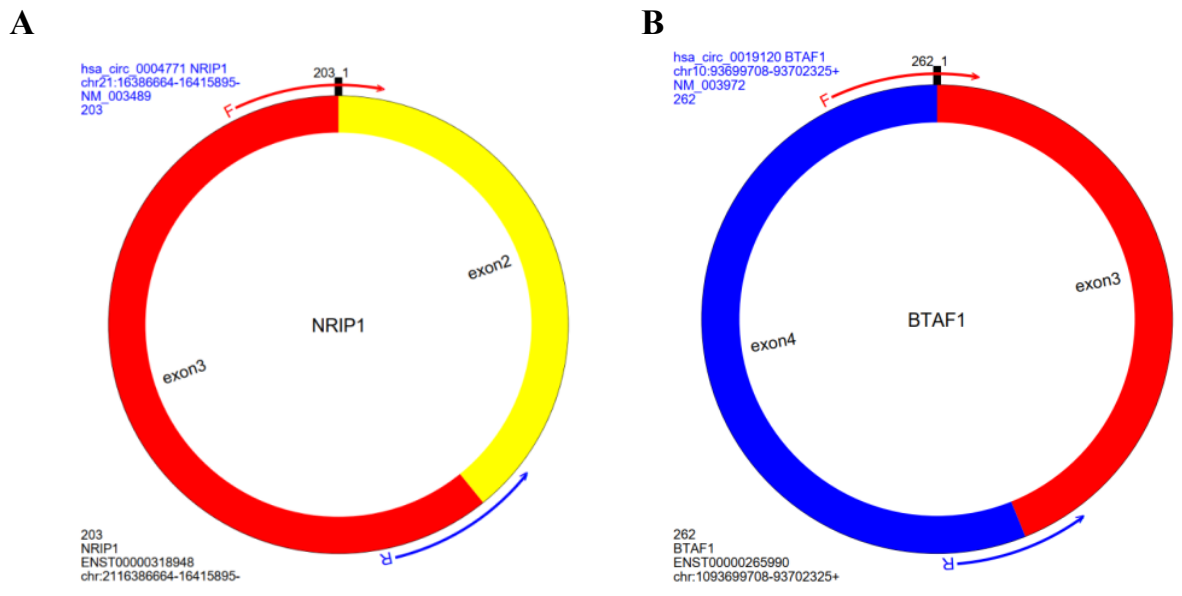
Specific primers for hsa_circ_0004771 and hsa_circ_0019120 were designed using CircPrimer (29) and the software NCBI primer BLAST tool (http://www.ncbi.nlm.nih.gov/tools/primer-blast/). The primer sequences can be found in Table 1 and Figure 1. In platelet samples, ACTB served as the endogenous control, and the relative expression level of hsa_circ_0004771 and hsa_circ_0019120 was calculated using the $2^{-\Delta\Delta Ct}$ formula.

The RT-qPCR was conducted using the Amplicon SYBR Green PCR Kit (Amplicon, Denmark) on the Applied Biosystems StepOne Real-Time PCR System (Thermo Fisher Scientific). The PCR conditions included an initial denaturation at 95 °C for 15 minutes, followed by 40 cycles of denaturation at 95 °C for 10 seconds and annealing/extension at 59 °C for 30 seconds.

Table 1. Primer sequences for hsa_circ_0004771 and hsa_circ_0019120 and ACTB gene.

circRNA		Primer Sequence (5'->3')	Length	Product length
hsa_circ_0004771 (Divergent)	Forward	GCTTGGAGACAGACGGAAGT	20	108
	Reverse	GTCAAGTGTGCATCTTCTGGC	21	
hsa_circ_0019120 (Divergent)	Forward	GTCCAAGATGAAAAATCAGGTGTTG	25	146
	Reverse	CGGAAGTAGGTTCTTGTCTGGTT	23	
ACTB	Forward	CCTCGCCTTTGCCGATCC	18	73
	Reverse	GAGCGCGGCGATATCATCA	19	

Fig. 1. A: Primer design position for hsa_circ_0004771 **B:** Primer design position for hsa_circ_0019120.



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Statistical analysis

The study employed the nonparametric Kruskal-Wallis test followed by Dunn's multiple comparisons test to assess differences among the normal, CRC, and polyp groups. The diagnostic performance of circRNAs was evaluated through receiver operating characteristic (ROC) curves and the calculation of the area under the curve (AUC). The Youden index was used to determine the optimal cutoff value for circRNAs. Statistical significance was defined for p-values less than 0.05. The statistical analyses were conducted using GraphPad Prism 8.0 and SPSS 22.0 software.

Results

Characteristics of the study population

A total of 25 individuals diagnosed with colorectal cancer (stage III and IV), 25 patients with advanced polyps, and 25 healthy individuals who underwent colonoscopy confirmation were included in the study. The groups were matched based on age and sex. The average age of the individuals in the CRC group was 68±10.8 years, 62±9.8 years in the advanced polyp group, and 63±9.2 years in the healthy group. The clinical characteristics of the study samples are detailed in Table 2.

Table 2. Clinical characteristics of patients and controls.

Variables	CRC N (%)	Advance polyp N (%)	Healthy people N (%)	
Sex	25	25	25	P values
Male	9 (36%)	15 (60%)	15 (60%)	0.146
Female	16 (64%)	10 (40%)	10 (40%)	
Age Range (mean ± SD)	41-88 (68±10.8)	33-87 (62±9.8)	37-80 (63±9.2)	0.159
Tumor site				
Descending colon	11 (44%)	-	-	
Ascending colon	8 (32%)	-	-	
Rectum	6 (24%)	-	-	
Stage				
III	14 (56%)	-	-	
IV	11 (44%)	-	-	

Hsa_circ_0004771 and hsa_circ_0019120 expression in the platelets

The analysis of hsa_circ_0004771 in the three groups, including CRC patients, advanced polyp patients, and healthy individuals, revealed upregulation of hsa_circ_0004771 in patients with advanced polyps and CRC. However, the upregulation was statistically significant only in CRC patients compared to healthy individuals (p-value = 0.0036*) (Fig. 2A). The comparison between the healthy and advanced polyp groups (p-value = 0.1191) and the advanced polyp and CRC groups (p-value = 0.7128) showed no significant differences.

Similarly, the expression level of hsa_circ_0019120 also indicated upregulation in patients with advanced polyps and CRC. This upregulation was significant in both patients with advanced polyps (P-value: 0.0175*) and CRC (p-value: 0.0356*) compared to healthy individuals. Subsequently, when comparing the expression levels of platelet-derived hsa_circ_0019120 between advanced polyp patients and CRC patients, no significant difference was observed (p-value > 0.99) (Fig. 2B).

Diagnostic potential of hsa_circ_0004771 and hsa_circ_0019120

A receiver operating characteristic (ROC) curve analysis of hsa_circ_0004771 between normal groups vs CRC groups showed an AUC of 0.784 (95% CI: 0.645 to 0.888) with a sensitivity of 84% and specificity of 64% (p-value = 0.0006*) (Fig. 3A). For

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hsa_circ_0019120 comparison between normal groups and the advanced polyp group, the AUC was 0.739 (95% CI: 0.591 to 0.856) with a sensitivity of 75% and specificity of 74% (p-value = 0.005*) (Fig. 3B). In comparison between normal groups and the CRC group the AUC was 0.717 (95% CI: 0.560 to 0.844) with a sensitivity of 65% and specificity of 74% (p-value = 0.0149*) (Fig. 3C).

To assess the collective efficacy of hsa_circ_0004771 and hsa_circ_0019120 biomarkers, their expression data in CRC groups versus normal groups by logistic regression were analyzed. The combined AUC was calculated to be 0.8348 (95% CI: 0.7131 to 0.9565) with a sensitivity of 84% and specificity of 80% (p-value = 0.0002*) (Fig. 3D).

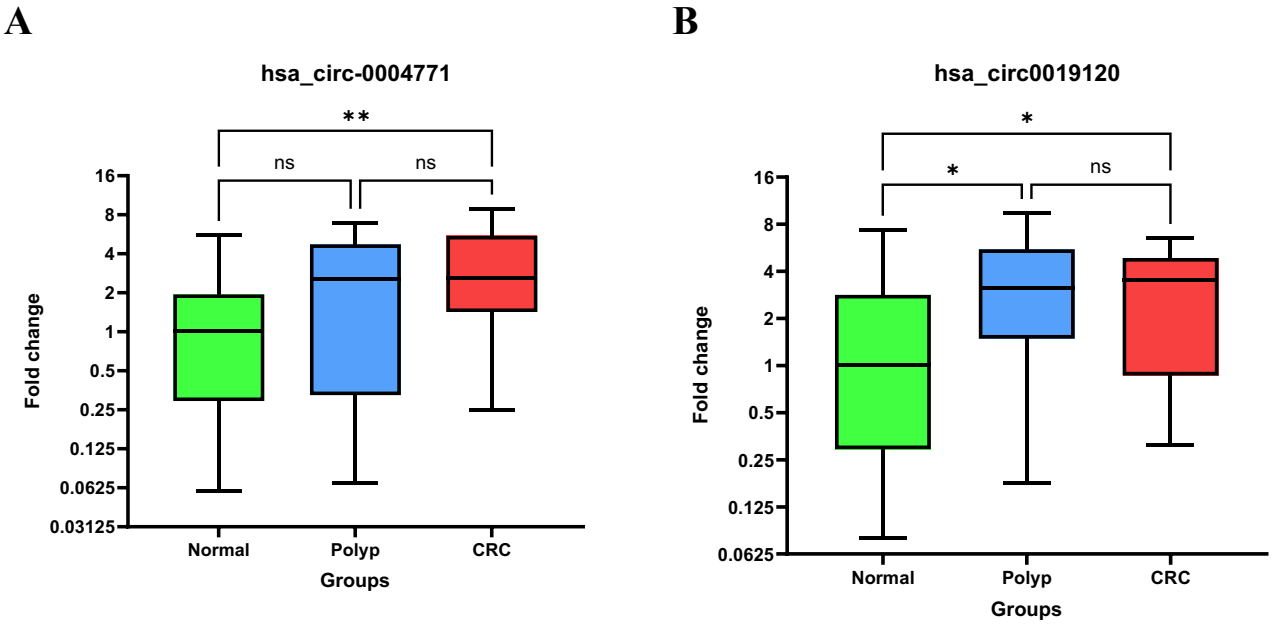
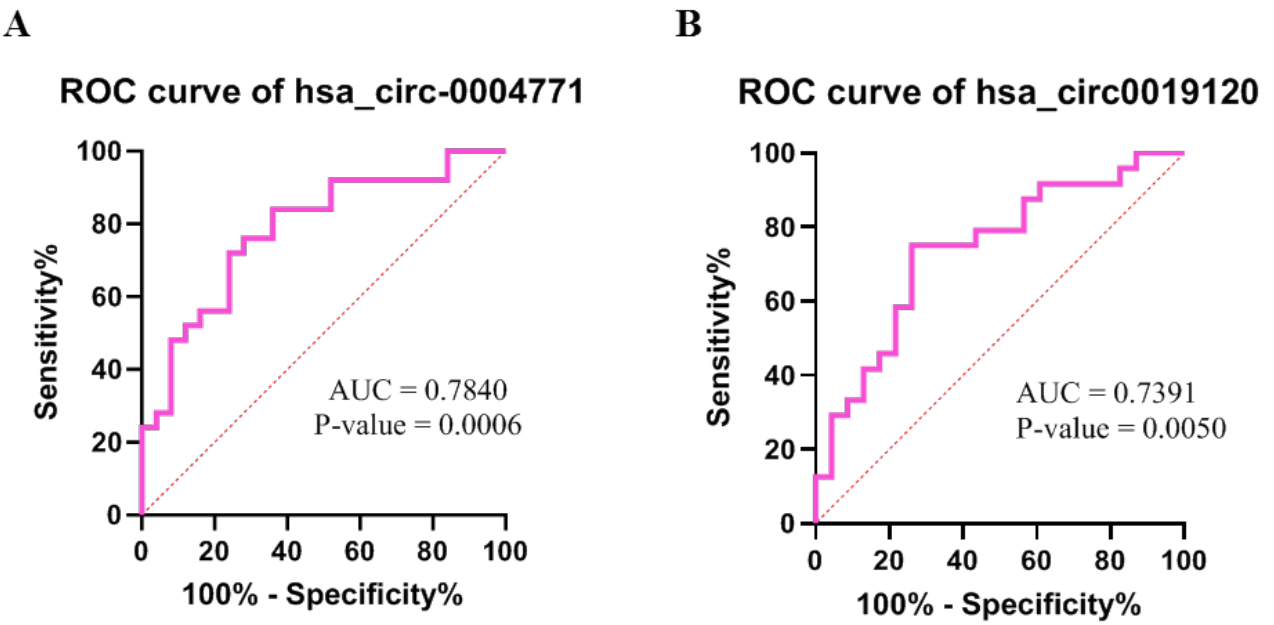


Fig. 2. Expression level of circRNAs in three groups. A: Comparison of the expression of hsa_circ_0004771 in the platelets of the colorectal cancer patient group, the advanced polyp group, and healthy individuals. B: Comparison of the expression of hsa_circ_0019120 in the platelets of the colorectal cancer patient group, the advanced polyp group, and healthy individuals. *: <0.05, **: <0.005.



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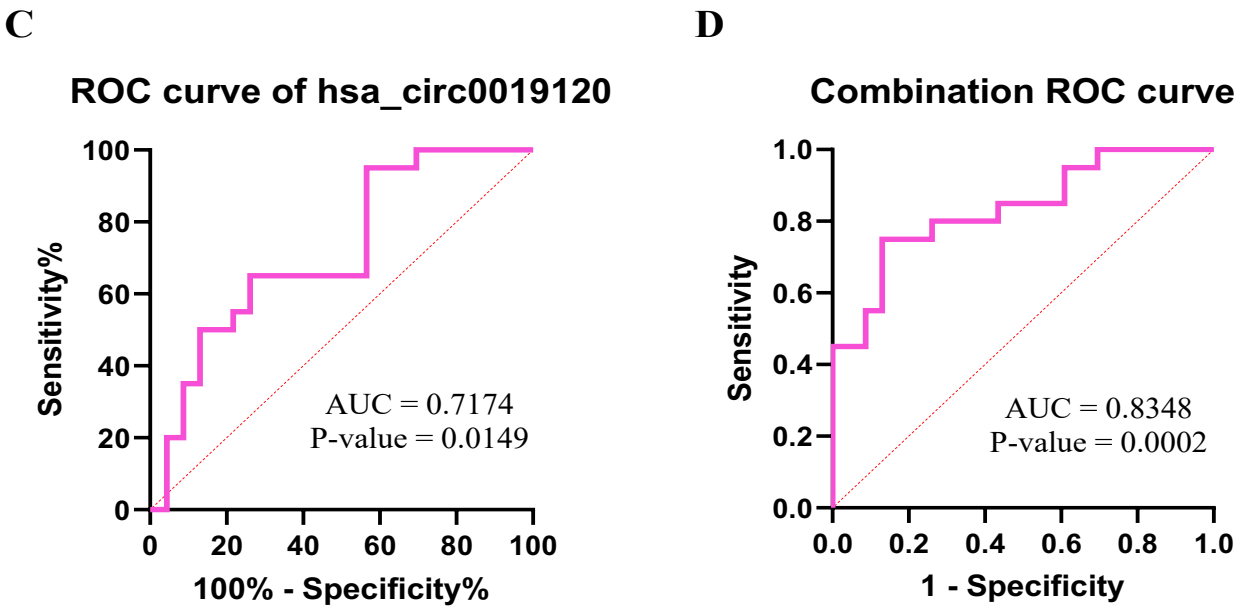

Fig. 3. Diagnostic potential of two circRNAs. A: ROC curve of hsa_circ_0004771 between the colorectal cancer group and the control group. B: ROC curve of hsa_circ_0019120 between the advanced polyp group and the control group. C: ROC curve of hsa_circ_0019120 between the control group and the colorectal cancer group. D: ROC curve related to simultaneous application of hsa_circ_0004771 and hsa_circ_0019120.

Discussion

In this investigation, we assessed the expression levels of two circular RNAs, hsa_circ_0004771 and hsa_circ_0019120, in three groups comprising individuals with colonic polyps, CRC patients, and healthy controls. The analysis unveiled upregulation of hsa_circ_0004771 in the CRC group and hsa_circ_0019120 in both the polyps and CRC groups.

Hsa_circ_0004771, also known as hsa_circNRIP1_007, is located on chromosome 21 (hg19: 16386664–16415895) and is produced from exons 2 and 3 of the NRIP1 (NM_003489) gene. NRIP1, a receptor-interacting protein, acts as a coregulator by modulating various receptor transcription factors. Although its exact role is not fully understood, NRIP1 interacts with transcription factors like E2F transcription factor 1 (E2F1), thyroid hormone receptor (TR), and estrogen-related receptor (EER) (30, 31). The biogenesis of hsa_circ_0004771 is facilitated by the binding of the RBP Quaking (QKI) to the introns surrounding the exons for circularization, predominantly in the cytoplasm (32). Its expression has been studied in three research projects involving CRC patients. Pan et al. demonstrated a notable rise in hsa_circ_0004771 levels in serum exosomes from early CRC patients, indicating its potential as a biomarker for early CRC diagnosis and prognosis (26). They reported AUC values of 0.86 (95%CI, 0.785–0.933) for stage I/II CRC patients and 0.88 (95%CI, 0.815–0.940) for CRC patients. Furthermore, hsa_circ-0004771 in serum-derived exosomes was found to contribute to 5-FU resistance in CRC patients through the regulation of the miR-653/ZEB2 signaling pathway (24). Consistent with prior studies, our research observed increased levels of hsa_circ_0004771 in advanced polyps and CRC patients (Figs. 2 & 3), with statistical significance evident only in CRC patients. The AUC for CRC patients was 0.784 (95% CI: 0.645 to 0.888), highlighting the significant role of hsa_circ_0004771 on malignant tumors and its clinical relevance.

As well as in this study, we investigated the expression of hsa_circ_0019120 in the platelets. hsa_circ_0019120, or hsa_circBTAF1_002, is located on chromosome 10 (hg19: 93699708-93702325) and is generated by exons 3 and 4 of the BTAF1 (NM_003972) gene. This gene codes for a TAF (TATA box-binding protein-

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associated factor) that interacts with TBP (TATA box-binding protein) to create the B-TFIID complex which is necessary for the initiation of gene transcription by RNA polymerase II. (33). There is only one study that showed an elevated level of hsa_circ_0019120 in CRC patients (27). Integrated analysis of exosomal circRNAs in colorectal cancer showed that hsa_circ_0019120 was significantly upregulated in serum exosome of CRC patients (27). Our study showed statistically elevated levels of hsa_circ_0019120 in advanced polyps and CRC patients (Figs. 2 & 3). This finding further indicates the important influence of hsa_circ_0019120 on malignant tumors, as well as its clinical significance.

The heightened presence of these examined circRNAs in the platelets of individuals with colorectal cancer and advanced polyps may result from platelet-tumor cell interactions during tumor progression and metastasis. Therefore, these two circRNAs could serve as potential biomarkers for assessing the risk of developing advanced polyps and colorectal cancer. However, the study is limited by the small sample size, and additional clinical cases are needed to validate the relationship between hsa_circ_0004771 and hsa_circ_0019120 expression with CRC.

This study identified differential expression of two circRNAs, hsa_circ_0004771 and hsa_circ_0019120, in the platelets of individuals with advanced polyps and colorectal cancer. These findings suggest their potential as non-invasive biomarkers for CRC risk assessment and early detection. Additional studies with larger sample sizes are required to confirm these results and investigate the clinical implications of these circRNAs.

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Conflict of interest

There are no competing interests or conflicts of interest present.

Ethics

This study was conducted in accordance with the ethical principles outlined in the Declaration of Helsinki. Prior to the study, ethical approval was obtained from the research ethics committee of Baqiatallah University of Medical Sciences (code of ethics No IR.BMSU.BLC.1402.043).

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