Potential diagnostic value of novel circular RNA hsa_circ_0060927 in human colorectal cancer

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Research

Keywords: Colorectal cancer (CRC), Circular RNA (circRNA), Biomarker, Hsa_circ_0060927, CYP24A1

Posted Date: February 19th, 2020

DOI: <https://doi.org/10.21203/rs.2.23975/v1>

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Abstract

Background CYP24A1 plays a role in strictly regulated vitamin D metabolism pathway and has been nominated as a prognostic biomarker for colorectal cancer (CRC). Increasing evidence suggests that circular RNAs (circRNAs) are involved in cancer initiation and progression; however, their diagnostic values in cancers as the new potential biomarkers are still not fully understood.

Methods and Results In the present study, by using quantitative real-time polymerase chain reaction (qRT-PCR), we demonstrated that CYP24A1 and hsa_circ_0060927 were only transcribed in 33 and 25 out of 83 CRC tissues, respectively. In these samples, we demonstrated that CYP24A1 was up-regulated in both linear and circular forms ($P < 0.05$). Furthermore, we showed that CYP24A1 and hsa_circ_0060927 transcription were induced after vitamin D treatment in HCT-116 and HT-29 colon cancer cell lines. Importantly, analysis of the ROC curve in expressed samples indicated that CYP24A1 and hsa_circ_0060927 could serve as the potential diagnostic markers of CRC, but due to the fact that these genes are not expressed in many samples, the diagnostic role of these genes could be rejected.

Conclusion Taken together, for the first time, the results of our study illustrated the expression levels hsa_circ_0060927 in CRC; however, further studies are required to validate the present results.

Background

According to the global cancer project (GLOBOCAN 2018) estimation, colorectal cancer (CRC) is one of the most common cancers representing third in terms of incidence and second in terms of mortality worldwide. In 2018, GLOBOCAN estimated that over 1.8 million new CRC cases and 881,000 deaths in the world [1]. Although many different altered molecular targets have been reported in CRC, only a few of them are clinically functional, and the exact mechanism underlying the development and progression of CRC remained generally unknown [2–4]. One of the most important altered pathways in CRC is the vitamin D metabolism pathway. Vitamin D decreases the epithelial cell proliferation and promotes differentiation in human colon cells [5]. Cytochrome P450 family 24 subfamily A member 1 (CYP24A1) gene encodes a mitochondrial enzyme that plays a role in vitamin D inactivation and is over-expressed in many cancers, including CRC [6]. A recent study emphasized that CYP24A1 serves as a potential biomarker for the progression and prognosis of CRC [7]; however, there is no evidence concerning the role of CYP24A1 related circular RNAs (circRNAs) in the CRC. circRNAs are an abundant newly accepted class of widely expressed non-coding RNAs in the eukaryotic transcriptome. These molecules are highly conserved and feature tissue and cell-type specificity and relative stability [8, 9]. Therefore, it is valuable to find stable and ideal circRNAs as biomarkers in colorectal cancer. Recent studies have shown that circRNAs are closely associated with several diseases, including CRC and participate in biological processes such as cell proliferation, differentiation, and apoptosis [10, 11]. For instance, Wang J et al. showed that hsa_circ_0000567 expression is down-regulated in CRC tissues and cell lines, therefore, could be served as a diagnostic biomarker. Furthermore, they showed that knockdown of hsa_circ_0000567 promoted CRC cells proliferation and migration in vitro [12]. In the present study, after

assessment of CYP24A1 gene expression, we, for the first time, estimated the expression level of hsa_circ_0060927 in CRC tissues. To determine the expression of CYP24A1 and hsa_circ_0060927 is influenced by vitamin D, we assessed the expression level of these genes in HCT-116 and HT-29 colon cancer cell lines after vitamin D treatment. Consequently, the aims of the present study were the assessment the expression levels of CYP24A1 and hsa_circ_0060927 in colorectal cancer tissues compared to control noncancerous tissues in order to determine that these genes are good diagnostic biomarkers or not. Furthermore, we investigate the correlation between expression levels of CYP24A1 and hsa_circ_0060927 and clinicopathological data of CRC patients.

Methods

Ethics, consent, and permissions

This study was approved by the Ethics Committee of Baqiyatallah University of Medical Sciences (Code: IR.BMSU.REC.1398.217). Written consent was obtained from all patients who were informed that the data would be used for research. CRC was confirmed based on clinical examination, colonoscopy, and histopathology tests on isolated biopsies. We excluded the patients who had undergone chemotherapy, radiotherapy or have other cancers or other diseases that affect the digestive system. Baqiyatallah Research Center for Gastroenterology and Liver Diseases (Baqiyatallah University of Medical Sciences) approved all aspects of this study.

Bioinformatics Analysis

We used circBase on-line database (available at: <http://www.circbase.org/>) to identify the predicted CYP24A1 related circRNAs. The hsa_circ_0060927 had been selected based on pervious studies [8, 13, 14]. Next, we retrieve the sequence of hsa_circ_0060927 from circBase for primer design using CircPrimer software [15]. We used the miRNAs predicted target list reported by Zhong Zh et al. They showed that hsa_circ_0060927 was up-regulated in bladder carcinoma by microarray assay. They also predicted that hsa-miR-224-3p, hsa-miR-29b-1-5p, hsa-miR-522-3p, hsa-miR-661 and hsa-miR-1264 as hsa_circ_0060927 targets [14]. Then we filtered and set the shared value of these miRNAs by TargetScan (http://targetscan.org/vert_71/). Subsequently, DIANA-miRPath (<http://diana.imis.athenainnovation.gr/DianaTools/index.php?r=mirpath/index>) and DAVID (<https://david.ncifcrf.gov/>) functional annotation online tools were used to identify target genes and pathways.

Patients And Tissue Specimens Collection

A total of 83 fresh CRC samples and paired adjacent normal tissues were obtained from the Baqiyatallah Research Center for Gastroenterology and Liver Diseases between January 2016 and January 2017

(Baqiyatallah University of Medical Sciences). Tumor samples were taken from the CRC tissue during surgical procedure. All samples were immediately frozen in liquid nitrogen and stored at -80°C .

Cell Lines And Culture Conditions

The human colon cancer HT-29 and HCT-116 cell lines were purchased from Pasteur Institute (Tehran, Iran). Cell lines were grown in RPMI-1640 medium (Gibco, USA) supplemented with 10% (v/v) fetal bovine serum and 1% penicillin/streptomycin (Sigma, St. Louis, MO). All cells were cultured in a humidified incubator containing 5% CO_2 at 37°C . In brief, HT-29 and HCT-116 cells (1×10^5 /well) were seeded into 6-well plates and treated with $10 \mu\text{M}$ $1, 25\text{-(OH)}_2\text{D}_3$ and harvested at 48 hours post-treatment.

RNA extraction and reverse transcription

Total RNA was directly extracted from the colorectal tissues, normal mucosa, and cell lines using a GeneAll Hybrid-R™ RNA purification kit (Geneall Biotechnology Co. Ltd, Seoul, Korea) as described by the manufacturer's protocol. RNA was visualized on 1% agarose gel to evaluate the RNA integrity. Additionally, the quantity and the quality of the total extracted RNA was estimated using NanoDrop® ND-1000 spectrophotometer (Thermo Fisher Scientific, USA). The RNA purity was evaluated according to the A260/A280 ratio. First-strand cDNA was synthesized from $1 \mu\text{g}$ of extracted total RNA using a Revert Aid First-Strand cDNA Synthesis Kit with random primers according to manufacturer-provided instruction (Thermo Fisher Scientific, USA).

Quantitative real-time PCR (qRT-PCR) condition and gene expression analysis

The qRT-PCR analysis was performed on an ABI StepOnePlus™ Real-Time PCR System (Applied Biosystems, Foster City, CA) using 2.0X RealQ-PCR Master Mix® with SYBR Green (Ampliqon, Odense, Denmark). The primer sets for CYP24A1 and Beta-2-microglobulin ($\beta 2\text{M}$) genes were designed by Allele ID 6 software (Premier Biosoft, Palo Alto, USA) (Table 1). Each reaction consisted of $10 \mu\text{l}$ 2X RealQ-PCR Master Mix®, $1 \mu\text{l}$ cDNA (10 ng), $1 \mu\text{l}$ of each primer (10 pmol) and $7 \mu\text{l}$ of nuclease-free water to conduct PCR in a $20 \mu\text{l}$ of reaction mixture. We carried out the reactions in duplicate and $\beta 2\text{M}$ gene was used as normalization control. This gene was selected according to previous research for identification of housekeeping control genes in colorectal cancer [16]. Two-step thermal cycling and real-time data acquisition were performed using the following conditions: 95°C for $15 \text{ min} \times 1 \text{ cycle}$, and 95°C for 15 s , followed by 60°C for $1 \text{ min} \times 40 \text{ cycles}$ followed by melt curve stage assessment. The melting curve profile and agarose gel electrophoresis were performed to verify the specificity of primers and the authenticity of the PCR products.

Table 1
qRT-PCR primer sequences

Genes	Primers	Sequences	Amplicon size (bp)
CYP24A1	Forward primer	CATTTGGCTCTTTGTTGGATTGTC	145
	Reverse primer	CACCATCTGAGGCGTATTATCG	
hsa_circ_0060927	Forward primer	TAATACGCCTCAGGGAAGG	196
	Reverse primer	GACCATTTGTTTCAGTTCGCT	
Beta-2-microglobulin	Forward primer	TGTCTTTCAGCAAGGACTGGT	143
	Reverse primer	TGCTTACATGTCTCGATCCCAC	

Statistical analysis

The qRT-PCR amplification efficiency was assessed using LinRegPCR software (version: 2017.1) and for each sample, the cycle threshold (Ct) and mean PCR efficiency were determined. We analyzed the experimental data using GraphPad Prism 7.0 (GraphPad Software, La Jolla, CA). All results are expressed as the mean $\pm$ SD of two independent experiments. The correlations between CYP24A1 and hsa_circ_0060927 expression levels and the clinicopathological factors of the CRC patients were assessed using a two-tailed t-test. The receiver operation characteristic (ROC) curve was established to estimate the diagnostic values of CYP24A1 and hsa_circ_0060927 expression levels. $P < 0.05$ was considered statistically significant.

Results

Hsa_circ_0060927 existence in colorectal cancer cells and tissues

We analyzed the hsa_circ_0060927 existence in CRC tissues. In addition, the existence of hsa_circ_0060927 was analyzed in human HT-29 and HCT-116 colon cancer cell lines before and after treatment with the vitamin D. Divergent primers were used to amplify hsa_circ_0060927 (Fig. 1). The Sanger sequencing results were completely consistent with the hsa_circ_0060927 sequence. These results validated the existence of hsa_circ_0060927 in the cells and CRC tissues.

CYP24A1 and hsa_circ_0060927 expression levels in colorectal cancer tissues and cell lines

We did not find any CYP24A1 and hsa_circ_0060927 expression in 60% and 70% of the tissue samples, respectively, but in the rest, CYP24A1 and hsa_circ_0060927 expression analysis results revealed up-

regulation of both transcripts in CRC tissues compared with adjacent tissue (Fig. 2). Similar to previous researches, the CYP24A1 gene expression is generally over-expressed ⁶, but, in our study, it was not expressed in all colon tissues. Surprisingly, in HT-29 and HCT-116 cell lines, CYP24A1 and hsa_circ_0060927 were not expressed before vitamin D treatment; however, both were transcribed after treatment.

Potential diagnostic values of CYP24A1 and hsa_circ_0060927 in colorectal cancer

Since we observed CYP24A1 and hsa_circ_0060927 over-expression in 40% and 30% of tissue samples, respectively, we further analyzed the potential diagnostic value of these molecules in CRC. The potential diagnostic value was evaluated by ROC curve. The larger the area under the ROC curve (AUC), the higher diagnostic value. The area under the ROC curve for CYP24A1 and hsa_circ_0060927 have shown in Fig. 3A and 3B, respectively. Besides, we analyzed the correlation between clinicopathological data and the expression levels of CYP24A1 and hsa_circ_0060927. Our results showed that the hsa_circ_0060927 expression level was correlated with and gender (P = 0.04). However, no association was found between CYP24A1 and hsa_circ_0060927 levels and other clinicopathological features such as clinical age and clinical stages (Tables 2 and 3).

Table 2
The correlation of CYP24A1 expression levels (Δ Ct) and clinicopathological features of the CRC patients.

Factors	No. of patients (%)	Mean $\pm$ SD	P value
Age (years)			
> 60	17 (0.51)	11.82 $\pm$ 4.1	0.90
$\leq$ 60	16 (0.49)	11.65 $\pm$ 3.92	
Gender			
Male	19 (0.58)	12.19 $\pm$ 3.33	0.42
Female	14 (0.42)	11.05 $\pm$ 4.67	
Clinical stage			
I & II	30 (0.91)	9.23 $\pm$ 5.18	0.79
III & IV	3 (0.09)	8.34 $\pm$ 4.31	

Table 3

The correlation of hsa_circ_0060927 expression levels (ΔCt) and clinicopathological features of the CRC patients.

Factors	No. of patients (%)	Mean ± SD	P value
Age (years)			
> 60	13 (0.52)	12.81 ± 4.49	0.94
≤ 60	12 (0.48)	12.93 ± 3.65	
Gender			
Male	12 (0.48)	14.32 ± 3.73	0.04
Female	13 (0.52)	11.68 ± 4.16	
Clinical stage			
I & II	21 (0.84)	10.99 ± 3.47	0.42
III & IV	4 (0.16)	9.11 ± 4.21	

Discussion

Circular RNAs are formed by non-linear reverse splicing, which had been considered as the result of splicing errors [17]. By the development of bioinformatics and statistical methods, lots of circular RNAs were found [18]. Although the mechanisms underlying the function of circRNAs are not fully understood, it has been suggested that circRNAs could act as microRNA sponges, RNA-binding proteins, protein and/or RNA transport in order to regulate transcription [17, 19]. In this study, we investigated the expression level of CYP24A1 and its related cirRNA, hsa_circ_0060927, using 83 fresh CRC samples and adjacent normal tissues by qRT-PCR. The results showed that CYP24A1 and hsa_circ_0060927 expressed in 40% and 30% of the tissue samples, respectively. In these samples, the expression level of CYP24A1 and hsa_circ_0060927 was obviously up-regulated. Because these genes were not expressed in many specimens, the role of these genes as biomarkers can not be accepted. Contrary to our results, some studies introduce the CYP24A1 as the biomarker [7, 30]. The co-expression of CYP24A1 and hsa_circ_0060927 has given us the idea that these genes are under common regulation. So, we investigated the existence of hsa_circ_0060927 in human HT-29 and HCT-116 colon cancer cell lines before and after treatment with the vitamin D. The HCT-116 and HT-29 colon cancer cell lines did not show expression of CYP24A1 and hsa_circ_0060927, but after vitamin D treatment the CYP24A1 and hsa_circ_0060927 were transcribed. According to Lemay J et al. the CYP24A1 gene is the most responsive primary vitamin D target gene [28]. Our results also confirmed the role of vitamin D as a key contributor of the transcription for both hsa_circ_0060927 and CYP24A1. The hsa_circ_0060927 mechanism of function has not been studied, but as mentioned, circRNAs act as microRNA (miRNA) sponges, thus could be part of the competing endogenous RNA network. For instance, circ_001569 acts

as a miRNA sponge to bind and inhibit miR-145 activity in CRC cells and circHIPK3 sponges the miR-7 and promotes colorectal cancer growth and metastasis [10, 20]. A recent study predicted that hsa_circ_0060927 targets the hsa-miR-224-3p, hsa-miR-29b-1-5p, hsa-miR-522-3p, hsa-miR-661 and hsa-miR-1264 [14]. Considering this study, we predicted the miRNAs that target both hsa_circ_0060927 and CYP24A1 by TargetScan to specify the common miRNAs. The results showed mechanically, circ_0060927 acts as a miRNA sponge to directly inhibit hsa-miR-224-3p, hsa-miR-522-3p and hsa-miR-1264 (Fig. 4). These miRNAs have various functions and are dysregulated in some cancers. Previous reports displayed miR-224-3p up-regulation in colon cancer, cervical cancer, and hepatocellular carcinoma [21, 22]. Moreover, it was suggested that this miRNA induces cell proliferation and metastasis in lung cancer cells by inhibition of TNF- α -induced apoptosis [23]. Hsa-miR-1264 targets DNA methyltransferase-1 (DNMT1) and is over-expressed in ovarian cancer cells [24, 25]. miR-522 promotes cell proliferation in CRC and is over-expressed in colon cancer, hepatocellular carcinoma, non-small cell lung cancer, and glioblastoma [26, 27]. These data provide important clues that up-regulation of hsa_circ_0060927 may sponge and suppress the inhibitory role of these miRNAs and hence result in the up-regulation of the miRNA target genes such as CYP24A1 (Fig. 4). We predicted the hsa-miR-224-3p, hsa-miR-522-3p and hsa-miR-1264 targeted genes according to DIANA-miRPath online tool and then the DAVID functional annotation was performed for target genes analysis. The analysis showed that the nominated genes were significantly correlated with transcription regulation. To our best knowledge, this is the first report of the expression of hsa_circ_0060927 in CRC tissues and HCT-116 and HT-29 colon cancer cell lines. Due to some specific features of circRNAs such as highly conserved sequences and a high degree of stability compared to other non-coding RNAs, such as miRNAs and long non-coding RNAs (lncRNAs), these molecules may serve as the diagnostic markers in some diseases such as CRC [20]. Further studies concerning the relationship between circular RNAs and CRC have suggested hsa_circ_0000567 and hsa_circ_0001649 as promising diagnostic biomarkers in human CRC [12, 29]. We didn't find significant correlation between expression of CYP24A1 in colorectal cancer tissue and sex, age, and clinical stages; however, hsa_circ_0060927 up-regulation was correlated with gender ($P < 0.05$) (Tables 2 and 3).

Conclusion

Taken together, the findings of the present study indicated that hsa_circ_0060927 the same as CYP24A1 common transcript had different expression levels between CRC tissue and adjacent normal tissue but could not be served as diagnostic biomarkers for CRC, but future studies with larger sample size are needed to prove our results as a fact. Additionally, functional analysis in colorectal cancer cells and tissues are needed to uncover the exact underlying mechanisms of hsa_circ_0060927.

Declarations

Ethics approval and consent to participate

This study was approved by the Ethics Committee of Baqiyatallah University of Medical Sciences (Code: IR.BMSU.REC.1398.217).

Consent for publication

Written consent was obtained from all patients who were informed that the data would be used for research.

Availability of data and materials

The datasets during and/or analysed during the current study available from the corresponding author on reasonable request.

Competing interests

The authors declare no conflict of interest.

Funding

None.

Authors' contributions

Hossein Sadeghi (Performed experiments, Performed bioinformatic analyses, Analysed data and co-wrote the paper)

Mohammad Heiat (Supervised the research, Performed bioinformatic analyses, Designed experiments and co-wrote the paper).

Acknowledgements

The authors acknowledge the supports from the Baqiyatallah University of Medical Sciences for the current research project (Code: 98000112).

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Figures

Figure 1

Schematic presentation of circular RNA hsa_circ_0060927 biogenesis from back-splicing of pre-mRNA CYP24A1. Hsa_circ_0060927 is generated from exon 3-11 of CYP24A1 gene locus. Black arrows indicate the divergent primers.

Figure 2

The relative expression levels ($-\Delta\text{Ct}$) of CYP24A1 and hsa_circ_0060927 in colorectal cancer tissues and control noncancerous tissues. The figures suggest that CYP24A1 and hsa_circ_0060927 were over-expressed in CRC tissues compared with adjacent normal tissues. $**P < 0.01$, $****P < 0.0001$.

Figure 3

Potential diagnostic values of CYP24A1 and hsa_circ_0060927 in colorectal cancer. The sensitivity and specificity were 0.91 for CYP24A1 (A). The sensitivity and specificity were 0.68 and 0.83 for hsa_circ_0060927, respectively (B).

Figure 4

Hsa_circ_0060927 is frequently up-regulated in CRC and acts as miRNA sponges to directly bind and inhibit hsa-miR-224-3p, hsa-miR-522-3p and hsa-miR-1264, consequently, this mechanism increases the CYP24A1 gene expression, which is targeted by the same miRNAs.