Profiling of microRNAs Targeting Survivin in The Iranian Patients with Colorectal Cancer

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Abstract

Survivin is involved in the apoptosis of cells. Impaired levels of survivin have been associated with increased survival of tumor cells. In this study, we intended to profile the microRNAs (miRNAs) targeting survivin in the tumoral and marginal tissues obtained from Iranian patients with colorectal cancer (CRC). Fifty CRC patients with Iranian Azari ethnicity were recruited. The RNA content of the tumoral and marginal tissues was isolated and the transcript levels of miR-34a, miR-16, miR-150, and miR-203a and survivin were determined through quantitative Real-time PCR. The mRNA expression of survivin was significantly increased (fold change = 3.21, $P = 0.0029$) in the tumoral tissues in comparison to the marginal tissues. There was significant downregulation of miR-16 (fold change = 0.28, $P = 0.003$) and miR-203a (fold change = 0.36, $P = 0.014$) in the tumoral samples in comparison to marginal samples. There was an inverse significant correlation ($\rho = -0.81$; $P < 0.001$) between expression of miR-203a and mRNA expression of survivin in the tumoral tissues from CRC patients. mRNA expression of survivin was higher significantly in the tumoral tissues from CRC patients with lymph node metastasis in comparison to those without lymph node metastasis ($P = 0.020$). In addition, there was significantly higher miR-203 expression level in the tumoral tissues from CRC patients with lymph node metastasis in comparison to those without lymph node metastasis ($P = 0.011$). It is suggested that miR-203 play an oncogenic role in CRC cancer by regulating survivin and lymph node metastasis.

Introduction

Colorectal cancer (CRC) is ranked as the second most usual carcinoma in females and the third in males in the world (1). Several risk factors have been attributed to increase onset of CRC, such as; Older age, Alcohol intake, Obesity, A number of genetic variations and epigenetic modifications and Chronic Inflammatory Diseases of Gut (2, 3). A bulk of evidence has demonstrated that the pathogenic molecular mechanisms of CRC depend on several signaling pathways that modulate tumor survival, proliferation, metastasis, and invasion (4). Additionally, numerous investigations have disclosed that microRNAs (miRNAs) are involved in the modulation of signaling pathways that play a role in the etiopathogenesis of CRC (5).

MiRNAs are a group of non-coding RNAs with approximately 20–24 nucleotides in length that exert their roles through suppressing the transcription of target genes via degrading or repressing the mRNAs. Furthermore, evidence show that miRNAs possess protooncogenic or tumor-suppressor activities during the pathogenic process of carcinogenesis (6, 7).

Survivin is codified by *Baculoviral IAP repeat containing 5 (BIRC5)* gene that belongs to inhibitor of apoptosis gene family (IAP) and plays a critical role in the cell cycle, cell growth, and apoptosis (8). Aberrant expression of survivin has been demonstrated in several malignancies. Several researches have revealed that survivin is uniquely upregulated in approximately all human malignancies, but almost non-expressed in the healthy differentiated cells (9, 10). Survivin has been attributed with abnormal stimulation of a number of signaling pathways that play important roles in the carcinogenesis and cell

survival, including insulin-like growth factor-1 receptor (IGF-1R), epidermal growth factor receptor (EGFR), hypoxia-inducible factor-1 (HIF-1), mitogen-activated protein kinase (MEK)/mitogen-activated protein kinase (MAPK), signal transducer and activator of transcription (STAT), and mammalian target of rapamycin (mTOR) (9, 10). By blocking caspase-9, survivin exerts its anti-apoptotic action (11). Reports show that upregulation of survivin in the tumor cells associates with tumor recurrence, resistance to therapies, and poor clinical outcomes (12, 13).

Therefore, we intended to evaluate the transcript level of miR-34a, miR-16, miR-150, and miR-203a and survivin in the tumoral and marginal tissues from CRC patients in order to search for their potential to be used as biomarkers in the prognosis and diagnosis of CRC.

Materials And Methods

Patients and sampling

In this study, 50 CRC patients referred to surgery in the Imam Reza Hospital of Tabriz University of Medical Sciences, Tabriz, Iran were included. The tumoral and healthy marginal tissues were obtained from each subject during surgery procedure and then immediately were placed into RNAase inhibitor solution (Qiagene, Germany) and stored in -80 till RNA isolation. Informed consent forms were signed by each study subject, and the protocol of the study received approval from the local ethical committee of Tabriz University of Medical Sciences. Patients were diagnosed with CRC by a gastroenterologist using histopathological examinations (Table 1). American Joint Committee on Cancer (AJCC) staging system was used to categorize the stage of CRC in the patients (14). Exclusion criteria were familial history of cancers, co-occurrence of other malignancies, history of radiation therapy and chemotherapy.

Table 1
Baseline data and clinicopathological characteristics
of the CRC patients.

Characteristic	Category	N = 50 n (%)
Age	< 60	22 (44%)
	> 60	28 (56%)
Gender	Female	24 (48%)
	Male	26 (52%)
Lymph node metastasis	Positive	31 (62%)
	Negative	19 (38%)
Tumor histology	Good	25 (50%)
	Moderate	21 (42%)
	Week	4 (8%)
Tumor grade	T2	7 (14%)
	T3	13 (26%)
	T4	30 (60%)
Vessel involvement	Positive	31 (62%)
	Negative	19 (38%)
Stage (AJCC)	II, III	17 (34%)
	IV, V	33 (66%)
Liver metastasis	Positive	8 (16%)
	Negative	42 (84%)
AJCC, American Joint Committee on Cancer		

RNA extraction and cDNA synthesis

RNA was extracted by Tripura isolation reagent (Roche, Germany) according to the manufacturer's protocol. To determine the quality and quantity of the RNA samples, Nanodrop spectrophotometer at 260/280 nm (ND-2000C, Thermo Fisher Scientific, USA) was utilized. The reverse transcriptions of extracted RNA to synthesize complementary DNA (cDNA) were conducted using miRCURY LNA universal RT kit (Exiqon, Vedbaek, Denmark) according to manufacturer's guidelines.

Quantitative Real-time PCR

The expression level of miR-34a, miR-16, miR-150, and miR-203a was measured by miScript SYBR Green PCR Kit (Qiagen, Germany) and StepOne Plus Real-Time PCR (Applied Biosystems, Foster City, CA, USA) using the pre-designed primers (Exiqon, Vedbaek, Denmark). As the housekeeping gene, the U6 expression level was analyzed in order to normalize the expression levels of miRNAs. Each reaction mixture was composed of 8 µl cDNA, 12 µl of SYBR Green Master mix, 1 µl primers each, and RNase free water to the final volume of 25 µl. The thermocycling conditions for the quantitative Real-time PCR were as follows: initial 95°C for 10 minutes, 40 cycles of 95°C for 15 seconds, and 55°C for 30 seconds, and finally 65°C for 60 seconds.

In order to calculate the mRNA expression of survivin, the sequence of the primers was obtained from PrimerBank (<https://pga.mgh.harvard.edu/primerbank/>) as survivin-Forward: 5'-AGGACCACCGCATCTCTACAT-3' and survivin-Reverse: 5'-AAGTCTGGCTCGTTCTCAGTG - 3'. In addition, the transcript level of Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was measured as the housekeeping gene using GAPDH-Forward: 5'-AACTTTGGCATTGTGGAAGG-3', GAPDH-Reverse, 5'-ACACATTGGGGGTAGGAACA-3'. Determination of survivin mRNA amount in the tumoral and marginal tissues from CRC patients was performed exerting RealQ Plus Master Mix (AMPLIQON, Denmark) and StepOne Plus Real-time PCR machine (Applied Biosystems, Foster City, CA, USA). The mixture in each microtube contained 6 µl cDNA, 10 µl SYBR Green Master Mix, 1 µl primers each, and RNase free water to reach a final volume of 25 µl. The thermocycler conditions of the Real-time PCR were as: the holding step of 95°C for 15 minutes, 40 cycles of 95°C for 15 seconds, and 62°C for 30 seconds, and then 65°C for 45 seconds.

The relative transcript levels of miRNAs and survivin were measured upon normalization using the transcript level of the related housekeeping genes in each sample using the comparative Ct method ($2^{-\Delta\Delta CT}$) (15).

Statistical analysis

To perform statistical analysis, the SPSS software v.23 was used. Plotting data by graphs was done using GraphPad Prism software v.8 (GraphPad Software Inc., San Diego, CA, USA). The Wilcoxon signed-rank test or Kruskal–Wallis one-way analysis of variance was used to compare the scale data between groups. To perform correlation analysis between the numeral variables, the Spearman's rho test was used. Data were presented as mean ± Standard error of mean (SEM) or number (and percentages), where applicable. The significant level in the statistical comparisons was determined to be less than < 0.05.

Results

Expression levels of miRNAs and survivin

The mRNA expression of survivin was significantly increased (fold change = 3.21, P = 0.0029; Fig. 1) in the tumoral tissues in comparison to the marginal tissues from CRC patients.

The transcript levels of all miR-34a, miR-16, miR-150, and miR-203a were downregulated in the tumoral tissues compared to marginal tissues from CRC patients. It was detected that there was significant downregulation of miR-16 (fold change = 0.28, $P = 0.003$; Fig. 2A) and miR-203a (fold change = 0.36, $P = 0.014$; Fig. 2B) in the tumoral samples in comparison to marginal samples. Nonetheless, the underexpression of miR-34a (fold change = 0.67, $P = 0.193$; Fig. 2C) and miR-150 (fold change = 0.71, $P = 0.250$; Fig. 2D) was not statistically significant between tumoral and marginal tissues from CRC patients.

Correlation analysis

The correlation analyses were conducted between transcript level of miRNAs and survivin mRNA expression. It was detected there was an inverse significant correlation ($\rho = -0.81$; $P < 0.001$) between expression of miR-203a and mRNA expression of survivin in the tumoral tissues from CRC patients. The correlations between miR-16 ($\rho = -0.21$; $P = 0.197$), miR-34a ($\rho = -0.33$; $P = 0.069$), and miR-150 ($\rho = -0.16$; $P = 0.540$) and mRNA expression of survivin were not statistically significant.

Association analysis

It was detected that mRNA expression of survivin was higher significantly in the tumoral tissues from CRC patients with lymph node metastasis in comparison to those without lymph node metastasis ($P = 0.020$). In addition, there was significantly higher miR-203 expression level ($P = 0.011$) in the tumoral tissues from CRC patients with lymph node metastasis in comparison to those without lymph node metastasis (Table 2).

Table 2
The association of transcript levels of miRNAs and survivin in the tumoral tissues with demographic and clinicopathological data of the CRC patients.

Characteristic	Category	Survivin	miR-16	miR-203	miR-34a	miR-150
Age	< 60	0.415	0.254	0.141	0.307	0.651
	> 60					
Gender	Female	0.511	0.620	0.108	0.241	0.311
	Male					
Lymph node metastasis	Positive	0.020	0.099	0.011	0.297	0.322
	Negative					
Liver metastasis	Positive	0.395	0.211	0.654	0.980	0.541
	Negative					
Tumor histology	Good	0.244	0.881	0.076	0.650	0.407
	Moderate					
	Week					
Tumor grade	T2	0.418	0.065	0.550	0.904	0.640
	T3					
	T4					
Vessel involvement	Positive	0.302	0.144	0.325	0.144	0.604
	Negative					
Stage (AJCC)	II, III					
	IV, V					
AJCC, American Joint Committee on Cancer						
* Bold values show statistically significant differences.						

Discussion

Survivin possesses an action in interrupting apoptosis and modulating cell cycle. A bulk of investigations have disclosed that survivin is specifically overexpressed in about all types of human tumors and its upregulation interferes with therapeutic approaches and causes poor cancer diagnosis and outcomes. The contrasting transcription of survivin in the tumoral cells compared to the normal cells might confer a tool to devise survivin-targeted therapeutics for treatment of different human malignancies. That notwithstanding, the precise molecular mechanisms modulating transcription of survivin in the

cancerous cells remain obscure. However, several reports have illustrated a role of miRNAs in controlling the survivin level in various tumors. As a consequence, it seems to be essential to determine the profile of cancer-related miRNAs and divulge the underlying molecular mechanisms of these miRNAs in specific malignancies. Here in this study, we assessed the transcript level of miR-34a, miR-16, miR-150, and miR-203a and survivin in the tumoral and marginal tissues from CRC patients in order to search for their potential to be used as biomarkers in the prognosis and diagnosis of CRC.

Studies show that miR-16 modulates the p53/survivin signaling pathway, leading to suppression of CRC tumor cell growth and stimulation of apoptosis. Evidence suggests that survivin is particularly transcribed during the G2/M phase of cell cycle. As a result, interrupting the expression of survivin can arrest the cell cycle at the G2/M phase (16). Researches also demonstrated that miR-16 was downregulated in different tumor types, leading to increased levels of survivin and tumor progression (17). Our study also indicated that miR-16 was downmodulated in the tumoral tissues from the CRC patients in comparison to the normal marginal tissues. However, we did not observe a correlation between miR-16 and survivin expression. Additionally, transcript level of miR-16 was not associated with pathological findings in the CRC patients. This may be due to targeting other molecules except than survivin by miR-16. It was seen that miR-16 was able to target the mRNA transcribed from *methyltransferase like 13 (METTL13)* gene, encoding for FEAT (faint expression in normal tissues, aberrant overexpression in tumors) protein, in lung cancer, breast cancer, and hepatocellular cancer tissues. miR-16 was able to control apoptosis in tumor cells by regulating the levels of FEAT protein (18).

MiR-203 is considered as one of the frequently investigated survivin-targeting miRNAs in the context of cancers. Studies show that miR-203 directly targets survivin mRNA and is involved in the metastasis and progression of prostate cancer. According to a study by Saini *et al*, it was observed that there was an upregulation of miR-203 in the prostate cancer cell lines obtained from bone metastasis as well as metastatic bone tissues (19). *In vitro* experiments revealed that upregulation of miR-203 in prostate cancer cell lines interrupted the epithelial-mesenchymal transition (EMT) development as well as the invasive and metastatic traits of the cells. Furthermore, miR-203 overexpression triggered the arrest of cell cycle at G1 phase and suppressed apoptosis of cells. By suppressing the transcription of survivin, miR-203 indicated anti-metastatic characteristics. Furthermore, miR-203 modulated several genes involved in the metastasis of cells, such as Smad4, ZEB2, and Bmi1 (19). Our experiments also indicated downregulated levels of miR-203 in the tumoral tissues compared to marginal tissues from CRC patients. An inverse correlation was detected between miR-203 and survivin expression, implying to the matter that miR-203 targets survivin in the CRC tumor cells. Additionally, the tumoral tissue expression of miR-203 was significantly lower in patients with lymph node metastasis. However, in patients with lymph node metastasis, there was a significant upregulation of survivin in the tumoral tissues. All these indicate that miR-203, by regulating survivin, is involved in the metastasis of tumor cells in the CRC patients. It seems that due to anti-metastatic properties of miR-203, it might be a promising miRNA in developing therapeutic tools in CRC patients.

Conclusions

In conclusion, we demonstrated that the mRNA expression of survivin was significantly increased in the tumoral tissues in comparison to the marginal tissues. There was significant downregulation of miR-16 and miR-203a in the tumoral samples in comparison to marginal samples. There was an inverse significant correlation between expression of miR-203a and mRNA expression of survivin in the tumoral tissues from CRC patients. mRNA expression of survivin was higher significantly in the tumoral tissues from CRC patients with lymph node metastasis in comparison to those without lymph node metastasis. In addition, there was significantly higher miR-203 expression level in the tumoral tissues from CRC patients with lymph node metastasis in comparison to those without lymph node metastasis. It is suggested that miR-203 play an oncogenic role in CRC cancer by regulating survivin and lymph node metastasis.

Declarations

Funding

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Conflict of interest

The authors declare that they have no conflicts of interest pertaining to this work

Availability of data and material

Data and materials used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

All authors contributed to the study conception and design. Material preparation, data collection, and analysis were performed by HCh, SB, MA, RD, DSh and MR. All authors contributed to interpretation of data. The first draft of the manuscript was written by HCh, and all authors commented on the drafts of the manuscript. All authors read and approved the final manuscript.

Ethics approval

This study was confirmed in the Ethics Committee of Tabriz University of Medical Sciences as a research project (Permit number: IR.TBZMED.REC.1397.441).

Consent to participate

All patients were signed consent forms, and all personal information are confidential.

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Figures

Figure 1

Bar graphs show the mRNA expression level of survivin in the tumoral and marginal tissues from CRC patients. This study included 50 CRC patients with Iranian Azari ethnicity. The RNA content of the samples was isolated and the mRNA expression level of survivin was measured using quantitative Real-time PCR (FC, fold change).

Figure 2

Bar graphs show the transcript level of the miR-16 (A), miR-203a (B), miR-34a (C), and miR-150 (D) in the tumoral and marginal tissues from CRC patients. This study included 50 CRC patients with Iranian Azari ethnicity. The RNA content of the samples was also isolated and the transcript levels of miRNAs were measured using quantitative Real-time PCR (FC, fold change).