Importance of mir-411-5p in colorectal cancer

Alavieh Fateh, Mohammad Ali Hosseinpour Feizi, Reza Safaralizadeh, Shirin Azarbarzin

Department of Animal Biology, Faculty of Natural Science, University of Tabriz, Iran

Abstract

The abnormal expression of microRNAs (miRNAs) plays a key role in colorectal cancer (CRC). The present study attempted to identify the potential miRNA biomarkers of CRC due to the important role of microRNAs within the DLK1-DIO3 genomic region, especially the role of mir-411-5p in other cancers. This prospective study characterized the contribution of mir-411-5p to CRC tumorigenesis. The Real-time quantitative reverse-transcription–polymerase chain reaction was used to examine miR-411-5p expression levels prospectively in 40 pairs of CRC tissues and adjacent noncancerous tissues (>2 cm from cancer tissue). Also, the relationship between mir-411-5p expression levels and clinicopathological characteristics was explored. The capability of mir-411-5p to function as a tumor marker in CRC was also examined. MiR-411-5p was significantly downregulated in a group of CRC samples compared with matched noncancerous tissues. A receiver operating characteristic (ROC) curve also showed ROC area of 68% for miR-411-5p (P value=0.006) with 70% and 65% sensitivity and specificity, respectively. According to the survey results, miR-411-5p might be considered as a tumor marker in CRC and it might be a promising therapeutic option which may help prevent CRC.

Introduction

Each year, 1.23 million individuals are affected by colorectal cancer (CRC). It should be noted that this type of cancer is placed as the third most common cancer type among males and the second among females. Moreover, it is the fourth most predominant cause of cancer death, universally. In northern America and Europe, the frequency of CRC has been assessed to be 30-50 cases for every 100,000 people. In Middle East, this rate is estimated to be 3 to 7 cases for every 100,000. Predictably, this cancer type acts as one of the most fatal disorders among western populations. Prior research findings indicated that the frequency of CRC is assumed to rise each year in developing countries, especially in Asian ones throughout the next two decades. The growing number of CRC diagnosis in the previous three decades has proven CRC to be an important public health problem in Iran. Furthermore, a high frequency of cancer in gastrointestinal tract has been reported in East Azerbaijan (located in North West of Iran). Consequently, there is a possibility to decrease the number of CRC affliction by accumulating knowledge about the biology and nature of CRC; therefore, designing effective prognostic, diagnostic, and treatment plans.

MicroRNAs (miRNAs) are defined as tiny, non-coding, single-stranded RNAs with the approximate length of 18-25; they are nucleotides that are expressed endogenously. Moreover, through binding to 3' untranslated region (3' UTR) of target mRNAs, MicroRNAs can post-transcriptionally control gene expression. The importance of MicroRNAs in regulating many biological processes (e.g., cell cycle, proliferation, differentiation, apoptosis, and invasiveness) cannot be underestimated. The results of previous studies demonstrate that miRNAs can suppress tumors and oncogenes. MiRNAs have the status of attractive molecules in cancer development diagnostics and therapeutics. Therefore, some of miRNAs identified in this pathway may be used as diagnostic and prognostic markers or therapeutic targets.

MiR-411-5p is located in the imprinted Dlk1-Dio3 region on chromosome 14q32.31. A large miRNA of this cluster is situated within a parentally imprinted chromosome. Various species highly conserve the Protein-coding genes within the Dlk1-Dio3 region, but only mammals conserve the miRNA cluster. The expression of this cluster has been observed in human cancers including melanoma, ependymoma, neuroblastoma, osteosarcoma, gastrointestinal stromal tumors, hepatocellular carcinoma, uterine osteosarcoma, and ovarian cancer. After all, its expression arrangement, clinical relevance, and functional role in CRC still remain unknown. Consequently, the researchers in this study tried to examine the miR-411-5p expression levels in CRC tissues. A study in this field demonstrated the over expres-
sion of miR-411 in the lung cancer cells. In a study on breast cancer, it was revealed that there were significant differences regarding miR-411 expression in metastatic breast cancer patients compared to control group; as a matter of fact, it’s down regulation was observed in metastatic breast cancer.

Materials and methods

In our study at Imam Reza Hospital (Tabriz, Iran), after colonoscopy and sigmoidoscopy in this hospital, all CRC samples and normal adjacent tissues were collected from 40 patients diagnosed with CRC. Imam Reza hospital is considered as the first affiliated hospital of Tabriz University of Medical Sciences. All samples studied were collected during a period of time between November 2014 and June 2015. From a piece of resected specimen at the furthest distance from tumor (>2 cm from tumor), the researchers obtained the non-tumor tissue. All study participants were born in East Azerbaijan, Iran. The Research ethics Committee of Imam Reza Hospital accepted this research in line with institutional protocol and all patients signed the informed consents. Consistently, the researchers processed all specimens resected for histopathological assessment.

Sample preparation and RNA isolation

It should be noted that regarding the sample preparation and RNA isolation till RNA Extraction, the researchers instantly flash froze all the tissue samples in liquid nitrogen and stored at –80°C. TRIzol reagent (Invitrogen Carlsbad, CA) was used to execute the Phenol based RNA extraction. To sum it up, 1 ml TRIzol LS reagent was added into homogenized tissue sample, then the mixture was pipetted up and down many times; finally, it was incubated at room temperature for 5 minutes. The next step was adding 200 µL chloroform and strongly shaking it for 15 minutes; then, the sample was incubated at room temperature for 2-15 minutes. For another 15 minutes the sample was centrifuged at 12,000 rpm at 4°C. The next step was transmitting the aqueous phase into a new Eppendorf tube and adding 500 µL of 100% isopropanol. After that, during the night, the mixture was centrifuged for 10 minutes at 13,000 rpm at 4°C with the aim of pelleting the nucleic acid. Then, after removing the supernatant, the pellet containing RNA was washed adding 1 ml of 75% ethanol. The next step was pipetting the sample at 7500 rpm for 5 minutes at 4°C. After adding 25 µL RNase free water to the RNA pellet, the mixture was pipetted up and down for many times. Finally, for 10 to 15 minutes, the mixture was incubated in a water bath at 55-60°C. The picodrop 2000 (Bob Battie International [BB], UK) was utilized to quantify the amount of isolated RNA concentration. Until cDNA synthesis, the extracted RNAs were stored at –80°C. Here, a 10 µL DNase I treatment reaction was performed in order to degrade any DNA contamination in extracted RNAs (Fermentas, Canada).

Reverse transcription and quantitative Real-time PCR

In a final volume of 10 µL reaction systems, both the Reverse transcription and quantitative Real-time PCR Reverse transcription were executed on 120 ng of total RNA. The 10 µL RT reaction mixture was incubated at 37°C for 60 minutes, 85°C for 5 seconds, and then held at 4°C using the Prime Script(R) miRNA cDNA Synthesis Kit (ParsGenome, Iran) according to the instructions given by the manufacturers. Moreover, in order to dilute the RT product, the researchers added 90 µL of the RNase free water.

After that, SYBR® Green was used to carry out the Real-time PCR and 4 µL diluted RT product was added into a 10 µL PCR reaction, which also contained 10 µL SYBR Green, 1 µL primer mix (purchased from ParsGenome, Iran), and 1 µL RNase-free water. MiR-411-5p and 5s rRNA (5srRNA was selected as a housekeeping gene used for normalization and data analysis) primers were also purchased from ParsGenome, Iran. In spite of using Rotor-Gene Q - QIAGEN Real-time PCR Detection System, the researchers administer all PCR reactions, including non-template controls, in triplicate. Finally, using the REST2009 Software, the raw data were examined. It should be mentioned that the researchers processed all samples in triplicate. According to the definition given in the literature, the threshold cycle (CT) is the cycle number at which the fluorescence passes the fixed threshold. Each experiment included a control without a template. Polyacrylamide gel electrophoresis (PAGE) validated the final products of real-time PCR.

Normalization and statistical analysis

The relative expression analysis of miR-411-5p was performed by a randomization test using the Relative Expression Software Tool (REST) 2009 (http://gene-quantification.com/rest-2009.html). $2^{-\Delta\Delta CT}$ method was employed to analyze the expression levels of miR-411-5p in CRC tissues relative to their matched non-tumor counterparts. The threshold cycle (CT) of fluorescence for each sample was determined. ΔCT indicated the difference in expression levels with the Ct value between miR-411-5p and 5s rRNA (ΔCT=Ct miR-411-5p - Ct 5s). ΔACT indicated the difference in the ΔCT value between the cancer tissue and the matched control (ΔACT=ΔCT cancer-ΔCT control). The $2^{-\Delta\Delta CT}$ value (fold value) was also calculated. It was found that when the fold value was <1, there was a low expression of miR-411-5p in the cancer tissues compared to their non-tumorous counterparts. Here, decreased expression was defined as the fold change less than one in expression. SPSS 18.0 software was utilized to carry out all the analyses (Chicago, IL, USA). All P-values cited contained two sides. It should also, be noted that P-values<0.05 were estimated to be statistically significant. Receiver operating characteristic (ROC) curve was also constructed to evaluate the specificity and sensitivity of predicting CRCs and normal tissues by miR-411-5p expression levels. Moreover, the sensitivity/specificity at various cut off values was calculated using Sigma Plot 12.5. A statistically significant difference was indicated by P values<0.05.

Expression levels of miR-411-5p in CRC and normal tissue

All the samples’ Ct values were entered in the REST 2009 software with the aim of comparing the miR-411-5p expression’s total level in CRC connected to normal tissues. The results of the randomization test showed that miR-411-5p expression in tumor samples decreased 6.5 times more than in normal tissues (Figure 1).

Clinicopathological characteristics and their association with miR-411-5p expression

Considering clinicopathological characteristics, no significant relationship was reported between the miR-411-5p expression and gender (P=0.703), age (P=0.408), histological grade (P =0.053), and tumor location (P=0.375), (Table 1).

Capability of miR-411-5p to function as a CRC tumor marker

Receiver operating characteristic (ROC) curve was constructed and the area under the curve (AROC) was calculated to evaluate
the specificity and sensitivity of predicting CRCs and normal tissues by miR-411-5p expression levels. Based on the analysis of ROC curves, miR-411-5p showed a ROC area (AROC) of 68%. The expression of miR-411-5p with a value of 0.68, compared with 1.0, conveyed that this microRNA is almost highly sensitive and specific; therefore it has the capability to distinguish tumor samples in CRC; subsequently, it can be viewed as a tumor marker (Figure 2).

Discussion

In physiological and pathological processes, MiRNAs are recognized as essential post-transcriptional gene expression regulators. Their expression is deregulated in many diseases including cancer.\textsuperscript{12} MiRNAs can be used as biomarkers for the diagnosis and prognosis of several malignancies and can recognize cancer subclasses of different clinical management.\textsuperscript{13} The DLK-DIO3 region was selected because this region hosts 53 miRNAs and the findings reported here indicate that these miRNAs are involved in a wide spectrum of human diseases, especially cancers. They may modulate important signaling pathways like MAPK (mitogen-activated protein kinase) and p53; besides, they are related to cytokine

Figure 1. Differential expression of miR-411-5p. miR-411-5p expression in tumor samples showed a significant decrease (6.5 times) compared to normal samples with confidence interval of 95% (CI=95%), P Value=0.006, and P<0.05.

Figure 2. Receiver Operating Characteristic (ROC) for biomarker in detection of CRCs. The ROC curve was automatically generated from 40 points of cutoff values set by the software SigmaPlot 12.5. The area under the ROC curve (AROC) is 0.68 out of 1, P Value=0.006 (P<0.05), with 70% and 65% sensitivity and specificity respectively.

Table 1. Relationships between miR-411-5p expression levels in cancer tissue samples from patients with CRC and clinicopathological characteristics.

<table>
<thead>
<tr>
<th>Variable</th>
<th>N°</th>
<th>miR-411-5p relevant expression ($2^{-\Delta\Delta Ct}$)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gender</td>
<td></td>
<td></td>
<td>0.70</td>
</tr>
<tr>
<td>Male</td>
<td>18</td>
<td>20.31±4.26</td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>22</td>
<td>18.91±3.91</td>
<td></td>
</tr>
<tr>
<td>Age</td>
<td></td>
<td></td>
<td>0.05</td>
</tr>
<tr>
<td>&lt;60</td>
<td>17</td>
<td>19.51±4.05</td>
<td></td>
</tr>
<tr>
<td>≥60</td>
<td>23</td>
<td>19.56±4.19</td>
<td></td>
</tr>
<tr>
<td>Histological grade</td>
<td></td>
<td></td>
<td>0.38</td>
</tr>
<tr>
<td>Well differentiated</td>
<td>19</td>
<td>19.36±3.87</td>
<td></td>
</tr>
<tr>
<td>Moderate differentiated</td>
<td>17</td>
<td>20.06±4.13</td>
<td></td>
</tr>
<tr>
<td>Poorly differentiated</td>
<td>4</td>
<td>18.18±5.60</td>
<td></td>
</tr>
<tr>
<td>Tumor location</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Colon</td>
<td>16</td>
<td>18.66±4.11</td>
<td></td>
</tr>
<tr>
<td>Sigmoid</td>
<td>22</td>
<td>20.51±4.28</td>
<td></td>
</tr>
<tr>
<td>Rectum</td>
<td>12</td>
<td>19.74±3.93</td>
<td></td>
</tr>
</tbody>
</table>

Data presented as mean±SD; P values obtained using ANOVA test. No significant relationship was found between miR-411-5p expression levels and clinicopathological characteristics.
signaling cascades, DNA methylation, oncogenes, and many others. Mir-411-5p is known as a member of the miR-379 family. This family is known to be placed in the miR-379/miR-656 cluster inside the DLK-DIO3 region on human chromosome. Placental mammals conserved the miR-379/miR-656 cluster to a great extent. Based on prior research studies, the vital role of miR-411-5p in different biological processes in various human cancer cells has been proven. Mir-411 was reported to be upregulated in focioscapulohumeral muscular (FSHD) dystrophy and to suppress myogenic factors.

In Another study, it was revealed that mir-411-5p possesses an inverse correlation among TGF-β1/SPRY4 and levels of miR-411-5p. Moreover, it was indicated that Re-expression of miR-411-5p can prevent in vitro rhabdomyosarcoma (RMS) cell proliferation as well as in vivo tumorigenicity. Here, the researchers accentuated an anti-oncogene role for mir-411-5p. Mir-411 cluster represents another set of transforming growth factor-beta1 (TGF-β1)-suppressed miRNAs in RMS, and miR-411-5p expression was negatively regulated by TGF-β1 in RMS. Studies also investigated the effects of TGF-β1 on human CRC. For instance, it has been revealed that TGF-β1 plays an important role in CRC, and TGF-β1 expression might be a complementary mechanism in the onset of CRC; that is to say, it can have a major effect on the prognoses of patients. TGF-β1 acts both as an inhibitor of tumor growth and as a promoter of tumor progression. Furthermore, a negative regulatory effect for Sprouty homolog 4 (SPRY4) (i.e., a direct target of miR-411-5p) has been reported on: i) Activation of protein kinase C (PKC) generated by PKCα-mediated Mitogen; ii) Activated protein kinases (MAPKs); iii) Vascular endothelial growth factor-A26 activation propel to growth arrest of RMS. In CRC, the link between MAPK pathway signaling and cell adhesion, angiogenesis, invasion, and metastasis is entrenched. SPRY proteins have major roles in regulating tubular morphogenesis, such as angiogenesis, as well as in lung, placenta, and kidney development. The reason why SPRY proteins are regarded as tumor suppressors is their ability to be repressed in some malignancies. Another study on colon cancer showed that epigenetic silencing and loss-of-function mutations of SPRY4 could lead to tumorigenesis. The documents at hand indicate that in RMS there is an autoregulatory loop among TGF-β1/miR-411-5p/SPRY4 and MAPK (especially p38MAPK) pathway.

Further, it was shown that in hepatocellular carcinoma (HCC) cells, the expression of Mir-411 is upregulated. The findings of the previous body of research demonstrated that ectopic expression of miR-411 leads to downregulation of ITCH, which results in cyclin D1 and c-Myc upregulation. This should not be forgotten that this upregulation has a vital role in carcinogenesis of human cancer which results in an increase in the proliferation of HCC cells. Overexpression of miR-411 in lung cancer regulates G1/S transition with cell cycle regulators such as cell cycle inhibitors p21 and p27; it also upregulates cell cycle regulator cyclin D1. Nevertheless, the role of miR-411 in cell cycle in CRC is still unknown.

Various studies in this field proved that another target for miR-411 was fork head box O1 (FoxO1), which has a regulating role for angiogenesis, apoptosis, cell invasion and metastasis, cell metabolism, oxidative stress, immune regulation, and self-renewal and stem cells discrimination. In addition, the findings revealed that FoxO1’s expression can be prevented by the over expression of miR-411 in lung cancer. Expression of FoxO1 correlated with autophagic capacity and tumor development in human colon cancer cells.