MiR-520c and MiR-519d Function as Oncogenes in Esophageal Cancer

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ABSTRACT

Objectives: Esophageal cancer is a poorly characterized deadly cancer with a malignancy ratio of 2/3 among Iranian males and females, respectively. Recent studies on miR-520c and miR-519d have evidenced their either oncogenic or tumor suppressing roles in different cancers. The goal of this study was to assay the altered expressions of miR-520c and miR-519d in esophageal tumor tissues and adjacent non-tumor tissues in patients suffering from esophageal cancer.

Methods: RNA was extracted from 36-pair paraffin-embedded tumor and adjacent non-tumor tissues using TRIZOL kit and relative expressions of miR-520c and miR-519d were quantified by Real Time and RT-PCR using specific predesigned primers. CT method ($2^{-\Delta\Delta CT}$) and SPSS16 were used for statistical calculations.

Results: Statistical analysis has revealed spectacular upward expression increases of 10.4 and 24.5 folds of miR-520c and miR-519d adjacent in Esophageal Squamous Cells compared to their non-tumor tissues ($P < 0.05$).

Conclusion: In general, the present study being the first report on the evaluation of miR-520c and miR-519d expressions in ESCC, is able to highlight the oncogenic roles of the two miRNA in ESCC and introduce them as appropriate potential alternatives to be utilized for further research on clinical treatment.

Keywords: Oncogene, MiR-520c, MiR-519d, Esophageal cancer

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INTRODUCTION

Cancer has remained one of the most common leading causes of death worldwide. As estimated, there will have been an increase of 45% in various types of cancer in developing countries by 2030 (1). Esophageal cancer (EC) is the sixth leading fatal cancer around the world (2). Squamous cell carcinoma (ESCC) and Adenocarcinoma (ADC) are two distinctive histological subtypes of EC (3). In this respect, Golestan Province, in Iran, is one of the regions with highly prevalent incidences of EC with over 90% of ESCC, worldover (1).

Despite surgery and advances in the treatment of EC, the prognosis and survival rates for both types of this cancer are still dismal(4). Otherwise stated, Proportional to the time of cancer detection, life expectancy for the next five years will be less than 15% in patients with advanced tumor compared to 90% for those diagnosed at the first stage and underwent surgery(3,5). Therefore, as conventional diagnostic strategies are not able to detect ESCC in the earliest stages, there is an urgent request for novel biomarkers which are less invasive and more powerful, and are capable of detecting ESCC at early stages (3,4).

Recent innovations in miRNAs profiling technology have supported its great potentials in cancer diagnosis and treatment monitoring (6,7). Small non-coding RNA molecules known as miRNA are single-stranded, highly conserved RNAs with approximate nucleotide length of 22, which are able to modulate the stability, translation efficiency and regulation of target gene expression through binding to the 3'un-translated regions (UTR) of their messenger RNAs (8,9). Due to perfect or imperfect pairing of miRNA with target site in the massager RNA, these miRNAs will be able to regulate hundreds of individual mRNAs (10–12).

Although the gene networks and molecular mechanisms orchestrated by miR-519d and miR-520c are not completely recognized, expressive alterations of these two miRNA have been

characterized in several cancers (13,14). According to these studies, in hepatocellular carcinoma and prostate cancer, miR-519d performs its roles by targeting mki67 and p21, both of which are crucially important in cell cycle, tumorigenesis, and development and survival rate of EC (2,8,9,15–18). Expressive alterations of miR-520c have been reported in different cancers including thyroid adenoma, liver, breast and fibroblast cancer, as well. So far, none of these two miRNAs has been investigated in EC (5,6,14,19–24). The goal of this study was to assay the expressive alterations of miR-519d and miR-520c in esophageal tumor and adjacent non-tumor tissues, in order to discover putative novel biomarkers for detecting, screening and surveillance of EC.

MATERIALS AND METHODS

Tissue collection and processing

Thirty-six pairs of formalin-fixed paraffin-embedded (FFPE) esophagus tissues, consisting human ESCC and normal adjacent tissues from the same patients were obtained from the archives of Jorjani and Shariati Hospitals (Iran). Moreover, the patients' age and gender information were gathered from those Hospitals. H-E stained slides were reviewed by an experienced pathologist to identify the tumoral ESCC regions as well as its corresponding adjacent normal specimens for the cores to be carefully cut from FFPE blocks and placed in RNase free 2.0 ml micro tubes for RNA isolation. This study was approved by the Golestan University of Medical Sciences Ethical Board.

RNA isolation from FFPE specimens

RNA was extracted from 72 samples by tissue weight of about 20 mgr. Initially, samples were trimmed of exceeding wax and deparaffined by three repeated rinses of 1ml 100% xylen for 10-

second vortex, followed by 3-minute centrifugation at 13000 xg in 22°C. The same steps were repeated three times for 100% ethanol. The pellet was also allowed to air dry in room temperature.

The samples were sub-merged in 200 µl 1x protease K digestion buffer containing 10µ of 20mg/ml protease K solution (Fermentase, Lithuania) and 190 µl PK buffer (1 mM EDTA, 1Mm NaCl, 5 Mm Tris-Hcl, PH=8), followed by the incubation of 4 hr at 56°C. Trizol solution (Invitrogen, USA) was then added to each sample tube, and purification was performed according to the manufacturing company's instructions. The quantified analysis of resulted RNA was performed by Pico drop spectrophotometer and OD 260/280 nm ratio.

Reverse Transcription and Quantitative Real-Time PCR

Using PARSGENOME miR-AMP kit (Iran) 1-2 ng of total RNA was reversely transcribed and amplified according to the manufacturer's protocol. Specific poly-A primers designed by PARSGENOME miR-AMP company were also used for the detection of miRNAs by quantitative Real-Time PCR using SYBR Green. The method is based on three following steps. Firstly, the length of miRNAs was extended with Poly-A enzyme. Secondly, the first-strand cDNA was synthesized with specific designed primers and, finally, it was amplified with ABI-7300 Real-Time PCR system (Applied Biosystem, USA).

The evaluations of miR-519d and miR-520c, target genes, expressions and miR-16 as endogenous were performed using SYBER Green. In this method, to perform Real-Time quantitative PCR, thermal cycling comprised with an initial incubation of 95°C for 3 min, which was followed by 40 cycles of denaturation at 95°C for 5 seconds. It was preceded by annealing at 63°C for 15 seconds and proliferation at 72°C for 30 seconds.

PCR was prepared in a final volume of 20µl and each measurement was performed in triplets. Real-Time PCR electrophoresis was performed on 3% agarose gel for further validation. To compare each miRNA expression among different specimen groups (tumor/non-tumor), normalization was performed based on miR-16 gene expression. Relative expression of target miRNAs was calculated by $2^{-\Delta\Delta C_t}$, $-\Delta\Delta C_t = -(\Delta C_t \text{ sample} - \Delta C_t \text{ control})$ (25).

Calculation

To compare the groups, T-test was used. For further calculations, CT method and SPSS 16 were used, and P values of $0 < 0.05$ were considered statistically significant. Pearson coefficient was performed to analyze the correlation of gene expressions.

RESULTS

In this study, the evaluation of miR-519d and miR-520c expressions was performed in 36 pairs of tumor/non tumor ESCC tissues by Real-Time PCR. Baseline clinical characteristics patient samples are summarized in Table 1.

Table 1: Baseline clinical characteristics of patients' samples

Parameter	Frequency	Percent (%)
Sex		
Male	19	52.7
Female	17	47.2
Age (Age range : 39_84, Median age: 61)		
>60	25	69.4
<60	11	30.6
Differentiation		
Well differentiation (low grade)	30	83
Poor differentiation (high grade)	6	17

Statistical analysis revealed significant expression rises of 10.4 and 24.5 folds of miR-519d and miR-520c in esophageal squamous cells compared to their adjacent non-tumor tissues, 95% confidence interval and P value < 0.05 were considered statistically credited (fig1).

Fig. 1: Evaluation expression of miR-519d and miR-520c in esophageal tumor tissue compared with non-tumor adjacent tissue

Real-Time PCR demonstration figures of negative control sample and three miRNAs (miR-519d, miR-520c, and miR-16) as well as CT and Melting Curve graphs are shown in fig 2-4.

Fig. 2: melting curve graphs corresponding to the miR-16, miR-519d and miR-520c's genes expression

Fig. 3: melting curve graphs corresponding to negative control sam

Fig . 4: Ct graph related miR-16, miR-519d and miR-520c's genes expression

Further analyses on patients' age and gender did not show any significant differences between males and females, and also between patients older and younger than 60 years old ($P > 0.05$). Furthermore, further analysis on tumor differentiation (grade) did not show any significant differences between tumoral group tissues ($P > 0.05$). The Pearson test was performed to investigate the correlation of miR-519d and miR-520c expression levels. The results signified a linear correlation between these two miRNAs with $R = 0.4$ and $P \text{ value} < 0.02$ (fig5).

Fig. 5: linear correlation of miR-519d and miR-520c with $R = 0.4$ and $P \text{ value} < 0.02$.

Moreover, for further evidential documents, Real-Time PCR's product's electrophoresis on 3% agarose gel stained with cyber safe visualized miR-16, miR-519d and **miR-520c**'s length by 62, 64 and 65 bp, respectively.

DISCUSSION

EC is a poorly characterized deadly cancer with a 3-2 malignancy ratio among Iranian males and female, respectively (1,26). Recent studies on molecular alterations associated with cancers have revealed the crucial roles of miRNA in cancer initiation and progression (3,27). The present study provides evidence on sharp increases of 24.5 and 10.4 folds in the expressions of miR-519d and miR-520c in ESCC's tumoral tissues compared to non-tumor marginal tissues which could indicates oncogenic roles of these two miRNAs in ESCC .Over expression of miR-519d and miR-520c has so far been reported in several cancers including breast, liver, prostate, fibroblast and hepatocellular carcinoma (8,14–16,22–24,28–30). However, over expression of these two miRNAs has not been reported in ESCC yet.

According to former studies on miR-519d in hepatocellular carcinoma and prostate cancer, this miRNA performs its oncogenic roles by targeting P21, P53, AKT3 and TIMP2 (8,15,17). Considering the oncogenic role of miR-519d in current study and further investigations based on certified database sources such as Pictar, mirWalk and Target scan and also reviewing the molecular mechanisms involved in the esophageal carcinogenesis process; P53, RAD23B, BID, P21, mKi67, PTEN and TIMP2 were identified as potential miR-519d's target gene in EC. Studies on P21 and Mki67 gene in EC point out their crucial role in cell cycle, prognosis, survival rate, cancer return and chemotherapy response in EC (2,9,16,18,20,21,31–34). Overall, these studies raised the hypothesis that P21 and mKi67 could

be main target genes of miR-519d in EC that still has not been investigated in this cancer. In addition, since samples collected in this experiment were from patients in Golestan Province, and also, due to the proven role of P53 in the pathogenesis of EC and extremely high mutation rate of P53 in ES patients in this area, this gene could well be considered as a target gene of miR-519d. To confirm this, however, further functional studies are needed . (35–37).

It has been previously reported that miR-520c has shown its oncogenic effects on stem cell, breast and prostate cancer by targeting P21 or CD44(22–24,38). It is worth adding that Correlation expression of these two miRNAs is able to indicate their oncogenic role through common carcinogenetic functional pathways like P21. Regarding the carried out investigations on miRNA target genes on previous reports which has shown that breast cancer is accompanied by increased risk of EC, it seems that CD44 can act as a target gene in EC as well, although more functional studies are needed to prove the hypothesis(6). So far, none of the genes mentioned above has been investigated as a miR-519d target gene in EC.

CONCLUSION

Suffice to say, to our knowledge, the present research is the first report on the spectacular increases in the expressions of miR-519d and miR-520c in ESCC, and supports the hypothesis of their oncogenic roles in ESCC. It also suggests them as potential targets for more research in clinical use and personalized treatment fields.

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