Expression of miR-520c in intestinal type gastric adenocarcinoma

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Background: MicroRNAs are small non-coding RNAs that participate in post-transcriptional gene regulation in cells thereby playing active role in pathological conditions and have been nominated as new class of biomarkers in disease including cancer. miR-520c has been reported as potential oncogenic micro-RNA in several previous studies. Gastric cancer is the most common cancer of digestive tract and the fourth prevalent cancer worldwide with the intestinal-type gastric adenocarcinoma (IGA) the dominant type of gastric malignancies. This study aimed to explore miR-520c putative role, in IGA and patient's clinicopathological features.

Methods: Total RNA was first extracted from 42 pairs of IGA tissues and relevant non-tumorous adjacent tissues. cDNA was synthesized from extracted RNAs using specific primers for miR-520c. The level of miR-520c was quantified using SYBER Green Real-Time PCR master mix. The relationship between miR-520c expression and clinicopathological features were examined.

Results: Our study resulted in no differential expression of miR-520c in IGA. There was no significant correlation between miR-520c expression and clinicopathological features including tumor grade, genus and age groups.

Conclusions: To our knowledge, this is the first report about exploring miR-520c expression in IGA tissue samples. Our results do not verify miR-520c previously established oncogenic role in IGA.

Keywords: Biomarker; gastric adenocarcinoma; microRNA; miR-520c; oncogene

Introduction

Gastric cancer is the most common cancer of digestive tract and the fourth prevalent cancer worldwide (1). As the most lethal global malignant neoplasm after lung cancer (2), gastric adenocarcinoma is gaining more break-out among men especially in developing countries. However, this malignancy has hit second rank among Iranian men (3). Among several histological types of gastric cancer, intestinal-type gastric adenocarcinoma (IGA) is the more prevalent type of gastric malignancies (4). As the preliminary stages of gastric cancer are asymptomatic, early diagnosis in most cases is difficult. Abdominal pain, weight loss and nausea are the usual signs of progressive tumor (5). Currently, several treatment modalities including chemotherapy, radiotherapy and gastrectomy are applied for patients however, the cure and survival of patients are not satisfactory (6).

Increasing evidences represent link between gastric cancer and tens of oncogenes and tumor-suppressors.

Although disorders in cellular mechanisms including cell cycle, DNA repair, apoptosis, cell adhesion, differentiation and epigenetic dysregulation have been reported in this multi-gene related malignancy (7) however, exact molecular pathology remains unclear.

Recent studies have reported link between cancer and novel aspect of post-translational gene regulation. MicroRNAs are small single-stranded, non-coding RNAs ranging 18 to 24 nucleotides in length and participate in post-translational gene regulation in eukaryotes via base pairing to their target's 3'-untranslated region that results in degradation or translational repression of their target mRNAs. More than 2,500 mature miRNAs have been known in human and many are to be validated (available at http://www.mirbase.org/). miRNAs are known to involve in many cellular functions, including proliferation, apoptosis and differentiation (8). miRNAs may play tumor suppressor or oncogenic role in many cancer types. As reported in literatures, many of them are down-regulated or up-regulated in malignancies. Some miRNAs called metastamiRs are known to be involved in metastasis too (9).

miRNAs have been shown to be dysregulated in gastric cancer too. Many miRNAs have been described to be overexpressed or underexpressed in gastric cancer and many others are correlated with gastric cancer prognosis. Some are designated as circulating miRNAs in serum as markers for gastric cancer (10).

miR-520c is a member of *miR-515* gene family. *miR-520c* gene is located on chromosome 19 and encodes an 87-base long precursor miRNA that later would be processed to two mature miRNA called hsa-miR520c-3p and has-miR-520c-5p. hsa-miR-520c-3p that we would call it miR-520c in this article (its previous ID), is a 22-base long miRNA processed from 3' arm of its precursor, has been characterized as an oncogenic miRNA in several studies.

In this study we analyzed miR-520c probable divergent expression in IGA in 42 pairs of endoscopic samples compared with adjacent non-tumorous tissues from Azeri patients to examine its putative role as oncomiR and its eventual biomarker value in IGA.

Methods

Patients and biopsies

Tumor samples and adjacent non-tumorous tissues were obtained from 48 people referred to Imam-Reza Hospital and Shahid-Madani Hospital in Tabriz, Iran for endoscopy

from November 2013 to December 2015. Among them, 42 patients were diagnosed with IGA and selected for study. Patients often were suffering from abdominal pain, weight loss and nausea and had not received any previous medication, chemotherapy or radiotherapy. Endoscopy was performed by specialist physician and the samples were transferred to RNase free micro-tube. The samples were snap frozen in liquid nitrogen and transferred to –80 ℃ freezer for later RNA extraction. Also, corresponding samples were immediately fixed in 10% formalin for 24 hours and dehydrating, paraffin-embedding, microdissecting into 5 µm tick sections, and staining with H&E were done respectively to samples be diagnosed histologically according to Lauren's classification and tumor grade (well-differentiated, moderately-differentiated and poorly-differentiated) (11). Since the samples were endoscopy biopsies, the information for tumor size, lymph node involvement, depth of invasion and distant metastasis were not obtained.

Informed written consent was obtained from patients and Medical Ethics Committee of Imam-Reza Hospital approved the study (medical ethics number: tbzmed. irec.1394.517).

Total RNA preparation

Total RNA was isolated from tumor tissues and corresponding adjacent non-tumorous tissues using Trizol reagent (Invitrogen) according to the manufacturer's instructions with some modification. So that, after adding isopropanol, the solution was incubated for one hour in room temperature instead of 10 min to better precipitate miRNAs. Thirty µL of DEPC-treated water was added to RNA pellet and incubated in water bath at 55 ℃ for 15 min after pipetting through sampler tip. RNA quality and concentration were assessed using Picodrop Spectrophotometer (Picodrop Ltd.).

Quantification of miR-520c

RNA was first treated with DNase I enzyme to digest any probable DNA remained from RNA extraction procedure (12). The 10 µL treatment reaction contained 2 µg RNA, 1 µL/10× DNase I buffer, 1 µL (1 U) Thermo Scientific DNase I, RNase-free enzyme, 0.5 µL RiboLock RNase Inhibitor enzyme and DEPC-treated water. The mixture was incubated at 37 ℃ for 30 min in 96-well Biometra thermocycler. To inactivate DNase I enzyme, we

added 1 µL of 50 mM EDTA and incubated the mixture at 65 ℃ for 10 min (13).

Prior to miRNA quantification, the DNase I treated RNA was 3'-extended with poly-A tail using poly-A polymerase enzyme. The 20 µL reaction mixture contained 2 µg RNA (10 µL total volume from previous step), 2 µL/10× reaction buffer, 2 µL/10 mM ATP and 0.5 µL poly-A polymerase enzyme. The reaction was incubated for 10 min at 37 ℃.

Real-time revers transcription polymerase chain reaction (qRT-PCR) was performed using PARSGENOME MiR-Amp kit. Briefly, the miR-520c and U6 (as internal control) was reverse-transcribed from total RNA using specific primers. The 10 µL RT reaction mixture contained 2 µL/5× RT reaction buffer, 1 µL/10 mM dNTP mix, 0.5 µL RT enzyme, 0.5 µL each specific primers for miR-520c and U6 as internal control and 5.5 µL poly-A extended RNA. The reaction condition was as follows: incubation for 60 min at 43 ℃ and incubation for 1 min at 85 ℃ to inactivate the RT enzyme. The synthesized cDNA was diluted up to 2 times and stored at −80 ℃ for subsequent real-time PCR.

The relative real-time PCR quantification was performed on QIAGEN Rotor-Gene Q system using SYBER Green Real-Time PCR Master Mix. The 20 µL reaction mixture includes 10 µL SYBER Green Real-Time PCR Master Mix, 1 µL mix of specific forward primer and universal reverse primer, 8 µL ddH$_2$O and 1 µL RT product of total RNA. The reaction conditions were as follows: Initial denaturation at 95 ℃ for 1 min followed by 50 cycles of denaturation at 95 ℃ for 5 s, annealing at 61 ℃ for 15 s and extension at 72 ℃ for 30 s followed by final extension for 2 min at 72 ℃. All samples were processed in duplicate. Duplicate samples differing on Ct value more than 0.4 were repeated. Ct values were detected by thermocycler as the cycle at which the fluorescence absorbed by system passed the threshold. Standard curve was plotted for both miR-520c and U6 using serial dilutions of cDNA as 1, 0.2, 0.04, 0.008 and 0.0016. For each real-time PCR, melting curve was generated to monitor reaction specificity.

Data analysis

Relative expression ratio between tumor samples and adjacent non-tumorous tissues was analyzed using both Pfaffl method and delta-delta Ct method (ΔΔCt).

For Pfaffl method we applied REST 2009 Software Tool V2.0.13 (QIAGEN Gmbh). This software utilizes Pfaffl equation (14) as follows:

$$\text{Ratio} = \frac{(E_{\text{target}})^{\Delta Ct_{\text{target (tumor-control)}}}}{(E_{\text{ref}})^{\Delta Ct_{\text{refrence (tumor-control)}}}}$$

For ΔΔCt method (15), Paired-samples t-test was employed to compare the difference between mean expression of miR-520c in tumor and non-tumorous tissues. $\Delta Ct_{\text{target}}$ was calculated as $Ct_{\text{mir}} - Ct_{\text{U6}}$ for tumor samples. $\Delta Ct_{\text{normal}}$ was also calculated for non-tumorous tissues as $Ct_{\text{mir}} - Ct_{\text{U6}}$. Then the normalized miR-520c expression was calculated separately for tumor tissue and adjacent normal samples using equation:

miRNA normalized expression = $2^{\Delta Ct}$

The resulting values were the input data for SPSS software.

Moreover, statistical analysis between groups were analyzed using SPSS® 16.0 (SPSS Inc.) for Windows®. To analyze correlation between miR-520c expression and clinicopathological features, Spearman correlation coefficient was applied. Kolmogorov-Simonov test was done to examine whether the expression of miR-520c is normal among samples. To analyze the homogeneity of variances Leven test was performed.

Results

We evaluated the expression levels of miR-520c in 42 pairs of IGA tissues and adjacent non-tumorous tissues by real-time revers transcription PCR. The sharp melting-curves of miR-520c and housekeeping gene U6 and a single band on agarose gel electrophoresis of the qRT-PCR products confirmed the reaction specificity for miR-520c and U6. The analysis results by both REST and SPSS software showed that there is no significant expression change of miR-520c in tumor tissues in comparison to that in the adjacent normal tissues (*Figure 1*).

We further determined correlation between miR-520c expression and clinicopathological features of patients in IGA. As shown in *Table 1* any significant correlation was found between miR-520c expression and clinicopathological features of patients including gender, tissue grade and age.

Discussion

Recently, microRNAs have been found to involve in many cellular mechanisms. Their important roles in cell functions indicate their major involvement in pathological conditions. Since discovery of first miRNAs, ambitious researchers have endeavored to uncover this novel aspect of biology.
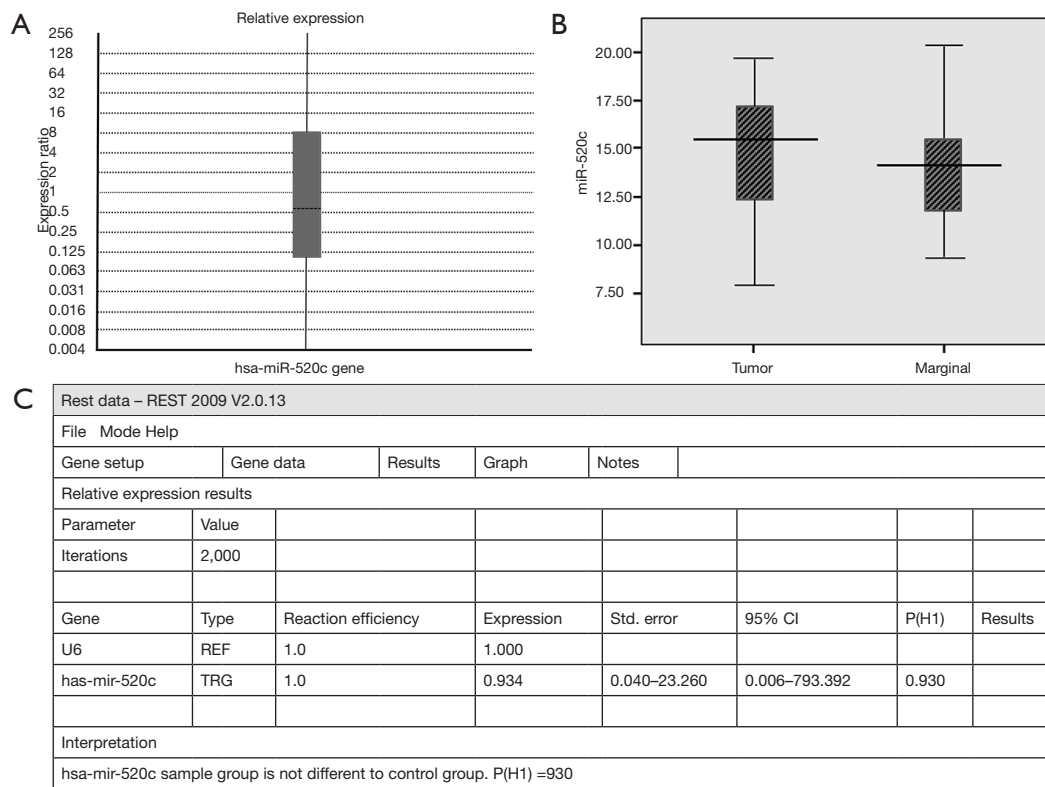
Figure 1 Mean expression of miR-520c in IGA samples compared with adjacent normal tissue. (A) REST Whisker-box plot for relative expression of miR-520c; (B) SPSS Box plot comparing miR-520c expression between tumor samples and adjacent normal tissues; (C) REST results for relative expression of miR-520c. IGA, intestinal-type gastric adenocarcinoma.

Table 1 The relationship between miRNA-520c expression levels and clinicopathological features of 42 Azeri patients with IGA

Clinicopathological variable	n	miR-520c mean ΔCt^a for tumor tissues	Statistical significance[b]
Gender			0.229 (NS[c])
Male	30	14.96	
Female	12	12.91	
Age group[d]			0.369 (NS)
1	22	13.70	
2	20	15.09	
Grade			0.483 (NS)
Poorly differentiated	24	13.85	
Well or moderately differentiated	18	14.95	

[a], $\Delta Ct = Ct_{miR} - Ct_{U6}$; [b], paired-samples *t*-test; [c], not statistically significant (P>0.05); [d], group 1 ≤66, group 2 >66. NS, not significant; IGA, intestinal-type gastric adenocarcinoma.

Along with other pathological conditions, cancer studies have benefited from miRNA discovery. So far, thousands of miRNAs have been detected to involve in cancer onset, development, metastasis and cancer relapse. These miRNAs misexpression may be as a result of their genes failure, like aberrant methylation of their promoter (16) and mutation in their genes (17), or misexpression may be as a consequence of other oncogenes or tumor-suppressors (18). Many miRNAs have been shown to down-regulate or up-regulate in certain types of cancers (19,20). This fact has opened another field in cancer-therapy studies (21) and elevated hopes to find more useful biomarkers for cancer prognosis and diagnosis (22).

Gastric cancer, as the second lethal cancer worldwide, is receiving more attention in miRNA related studies. Aberrant expression of many miRNAs has been detected in gastric cancer (23,24) and several miRNAs have been identified as biomarkers for this malignancy (25,26). More importantly, recent studies have identified miRNAs as non-invasive biomarker candidates in gastric cancer. Cai *et al.* identified that plasma miRNAs of miR-106b, miR-20a, and miR-221, may act as potential non-invasive biomarkers for early detection of gastric cancer (27). Also, Tsujiura *et al.* showed that plasma concentration of miR-17-5p, miR-21, miR-106a, miR-106b were significantly higher in gastric cancer patients than controls (28).

Several studies have proposed miR-520c as potential oncogene and metastasis driven microRNA that exerts its functions by targeting tumor suppressors. Huang *et al.* in a study applied trans-well migration assay to find out miRNAs with metastasis promoting potency using non-metastatic MCF-7 cell line that was transduced with a miRNA-expression library (29). They found that human miR-373 and miR-520c can stimulate cancer cell migration and invasion *in vitro* and *in vivo* by targeting 3'UTR region of *CD44* gene. Other study by Yang *et al.* showed that miR-520c and miR-373 exert pro-invasive effects in PC-3M cells by suppressing CD44 in protein level by binding its 3'UTR region (30). Liu and Wilson in another study explored miR-520c and miR-373 effects on MMP9 mRNA in human fibrosarcoma HT1080 cells. They described that these miRNAs upregulate *MMP9* expression, translation and activity by directly targeting the 3'UTR of mRNAs of *mTOR* and *SIRT1*, negative regulators of expression of *MMP9* via inactivating the Ras/Raf/MEK/Erk signaling pathway and transcription factor NF-κB activity and, thus suppressing translation levels of *SIRT1* and *mTOR* that ends in enhancing cell migration and cell growth in 3D type I collagen gels (31).

In this study, we explored expression of miR-520c in IGA, the major malignancy of stomach, to find out whether this miRNA plays mentioned oncogenic role and also to evaluate its value as biomarker for IGA detection. Our study on 42 pairs tissue samples of IGA and adjacent normal tissues showed any significant correlation between IGA and miRNA-520c, a finding that opposes previous findings of this miRNA. This finding suggests conflicting roles for miRNAs and different malignancy mechanisms in cancer. Since different molecular aberrations may result in gastric cancer, it is probable that miRNA-520c drives its oncogenic character via distinct procedure or in a specific stage of tumor formation and invasion as the above-mentioned studies concentrated on miR-520c as MetastamiR and invasion promoting microRNA.

Conclusions

All in all, our study resulted in conflicted role for miR-520c as an oncogene and denies oncogenic role for miR-520c in IGA and its capability as biomarker in gastric cancer.

Acknowledgements

Footnote

Conflicts of Interest: The authors have no conflicts of interest to declare.

Ethical Statement: Medical Ethics Committee of Imam-Reza Hospital approved the study (medical ethics number: tbzmed.irec.1394.517) and informed written consent was obtained from patients.

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