Original research **Journal of Medical Physiology. 2019; 4: e5.**

Tissue and plasma co-expression of microRNA let-7g-5p in colorectal cancer patients

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Received: April 2019; Accepted: May 2019

Abstract:

Background: Tumor-associated microRNAs (miRNAs) have been detected in cancer, though whether plasma miRNAs could be a potential biomarker for cancers need to be more clear. We aimed to determine tissue and plasma expression levels of miRNA let-7g-5p, potentially predicting the clinical characteristics of patients with colorectal cancer (CRC).

Methods: A total of 62 cancer tissue and peripheral blood samples and 32 controls were included in the study. After total RNA extraction, quantitative real-time PCR was used to detect the relative expression of let-7g-5p.

Results: The expression level of miR-let-7g-5p was down-regulated in CRC tissue compared to the non-tumor tissues (2.32±0.38 vs. 6.86±0.55, $p<0.001$). Moreover, a significant down-regulation of let-7g-5p was detected in plasma samples from CRC patients than in those of the controls (0.97±0.21 vs. 1.15±0.11, $p<0.001$). Further analysis showed a possible relationship between the tissue and plasma levels of let-7g-5p. Correlation analysis detected a significant association between expression levels of tissue and plasma let-7g-5p ($r=0.56$; $p<0.001$).

Conclusion: The down-regulation of let-7g-5p in plasma and tissue is matched in CRC and may be served promisingly as a potential biomarker.

Keyword: Colorectal Cancer; Let-7g-5p; MicroRNA; Plasma; Tissue

Cite this article as: Zare AA, Talebi A, Agah S, Masoodi M, Mokhtare M, Akbari A. Tissue and plasma co-expression of microRNA let-7g-5p in colorectal cancer patients. J Med Physiol. 2019; 4: e5.

1. Introduction

Colorectal cancer (CRC) is the third most common cancer and the fourth cause of cancer-related death in the worldwide. The prognosis of invasive disease is poor and long-term survival greatly depends on the clinical stages at the time of tumor detection (1-4). Notably, the routine CRC screening tests are usually not convenient and suffer from several limitations. For example, colonoscopy (as the gold standard) is invasive and costly (5, 6). Therefore, finding out new noninvasive biomarkers could impressively improve prognosis and diagnosis of CRC (7-11). MicroRNAs (miRNAs) are small, non-coding single-strand RNAs, 18–25 nucleotides in length (12). They regulate gene expression by binding to 3′ untranslated region (3′UTR) of target mRNAs (12, 13). There is increasing evidence indicating that miRNAs can act as tumor suppressors and oncogenes. In this regard, they play crucial role in the regulation of many biological processes, including cell proliferation, differentiation and apoptosis (13, 14). It has also been demonstrated that miRNAs are commonly dysregulated in CRC and have possible applications in cancer managenet (1, 7). For example, various studies showed that the expression level of various of miRNAs was correlated with cancer stage, distant metastasis and patient survival. The stability of miRNAs making them attractive as potential tools for cancer detection biomarkers (1, 7, 13, 15). Researchers have demonstrated that the plasma miRNAs were significantly dysregulated in CRC, discriminating CRC patients from healthy individuals (15, 16). Notably, analysis of the expression levels of specific miRNAs in plasma and matched tissues is helpful for investigating tumor-specific non-invasive biomarkers. Co-expression of miRNAs in

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tissue and plasma more likely reflects the presence of cancer specific miRNAs, functioning as valid cancer biomarkers in circulation(4, 17, 18). However, whether dysregulated expression of miRNAs in tissue or circulation is consistent has not been still well-identified and actually needs to be investigated.

One miRNA, named let-7g-5p, is known to regulate multiple aspects of cellular functions in cancer development and progression (19). In CRC, let-7g-5p has been revealed to remarkably be down-regulated compared to the adjacent noncancerous tissues. Functional studies have confirmed that let-7g could target RAS mRNA and negatively regulate tumorigenesis, indicating it as a potential tumor-suppressor. In addition, this miRNA was found to be associated with clinical outcomes of CRC (20). Nonethelesss, the consistency of let7-g-5p expression in tissue or circulation of CRC patients has not been investigated. The main aim of the present study was to evaluate and correlate the expression of miRNA let7-g-5p in tissue and plasma, which could potentially could be served as suitable diagnostic tool in CRC.

2. Method

2.1. MiRNA selection

By the virtue of being confirmed as colorectal cancer-associated miRNA acting as a tumor suppressor in CRC, let7-g-5p was selected from the Sanger Center miRNA Registry at: http://www.sanger.ac.uk/Software/Rfam/mirna/index.shtml. Moreover, other miRNA databases (miRDB, TargetScan, miRBase and miRTarBase) were used to predict the potential targets and biological crosstalking.

2.2. Study design and participants

In this case-control study, a total of 62 blood samples (31 with stage II, 27 with stage III and 4 with stage IV of CRC) and a subset of 62 matched tumor tissues were collected from CRC patients (Case group). In the healthy group, 32 blood samples were collected from subjects with no current malignancy or infectious disease (Control group). Written and signed informed consent forms were completed by all the participants. All the participants stated they had received no chemotherapy or treatment in the two months prior to the study.

The study was approved by the Ethic Committee of Iran University of Medical Sciences (Ethical code: IR.IUMS.REC 1394.26649).

2.3. Sample processing and RNA extraction

The tumor tissue specimens were collected in tubes containing RNALater (Thermo Fisher scientific, Germany) and stored at -20˚C. Peripheral blood was collected in vacutainer liquid EDTA 6 ml blood collection tubes, and plasma was separated by a density gradient separation, as described previously (8). The separated plasma was frozen at -80˚C until miRNA extraction.

2.4. MiRNA extraction

The plasma (200 µl) and tumor tissue samples (5 mg) were subjected to miRNA extraction. MiRNAs were extracted from plasma samples using a miRNA extraction kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. Tissue miRNAs were isolated by a modified TRIzol protocol as explained previously (14). Also, the quantity and quality of the extracted RNA were evaluated (21).

2.5. cDNA synthesis

A cDNA Reverse Transcription Kit (Ampliqon, Denmark) was used for reverse transcription of total RNA (1 µg) into cDNA (16, 17). Briefy, 1 µg of RNA was mixed with 1 µl of random hexamer primers and 1 µl M-MuLVreverse transcriptase (200 U/µl). The mixture was uped to a volume of 15 µl by nuclease-free diethyl pyrocarbonate (DEPC)-treated water. The mixture was incubated at 65˚C for 5 minutes and cDNA was synthesized by 7500 thermocycler (ABI) in following program; 5 minutes at 25˚C, 60 minutes at 42˚C, and 5 minutes at 70˚C (22, 23).

2.6. Quantitative real-time PCR (qRT-PCR) for let-7g-5p detection

The expression levels of let7-g in plasma and tissue specimens were evaluated by quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) technique (21) using poly A method, as explained previously (14). The primer sequences used for qPCR analysis in this study were indicated in Table 1. The reactions were carried out in duplicate and mean Ct (cycle threshold) data was calculated. 5SrRNA was used as an internal control for normalization of the relative expression, considering the equation: $\log_{10}(2^{-\Delta\Delta Ct})$, in which $\Delta\Delta Ct = Ct_{cancer} - Ct_{control}$.

2.7. Statistical analysis

The results were statistically described as mean±standard

Table 1: The primer sequences used in the study

MiRNA/gene	5´ to 3´ sequences
Let-7g-5p	Forward:GCACTGAGTTAGTAGGTGGT Reverse:GATCCAGTTTTTTTTTTTTTTTAACTATGC
5SrRNA	Forward: GCCCGATCTCGTCTGATCT Reverse: AGCCTACAGCACCCGGTATT

deviation in continuous variables. The normality of continuous variables was checked using the Kolmogorov-Smirnov test. Non-parametric statistics were applied for data analysis. The Mann–Whitney U-test was used to compare between two groups. Also, Spearman's rank correlation coefficient was used to assess the relationship between plasma and tissue levels of let-7g-5p. Level of significance for statistical tests was 0.05. The SPSS software version 24 and Microsoft Excell 2010 were applied for statistical analysis.

3. Result

3.1. Clinicopathological features of CRC patients

A total of 62 CRC patients and 32 healthy controls were included in the study. The median age of the patients was 58.7±10.2 years, while the median age of the controls was 59.1±8.5 years. No differences were detected between the groups (p=0.14). The other details concerning clinicopathological features of CRC patients have been presented in table 2.

3.2. Expression levels of tissue and plasma let-7g-5p

Either tissue or plasma levels of let-7g-5p were quantified using a quantitative PCR assay with RNU6B as the internal control. Our results showed that the let-7g-5p level was down-regulated in CRC tissue compared to the non-cancer tissues (2.32±0.38 *vs.* 6.86±0.55, p<0. 001). Moreover, a significantly lower let-7g-5p level in plasma samples from CRC patients than in those of the controls (0.97±0.21 vs. 1.15±0.11, p<0.001) was detected (Figure 1).

We also evaluated the let-7g-5p levels for each different clinical stage of CRC. It was found that both tissue and plasma let-7g-5p levels were lower in stage III/IV cases. Additionally, the levels of let-7g-5p in stage III, and IV were not significantly dysregulated compared to that of stage II (Figure 2). In the other word, no decreasing/increasing trend was found as the stages advanced.

Table 2: The clinicopathological characteristics of the patients

Variable	Number of patients (n=62)
Age	
≥55	28 (45.2%)
<55	34 (54.8%)
Gender	
Male	40 (64.5%)
Female	22 (35.5%)
TNM stage	
II	31 (50.0%)
III	27 (43.5%)
IV	4 (6.5%)
Tumor size	
<2	7 (11.3%)
2-3.5	28 (45.2%)
3.5-5	24 (38.7%)
>5	6 (9.7%)
Localization	
Colon	23 (37.2%)
Rectum	29 (46.8%)
LVI	
Positive	46 (74.2%)
Negative	28 (45.2%)
Differentiation	
Well	7 (11.3%)
Moderate	52 (83.9%)
Poor	3 (4.8%)

CRC= colorectal cancer; TNM= tumor-node-metastasis; LVI= lymphovascular invasion

3.3. The correlation between let-7g-5p expression levels in matched tissues and plasma

We also completed a further analysis to identify a possible relationship between the tissue and plasma levels of let-7g-5p. Data analysis showed a possible relationship between the tissue and plasma levels of let-7g-5p. Correlation analysis detected a significant association between expression levels of tissue and plasma let-7g-5p (r=0.56; p<0.001; Figure 3). Our results indicated that the plasma expression of let7-g may has a potential circulating biomarker for the early detection of CRC.

4. Discussion

MiRNAs have increasingly been investigated as diagnostic tools for the early detection of various malignancies, including CRC. Since the miRNA expression is tissue-

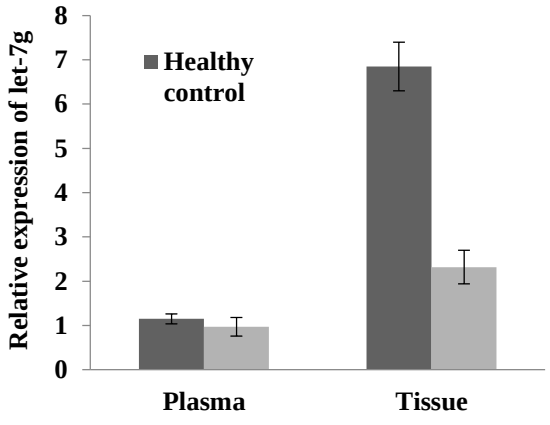

Figure 1: The miR-let-7g-5p expression levels in CRC tissues/plasma compared to the healthy group. The expression level of miR-let-7g-5p was down-regulated in CRC tissue compared to the non-tumor tissues (2.32±0.38 vs. 6.86±0.55, p<0.001). Also, there was a significant down-regulation of let-7g-5p level in plasma samples from CRC patients than in those of the controls (0.97±0.21 vs. 1.15±0.11, p<0.001).

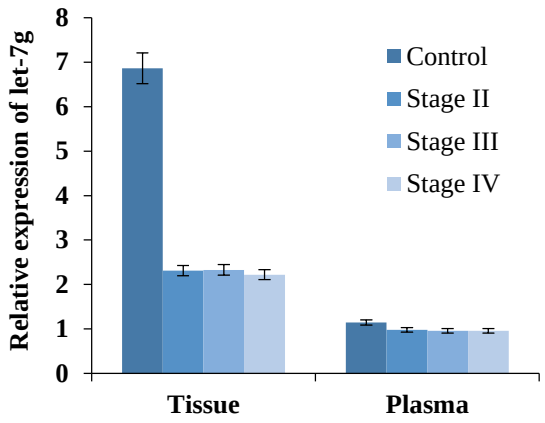

Figure 2: Differential expression of miRNAs in different clinical stages of CRC. The expression levels of let-7g-5p in stage III and IV were not significantly different compared to that of stage II ($p>0.05$).

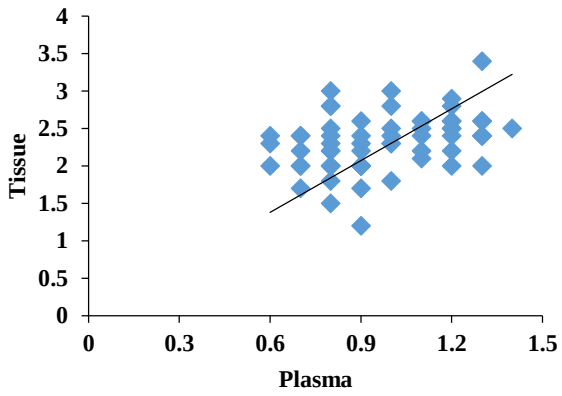

Figure 3: The correlation between expression levels of let-7g in tissue and plasma of CRC patients. Correlation analysis showed a significant association between expression levels of tissue and plasma let-7g-5p ($r=0.56$; $p<0.001$).

specific and relatively stable, they may be regarded as proper biomarkers for cancer diagnosis. Although a number of studies have investigated the potential application of miRNA as a biomarker, they commonly have limited clinical significance due to histological specimens.

In this regard, the analysis of miRNA pattern in plasma and matched tissues is cooperative. Co-expression of miRNAs in tissue and plasma may reflect the cancer specific miRNAs, purposing as cancer biomarkers (4, 17, 18). Given that cell-free miRNAs are stable in body fluids, we evaluated the tissue and plasma levels of miRNA let-7g-5p in CRC. According to the requirements for biomarkers in diseases, we speculated that both tissue and plasma let-7g-5p must to be investigated for evaluating the diagnostic values for CRC. Our study was the first to detect both tissue and plasma let-7g-5p in plasma of CRC patients by using qPCR. Here, we conducted a case-control study to identify the expression and association of both tissue and plasma let-7g-5p in CRC patients and estimated the possible correlation between them. In the current study, we decided to determine potential value of let-7g-5p in the diagnosis of CRC patients. Our data showed that levels of tissue and plasma let-7g-5p expression were significantly down-regulated in CRC compared to the corresponding non-cancerous specimens. Moreover, we found that the expression levels of let-7g-5p in tissue and plasma were significantly associated. This result was accordant with several previous studies, such as microRNA-375 in colorectal cancer (17) and miR-106b~25 expressions in patients with gastric cancers (18). The investigations of tissue- and plasma-specific miRNA signatures may answer some questions about the validity of circulating miRNAs as non-invasive biomarkers in cancers. Moreover, such analyses of the miRNA expression, could provide clinical biomarkers and some clues to develop miRNA-based therapies (17, 18).

A number of studies indicated that let-7g-5p was dys-regulated in various cancers, including lung cancer(24) gastric tumors (25), colon cancer (26), although the clinical impact was not noted. As in most previous studies, the clinical values of let-7g-5p on diferent cancers were analyzed in the tissues(26-28). It has been reported that plasma miRNAs were quite stable and could scarcely degraded by enzymes(29). However, as the importance of circulating miRNAs is considered in the diagnosis of cancer, we investigated the potential application of plasma let-7g-5p in CRC. In a previous study, the levels of let-7g-5p in cancer tissues or cell lines were signifcantly higher than in healthy tissue or non-cancer cells(26, 27). However, let-7g-5p levels in plasma were not reported in previous studies in CRC.

Let-7 miRNA has been demonstrated as a potential therapeutic target in cancer. Let-7 is able to suppress tumor proliferative activities regulating various oncogenes (28). Let-7g, as a potent suppressor, targets some oncogenes, including RAS, HMGA2, BCL2L1 and GAB2. This miRNA was found to regulate cell proliferation and apoptosis (7). The significant downregulation of let-7g has been reported in several tumors, including lung, liver, breast, gastric and colon cancer (7). Previous findings indicated a down-regulated expression of let-7g in CRC compared to normal samples, suggesting it as a tumor-suppressor (37). This miRNA also was reported to be associated with clinical outcomes of CRC. Notably, analysis of the expression levels of specific miRNAs in plasma and matched tissues is helpful for investigating tumor-specific non-invasive biomarkers. Co-expression of miRNAs in tissue and plasma more likely reflects the presence of cancer specific miRNAs, functioning as valid cancer biomarkers in circulation (4, 17, 18). However, whether dysregulated expression of miRNAs in tissue or circulation is consistent has not still well-identified and

actually needs to be investigated. Our work may be considered as a basis for supplementary investigations, that needs to relatively large-scale rationale with a more sample size, before plasma let-7g-5p approved as a potential clinical tool for CRC.

5. Conclusion

Our results indicated that the down-regulation of let-7g-5p in plasma and tissue is matched in CRC. The findings supported the hypothesis that plasma miRNAs pattern might reflect the expression pattern of their matched tissues. Taken together, plasma let-7g-5p may be investigated promisingly as a potential biomarker for CRC.

6. Acknowledgment

We thank the patients who participated in the study.

7. Conflict of interest

No conflict of interest was declared.

8. Funding source

This work was supported by Iran University of Medical Sciences (Grant No. 26649) and Iran Biotech Fund.

9. Author contribution

A-A. Z and A.A contributed to study design and conception. M.M, and S.A contributed to the selection of patients. A.A and M.M performed the experiments. A.A prepared the manuscript which M.M significantly revised. A.A assisted with analysis of the data.

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