Alterations in MicroRNA Gene Expression Profile in Liver Transplant Patients with Hepatocellular Carcinoma

Afsoon Afshari

Shiraz University of Medical Sciences, Transplant Research center <https://orcid.org/0000-0002-6086-3612>

Ramin Yaghobi

Shiraz University of Medical Sciences

Mohammad hossin Karimi (✉ karimimh@sums.ac.ir)

Shiraz University of Medical Sciences <https://orcid.org/0000-0002-2435-6277>

Jvad Mowla

Tarbiat Modares University Faculty of Biological Sciences

Research article

Keywords: LNA-Array, microRNA, Liver, Transplantation, Hepatocellular Carcinoma, qRT-PCR

Posted Date: December 9th, 2020

DOI: <https://doi.org/10.21203/rs.3.rs-42044/v3>

License: This work is licensed under a Creative Commons Attribution 4.0 International License.

[Read Full License](#)

Abstract

Background: HCC can lead to liver failure, rendering liver transplant. Since miRNAs can receive early evaluation before clinical symptoms are manifested, they might be detected as biomarkers of several disorders. Therefore, in the present study, alterations in miRNAs as biomarkers were detected in LT patients with HCC.

Methods: Fourteen tissue samples composed of 5 rejected and 9 non-rejected ones were used for studying the microRNAs expression pattern by LNA-array probe. The result was evaluated by qRT-PCR on 30 other tissue samples composed of 10 rejected and 20 non-rejected ones for the selected miRNAs. All samples were collected from liver transplanted patients with HCC.

Results: The study results revealed that in rejected patients compared to non-rejected ones, hsa-miR-3158-5p, -4449, -4511, and -4633-5p were up-regulated and hsa-miR-122-3p, -194-5p, 548as-3p, and -4284 were down-regulated.

Conclusion: It was concluded that the tissue levels of specific miRNAs (especially hsa-miR-3158-5p, -4449, -194-5p and -548as-3p) correlated well with the development of HCC, which could be further studied as biomarkers.

Background

Many diseases might lead to liver failure for which the main treatment approach is liver transplant (LT) ¹. Ranked as the fifth most common diagnosed cancer worldwide, hepatocellular carcinoma (HCC) refers to a multistage process, involving many genes ², which can render to hepatocyte turnover, oxidative DNA damage, and inflammation ³. Even though innovated therapeutic strategies are being established, handling advanced HCC is poorly efficacious and merely at the point of diagnosis. Apparently, transplantation seems to be the only known effective treatment approach for this disease ⁴.

A considerable part of human genome is consisted of non-coding regions, including non-coding RNAs, known as microRNAs (miRNAs) ¹. These regions encode dynamic miRNAs, regulating around 20-30% of mammalian genes that comprise nearly 5% of transcriptome ^{1,5}. MiRNAs are evolutionary conserved, made up of 18-25 nucleotides. They are also involved in various biological processes e.g. cell growth, apoptosis, hematopoietic lineage differentiation, and gene regulation ^{1,6,7}.

There are also many miRNAs with specific expression patterns in the liver at different stages ⁸. Investigations have demonstrated that some miRNAs are involved in regulating many metabolic pathways; hence, changes in their expression levels might reflect upon the underlying sources of inflammation ⁹. The research which was conducted by Murakami et al., in 2006 was one of the pioneer studies on investigation the role of miRNAs in HCC development ¹⁰. After that a number of studies have been investigated the miRNA changes in HCC ¹¹⁻¹⁵. As an illustration, it has been reported that miR-17-

92, miR-21, miR-221, miR-222, and miR-224 are frequently up-regulated in HCC. Furthermore, the importance of miRNAs in liver function has been broadly documented in the related literature¹⁶. Studies have also revealed that miR-122 as a blood marker changes in viral, alcoholic, and chemical injuries induced in the liver⁸. By the way, the importance of miRNAs is also, studied in LT rejection and it seems that some of miRNAs are playing specific role in this regard. Ectopic expression of serum miRNA signatures is known to be prognostically, diagnostically, and biologically related to liver allograft rejection¹⁷. Other sources showed that in rat model of LT rejection, miR-146a, 15b, 223, 23a, 27a, 34a and 451 were upregulated¹⁸.

Furthermore, different studies have been used the LNA-array technique for detection of miRNAs' level in different samples and discussed the advantages of this method over methods and the researchers have found that this method, due to its ability to produce high affinity hybridizations render to accurate discriminating signals^{5,19–21}.

There are attempts to find miRNAs as early detectors and biomarkers. Nowadays, pathological tests being used to detect transplant outcomes mainly through biopsy, are invasive and take several days. Thus, the necessity for a faster and less invasive method seems crucial. Therefore, LNA-array (locked nucleic acid-array) probe and (quantitative) real-time polymerase chain reaction (qRT-PCR) was utilized to detect alterations in miRNA expression in the tissues of LT patients with HCC. The qRT-PCR was employed to certify the LNA-array probe results in the tissue samples of LT patients with HCC. This study is tried to elucidate the role of miRNAs in controlling the rejection of liver transplant in HCC patients.

Methods

Patients and Samples

This study was fulfilled in the Pathology Ward of Namazi Hospital, Shiraz, Iran (2016-2018). To conduct this study, a total number of 44 formalin-fixed paraffin-embedded (FFPE) samples were collected from LT patients with HCC. Macro-dissection was also performed. In brief, there were attempts to adopt a standard procedure to dissect the middle region of the tumor to harvest a proper sample, surrounded by the cancerous cells. The harvesting procedure was then confirmed microscopically to ensure the nonexistence of non-cancerous cells in each sample. All of the participants were asked to completed a questionnaire on demographic characteristics, history of other cancers except HCC, alcohol use, and tobacco use. Those with a history of other cancers, alcohol use, or tobacco use were excluded from the study.

As this study was a retrospective one, the samples were collected and divided into acute rejected and nonrejected patients due to their pathology reports. The pathology results were closely examined by expert pathologists and all of tissue samples that were classified as rejected were at stages III and IV based on AJCC (American Joint Committee on Cancer) staging system²².

The samples were divided into two main groups. The 1st group (test group) was composed of 14 liver transplant samples (5 rejected and 9 nonrejected). The 2nd group (validation group) was composed of 30 samples (10 rejected and 20 nonrejected). For more clarification the sample division is shown in Figure 1. All the protocols applied in this study were in complete agreement with the Helsinki Declaration and its later amendment. The local Ethics Committee of Shiraz University of Medical Sciences also approved each stage of this study. After explaining the study objectives, written informed consent was further obtained from each patient.

De-Paraffinization of FFPE Samples, RNA Isolation and Quality Control

Using a microtome, 10 cuts of 5µm of each sample were prepared. All the samples were RNA isolated, using Trizol™ (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. Sample RNA Quality Control was further performed by an Agilent 2100 Bioanalyzer, to provide an electropherogram for each sample. In addition to the measurement of the rRNA ratio in a traditional manner (28S/18S), the Bioanalyzer provided an RNA Integrity Number (RIN) value (ranging from 0 to 10) and a reliable impression of RNA quality. The recommended RIN values were higher than 7 for good array performance. The NanoDrop instrument was also used to measure the concentration (A260), protein contamination (ratio A260/A280), and contamination with buffer components, or organic compounds (ratio A260/A230) accurately.

Sample Preparation for LNA-Array Probe

Labeling, Hybridization, and Normalization

Using the miRCURY LNA™ miRNA Hi-Power Labeling Kit (Exiqon, Denmark), 400ng total RNA were labeled by Hy3™ and Hy5™ fluorescent label for labeling the samples and the references, respectively to produce highly efficient and uniform labeling. The samples (labeled with Hy3™) and the references (labeled with Hy5™) were then mixed pairwise and recruited for hybridization. The hybridization procedure was performed using Tecan's HS 4800™ hybridization station (Tecan, Austria) followed by miRCURY LNA™ miRNA array instruction manual.

Later, the labeled specimens were hybridized by miRCURY LNA™ miRNA array 7th Gen (Exiqon, Denmark) using the manufacturer's protocol. The quantified signals of the background were also corrected (Normexp with offset value 10) and normalized using the global Locally Weighted Scatterplot Smoothing (LOWESS) regression algorithm.

Principle Component Analysis (PCA) Plot

In order to decrease the proportions of the bulky data and to explore sample classes arising naturally and according to the expression profile, the PCA plot was applied. The top 50 miRNAs that had major variations across all the samples were also included.

Array Slide Quality Control using Spike-Ins (Labeling Controls) and Normalization

The spike-in controls were added in various concentrations in both Hy3™ and Hy5™ labeling reactions, giving the opportunity to evaluate the labeling reaction, hybridization, and performance of array experiment in a general manner (The level correlation of the signal intensities across all the slides is shown in the Additional File, Figure A1).

The LOWESS also allows the correction of systematic deviations in the MA plot resulting in an intensity-dependent adjustment of the MA-data to a straight line. The positive effect of this normalization is illustrated in the MA plots for each slide, showing the plot before and after normalization (see Additional File 1, Figure A2).

qRT-PCR

Poly A Polymerization, cDNA Synthesis, and qRT-PCR

To this end, for study the expression level of selected miRNAs in the validation group of samples, 5µg of each isolated total RNAs was used for poly A polymerization by means of MiRNA cDNA Synthesis Kit (Parsgenome, Iran). Then, 2 µg of the product was applied for the next step, i.e., cDNA synthesis using specific reverse primers for each miRNA. Finally, these miR specific cDNAs were employed as templates for quantitative SYBR Green Real-time analysis (Parsgenome, Iran). The expression level of the selected miRNA molecules was further determined employing SYBR Green Real-time PCR (ABI, USA). In addition, U6 miRNA primers were included in the mentioned kit as an internal control.

The mix used for qRT-PCR for each reaction contained SYBR Green Premix (10µl of Ex taq, Takara, Japan), Rox reference dye (0.4µl), forward and reverse primers (1µl of 10pM mix of each primer pairs), and template (2µl of synthesized cDNA, between 0.1 to 100ng). Nuclease free water also added to this reaction up to 20µl total volume. The program employed for qRT-PCR was one cycle 95°C -5 minutes, followed by 40 cycles of 95°C- 5 seconds, 61°C- 20 seconds, and 72°C- 30 seconds, followed by melting curve analysis for specificity of each reaction.

Statistical Analysis

The data were coded and imported into the IBM SPSS Statistics software (version 24). The quantitative variables were then compared using the non-parametric Kruskal-Wallis test for a comparison between more than two groups and the Mann-Whitney U test was employed to compare two groups. The calculations for expression comparison in LNA-array tested samples were also executed by the R/Bioconductor software, using the LIMMA package. The fold changes in the validation group of samples were calculated by livak method ($2^{-\Delta\Delta Ct}$). The *P*-value cut-off score for a significant difference was considered less than 0.05.

Results

Descriptive Statistics

The test group was composed of 14 LT patients with the mean age of 47.4 ± 7.8 years (age range: 31-56) and included 9 (64.3%) males. These samples were used for LNA-array probe. More data is shown in Figure 1 and Table 1. The validation group consisted of 20 LT patients with no acute rejection of LT and 10 patients showing acute rejection. The mean age of nonrejected ones was 49 ± 15.3 years (age range: 14-69) and included 14 (70%) males. The rejected samples had mean age of 49.7 ± 17.1 years (age range: 13-68) and included 6 (75%) males.

PCA Plot Analysis

The top 50 miRNAs that had major variations across all the samples were used for PCA plot analysis. The results are depicted in Figure 2A, demonstrating variations of the biological patterns or technical factors in the test group ($n=14$). Accordingly, PC1 and PC2 describe variations related to sample groups or treatments, whereas PC3 and later PC's might describe underlying or less variable factors like sample preparation conditions, operator, storage time, etc.

The unsupervised PCA and the hierarchical clustering could clearly show a tight grouping of the control samples (i.e., the non-rejected), whereas the treated samples (namely, the rejected) differed both from the controls as well as from each other. A simple expression analysis by comparison did detect a subset of differentially expressed miRNAs including four up-regulated and four down-regulated miRNAs selected for further analyses.

Heat Map and Unsupervised Hierarchical Clustering

The heat map diagram shows the results of a two-way hierarchical clustering of miRNAs and the samples in the test group ($n=14$). The clustering was done using the complete-linkage method together with the Euclidean distance. A small subset of the miRNAs was also excluded from the heat map. As well, the normalized log ratio values were used for the analysis (Figure 2B).

MiRNA Expression Analysis for Rejected versus Non-Rejected in LNA-Arrayed Samples

An expression analysis for the rejected cases versus the non-rejected ones was performed using a simple comparison method in the test group ($n=14$). The miRNA profiling accordingly identified a subset of top miRNAs (where the absolute value of the log fold change was larger than 1) out of the total number of miRNAs analyzed (371) by the miRCURY LNA™ miRNA Array, differentially expressed in the rejected and the non-rejected samples. Table 2 provides a list of miRNAs with the most differential expression. The results showed that hsa-miR-122-3p, -548as-3p, -4284, and -194-5p had the highest levels of reduction amongst all the studied miRs, and hsa-miR-4511, -4449, -4633-5p, and -3158-5p were the most increased ones; respectively.

Selected miRNA Expression Level Comparison between Rejected and Non-Rejected Groups of Patients after LT

The expression level of the selected miRs was tested in the 2nd groups of patients (validation group) composed of rejected and non-rejected ones. The results of this selection are demonstrated in Figure 3A for the down-regulated miRs and Figure 3B for the up-regulated ones based on a fold change. The results also revealed that hsa-miR-548as-3p and -194-5p had significantly reduced and hsa-miR-4449 and -3158-5p had shown a significant rise in the patients with LT rejection.

Calculating Sensitivity and Specificity of Down-Regulated miRNAs between Nonrejected and Rejected Groups of Patients after LT

The sensitivity and the specificity of the down-regulated miRNAs between the non-rejected and rejected samples of patients in the validation group were calculated (Figure 4). The area under curve (AUC), p -value, and 95% confidence interval (95% CI) of each down-regulated miRNA are demonstrated in Table 3. Two of the miRNAs (i.e., has-miR194-5p and -548as-3p) had a significant difference ($p < 0.05$), making them candidates as promising biomarkers.

Calculating Sensitivity and Specificity of Up-Regulated miRNAs between Nonrejected and Rejected Groups of Patients after LT

The sensitivity and the specificity of the up-regulated miRNAs between the non-rejected and rejected samples of patients in the validation group were calculated (Figure 5). The AUC, p -value, and 95% CI of each up-regulated miRNA are demonstrated in Table 4. Two of the miRNAs (namely, has-miR4449 and -3158-5p) established a significant difference ($p < 0.05$), making them candidates as promising biomarkers.

Discussion

The functions of miRNAs can provide valuable diagnostic measurements as well as prognostic indicators for several diseases including types of cancer⁵. A healthy liver consists of different cells producing a variety of miRNA expression profiles according to complex intrinsic and extrinsic signals. Under pathogenic conditions, these cells can also alter the profile of miRNAs²³. Commonly, HCC is accompanied by loss of liver and the remaining treatment would be transplantation. Although many studies have been accomplished in order to find early detecting markers on the basis of miRNAs, still no effective biomarkers have been introduced in this regard (6 of 2019, Jin). By the way, as HCC renders to transplantation, the maintenance of the new liver and prevention from rejection is the priority of these patients. In the present research, it is tried to evaluate the alterations in miRNA expression profile of LT patients with HCC in order to introduce probable miRNA biomarker in early detection of rejection. In this sense, LNA-array probe was used to identify the expression profile of the miRNAs and the results of LNA-array was tested in other samples of liver transplanted patients with the same condition of underlying disease. Although it has been suggested that some of the circulating blood miRNAs have the potential to be used as miRNA-based blood biomarkers in cancer detection^{24,25}, it is noteworthy to investigate the possibility of introducing more accurate miRNAs in detecting rejection in the biopsy samples that are

taken commonly from patients. In a study done by Miyaaki et al, they used the same techniques as we did, for studying the miRNA profile of liver transplanted patients with HCV/HIV co-infection and confirmed that miR-101b and miR-149 are significantly decreased in co-infected group of patients comparing to HCV-infected ones ²⁶.

The findings revealed that miR-4449, miR-4511, miR-3158-5p, and miR-4633-5p had been up-regulated and miR-4284, miR-122-3p, miR-194-5p, and miR-548as-3p had been down-regulated in patients with LT rejection once compared with non-rejected ones. The results from LT patients were also in accordance with the LNA-array probe results. With reference to the results collected from the PCA plot, there was a significant difference between miRNA expression in the rejected LT patients and the non-rejected ones.

The miR-122-3p is a liver-specific miRNA, highly expressed, comprised of more than 70% of all cloned liver miRNAs, unlike other tissues ^{1,27}. Moreover, this miRNA plays a role in the liver metabolism, for instance, many genes interfering in lipid metabolism regulation can be down-regulated when using an antisense strategy to knock it down ²⁸. The sequence and the expression pattern of the miR-122-3p in the liver is also highly conserved ²⁹. In a study, HCC patients had shown a reduction in miR-122-3p expression ³⁰. MiR-122-3p, miR-26a, and miR-195 have been also detected as tumor suppressors in the liver ³¹. The fact that miR-122-3p reduces in HCC patients was accordingly certified in the present study. Other studies on liver transplant recipients with recurrent hepatitis C and acute cellular rejection demonstrated the importance of miR-122 and miR-194 in liver transplant rejection either ^{24,32}. Furthermore, the correlation between miR122-3p and -194-5p has been a subject of research studies. Therefore, this miRNA was analyzed between HCC non-rejected and rejected patients, indicating that the given miRNA could significantly ($p=0.041$) decrease in the rejected LT cases.

The miR-194-5p expression had been previously detected, reporting that such an miRNA could play a role in activation of stellate cells during liver fibrogenesis ^{33,34}. Another study had further profiled the expression of miR-194-5p as a marker of liver HCC metastasis by showing the overexpression of miR-194 in cancerous liver cell lines to down-regulate N-cadherin expression and to suppress migration, invasion, and metastasis ³¹. An increase in the expression of miR-194-5p in HCV-infected hepatoma cells has been also well documented although the miR-194-5p expression level had significantly reduced in HCC patients ³⁵. In addition, Farid et al. had demonstrated that the expression levels of miR-122 could significantly correlate with miR-194 among HCC patients ($r=0.322$, $p=0.007$). They had also reported no significant differences between non-rejected and rejected groups of patients against the expression of miR-194-5p ($p=0.134$). However, the difference in miR-122 expression level in their study was significant ($p=0.001$) ³⁶. In addition, a research in 2015 had found that miR-194-5p had been down-regulated in the tissues involved in HCC and they had reported that it was able to reduce cell viability and proliferation by inducing G1 arrest and apoptosis in HCC cells. Moreover, they believed that miR-194-5p, inhibiting MAP4K4, was a potential diagnostic and therapeutic marker for HCC ³⁷.

Some miRNAs might be originated from repetitive elements like transposable elements (TEs) ³⁸. Hsa-miR-548as-3p is also a member of a large human gene family, derived from transposable elements named Made1 ³⁹. Approximately 69 members are found in almost all human chromosomes, especially chromosomes 6, 8, and X (30.43%). The predicted targets for hsa-miR-548as-3p are also functional molecules in many biological processes like MAPK signaling, cell cycle, p53 signaling pathway, colorectal cancer (CRC), non-small cell lung cancer, B cell receptor signaling pathway, transforming growth factor β (TGF- β) signaling pathway, renal cell carcinoma, and many others ⁴⁰. TGF- β signaling pathway is also important in post-transplant inflammation. Besides, it is considered as one of the main mediators and inducers of fibrosis. Therefore, miRNA down-regulation during the rejection period renders to overexpressed SMAD4, enhancing the signaling pathway of TGF- β ⁴¹. In addition, it has been reported that Tg737 is a target gene for miR-548as-3p, whose down-regulation facilitates HCC cell proliferation in vivo and in vitro, followed by an increase in colony formation ⁴². The present study showed that the given miRNA had significantly ($p=0.041$) reduced in rejected LT patients with HCC, accounting for its critical role in rejection.

The miRNA-4248 is located on chromosome 7, but there is not much data published about it. One study had used cross-mapping to detect miRNAs and had reported hsa-miR-4284 in cross-mapping events ⁴³. In addition, another research team had reported that such miRNAs could be the most down-regulated ones in the tissue samples of ulcerative colitis patients, and they had further claimed that miR-4284 level correlated with the disease activity by regulating CXCL5 mRNA expression ⁴⁴. Another research had found that miR-4284 and -4484 could be considered as diagnostic biomarkers for diffuse large B-cell lymphoma (DLBL) disease ⁴⁵. The down-regulation of this miRNA was further detected in LT patients with HCC in the present study. Furthermore, the sensitivity and the specificity of analyses of the four down-regulated miRNAs showed that miR194-5p and miR548as-3p might have enough qualities (Table 3) for being biomarker candidates for rejection especially among HCC patients.

In this research, a profile of miRNA was demonstrated in which miR-194-5p, -548as-3p, -122-3p, and -4284 were down-regulated and miR-3158-5p, -4449, -4511 and -4633-5p were upregulated in HCC patients with rejected LT. MiR-3158-5p is also located on chromosome 10 and has more than 500 predicted targets. The bioinformatic studies here showed that *TGF- β 2* gene could be observed among its upper 95% target scores (see Additional File 1, Table A1; www.miRDB.org). TGF- β also plays an important role in preparing a suitable microenvironment for tumor cell growth in liver diseases. This factor causes a signaling pathway that can promote progression of HCC in two ways; first, this factor has an intrinsic activity as an autocrine or paracrine growth factor, and second, it has extrinsic activity inducing changes in microenvironments like variations in cancer-associated fibroblasts, regulatory T cells (T_{reg} cells), and inflammatory mediators ⁴⁶. The other up-regulated miRNA is miR-4449, located on chromosome 4 with 22 predicted targets in miRDB (see Additional File 1, Table A2). Among the predicted targets, the cyclin-dependent kinase 5 regulatory subunit 2, p39 (CDK5R2) is detected as the most highly scored one. Previously, P39 had been introduced as a potential clinical prognostic marker for HCC ⁴⁷. Also, it had been detected that miR-4449 was a potential blood-based marker in multiple myeloma ⁴⁸. The present study

suggested that these two mentioned miRNAs (i.e., miR-3158-5p and miR-4449) had significantly ($p=0.014$ and 0.012 respectively) up-regulated in HCC patients with LT rejection. Even in analyzing sensitivity and specificity, these two miRNAs had AUC greater than 0.8 (refer to Table 4 for further information); therefore, they were proposed as a biomarker candidate for rejection in HCC patients.

The third up-regulated miRNA in HCC patients with LT rejection was miR-4633-5p, located on chromosome 5 with more than 100 predicted targets in miRDB (see Additional File 1, Table A3). In 2015, a research group had further noted that miR-4633-5p could be significantly expressed in human epidermal growth factor receptor 2 (HER2)-positive breast carcinomas among 85 tested miRNAs versus normal people ⁴⁹. The last up-regulated studied miRNA in LT rejected patients with HCC is miR-4511, located on chromosome 15 with more than 400 predicted targets in miRDB (see Additional File 1, Table A4). There are also few studies on the role of miRNA in LT and further studies are warranted to detect the exact role of this miR and its targets. The results of the present study showed the up-regulation of these two miRNAs in HCC patients with rejected LT.

It is of note that more than 97% of liver biopsies are hepatocytes and biliary cells, and the rest are Kupffer cells and endothelial ones. In LT rejected biopsies, some macrophages, plasma cells, and lymphocytes are also added to their population ⁵⁰. Given the fact that the selected miRNAs are expressed in the non-rejected group of patients and the majority of cells in biopsies are hepatocytes, it can be concluded that alterations in the expression profile of the selected miRs might be due to the rejection process in hepatocytes.

Putting together, noticing the fact that there is an urgent need for detecting biomarkers in diagnosis and prognosis of HCC, the findings of this study might be the first step in this pathway and seems to be preliminary keys for further confirmation of their regulatory role in rejection in LT patients with HCC. For instance, among down-regulated miRNAs, miR194-5p and miR548as-3p, both showed significant difference between rejected and nonrejected samples and the ROC curve analysis of these two miRNAs showed significant high specificity and sensitivity. Also, among up-regulated miRNAs in this study miR-4449 and miR-3158-5p can be considered to have important roles in causing rejection in such patients. Besides the ROC curve analysis of these two microRNAs showed significant high specificity and sensitivity. Further studies on detecting the important targets of these miRNAs, obviously would be a large finding in this field.

Although finding miRNAs as biomarkers might be very helpful for more accurate prognosis and diagnosis of HCC, some limitations interfere in this type of researches like ours. One of them is the limitation that exist for samples size. As HCC is not a very common disease, collecting tissue samples from such patients takes time, and the other limitation is lack of follow ups due to the risky and invasiveness nature of biopsy procedure. Finally, according to mentioned points, it is not possible to take tissue biopsy samples from normal population. Due to tissue specificity of miRNAs, it would be helpful for a better comparison.

Conclusion

Even though the biological function of miRNAs is yet to be fully understood, it has been shown that the tissue levels of specific miRNAs correlate well with the pathological development of several different types of cancer. Previously, it has been suggested that some blood miRNA have the potential to be used as miRNA-based blood biomarkers in cancer detection. In summary, the identification of miRNAs and small RNA species seems to represent only the tip of the iceberg. In addition, the prediction of an individual miRNA, its target, and even its function is still one of the biggest challenges in research. In the present study, two down-regulated (i.e. miR194-5p and miR584as-3p) and two up-regulated (namely, miR-4449 and miR-3158-5p) miRNAs were introduced as candidate biomarkers for LT rejection in HCC patients which need to be confirmed by further studies.

Abbreviations

alanine transaminase (ALT)

aspartate transaminase (AST)

α -fetoprotein (AFT)

Hepatocellular carcinoma (HCC)

microRNAs (miRNAs)

3'-untranslated regions (3'-UTRs)

LNA-array (locked nucleic acid-array)

formalin-fixed paraffin-embedded (FFPE)

transposable elements (TEs)

Transforming growth factor β (TGF- β)

large B-cell lymphoma (DLBL)

regulatory T cells (T_{reg} cells)

colorectal cancer (CRC)

cyclin-dependent kinase 5 regulatory subunit 2, p39 (CDK5R2)

human epidermal growth factor receptor 2 (HER2)

Declarations

Ethics approval and consent to participate: All applied protocols in this study, were in complete agreement with the Helsinki Declaration and its later amendment. The local Ethics committee of Shiraz University of Medical Sciences approved every step of this study. After explaining the study objectives, written informed consent was obtained from each patient.

Consent for publication: written informed consent for publication was obtained.

Availability of data and material: The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests: The authors declare that they have no competing interests.

Funding: This work was supported by grants from Transplant Research Center, Shiraz University of Medical Sciences, with grant No. 91/181. Funding sources had no influence over study design, data collection, analysis or interpretation or manuscript preparation and submission.

Authors' contributions: AA performed the majority of experiments and analyzed the data; AA, KM, YR performed the molecular investigations; AA, KM, YR and MJ designed and coordinated the research; AA, KM, YR and MJ wrote the paper. AA and YR have equal role as first author.

Acknowledgements: The authors wish to thank Mr. H. Argasi at the Research Consultation Center (RCC) of Shiraz University of Medical Sciences for his invaluable assistance in editing this manuscript.

References

1. Chen XM. MicroRNA signatures in liver diseases. *World J Gastroenterol*. 2009;15(14):1665-1672. doi:10.3748/wjg.15.1665
2. Huang S, He X. The role of microRNAs in liver cancer progression. *Br J Cancer*. 2011;104(2):235-240. doi:10.1038/sj.bjc.6606010
3. Dragani TA. Risk of HCC: Genetic heterogeneity and complex genetics. *J Hepatol*. 2010;52(2):252-257. doi:10.1016/j.jhep.2009.11.015
4. Jemal A, Center MM, DeSantis C, Ward EM. Global Patterns of Cancer Incidence and Mortality Rates and Trends. *Cancer Epidemiol Biomarkers Prev*. 2010;19(8):1893-1907. doi:10.1158/1055-9965.EPI-10-0437
5. Castoldi M, Schmidt S, Benes V, et al. A sensitive array for microRNA expression profiling (miChip) based on locked nucleic acids (LNA) A sensitive array for microRNA expression profiling (miChip) based on locked nucleic acids (LNA). *RNA Soc*. 2006;12:913-920. doi:10.1261/rna.2332406.a
6. Barad O, Meiri E, Avniel A, et al. MicroRNA expression detected by oligonucleotide microarrays: System establishment and expression profiling in human tissues. *Methods*. 2004;14:2486-2494. doi:10.1101/gr.2845604.4

7. Song Q, Song J, Wang Q, et al. miR-548d-3p/TP53BP2 axis regulates the proliferation and apoptosis of breast cancer cells. *Cancer Med*. 2016;5(2):315-324. doi:10.1002/cam4.567
8. Lewis AP, Jopling CL. Regulation and biological function of the liver-specific miR-122. *Biochem Soc Trans*. 2010;38(6):1553-1557. doi:10.1042/BST0381553
9. Hayes C, Chayama K. MicroRNAs as Biomarkers for Liver Disease and Hepatocellular Carcinoma. *Int J Mol Sci*. 2016;17(3):280. doi:10.3390/ijms17030280
10. Murakami Y, Yasuda T, Saigo K, et al. Comprehensive analysis of microRNA expression patterns in hepatocellular carcinoma and non-tumorous tissues. *Oncogene*. 2006;25(17):2537-2545. doi:10.1038/sj.onc.1209283
11. Yang N, Ekanem NR, Sakyi CA, Ray SD. Hepatocellular carcinoma and microRNA: New perspectives on therapeutics and diagnostics. *Adv Drug Deliv Rev*. 2015;81:62-74. doi:10.1016/j.addr.2014.10.029
12. Gramantieri L, Ferracin M, Fornari F, et al. Cyclin G1 is a target of miR-122a, a MicroRNA frequently down-regulated in human hepatocellular carcinoma. *Cancer Res*. 2007;67(13):6092-6099. doi:10.1158/0008-5472.CAN-06-4607
13. Burchard J, Zhang C, Liu AM, et al. MicroRNA-122 as a regulator of mitochondrial metabolic gene network in hepatocellular carcinoma. *Mol Syst Biol*. 2010;6:402. doi:10.1038/msb.2010.58
14. Mizuguchi Y, Mishima T, Yokomuro S, et al. Sequencing and Bioinformatics-Based Analyses of the microRNA Transcriptome in Hepatitis B–Related Hepatocellular Carcinoma. Guan X, ed. *PLoS One*. 2011;6(1):e15304. doi:10.1371/journal.pone.0015304
15. Wang Y, Lee ATC, Ma JZI, et al. Profiling microRNA expression in hepatocellular carcinoma reveals microRNA-224 up-regulation and apoptosis inhibitor-5 as a microRNA-224-specific target. *J Biol Chem*. 2008;283(19):13205-13215. doi:10.1074/jbc.M707629200
16. Sui W, Lin H, Peng W, et al. Molecular dysfunctions in acute rejection after renal transplantation revealed by integrated analysis of transcription factor, microRNA and long noncoding RNA. *Genomics*. 2013;102(4):310-322. doi:10.1016/j.ygeno.2013.05.002
17. Shaked A, Chang BL, Barnes MR, et al. An ectopically expressed serum miRNA signature is prognostic, diagnostic, and biologically related to liver allograft rejection. *Hepatology*. 2017;65(1):269-280. doi:10.1002/hep.28786
18. Morita M, Chen J, Fujino M, et al. Identification of microRNAs involved in acute rejection and spontaneous tolerance in murine hepatic allografts. *Sci Rep*. 2014;4. doi:10.1038/srep06649
19. Castoldi M, V B, MW H, MU M. miChip: a microarray platform for expression profiling of microRNAs based on locked nucleic acid (LNA) oligonucleotide capture probes. *Methods*. 2007;43(2). doi:10.1016/J.YMETH.2007.04.009
20. Castoldi M, S S, V B, MW H, MU M. miChip: an array-based method for microRNA expression profiling using locked nucleic acid capture probes. *Nat Protoc*. 2008;3(2). doi:10.1038/NPROT.2008.4
21. Li W, K R. MicroRNA detection by microarray. *Anal Bioanal Chem*. 2009;394(4). doi:10.1007/S00216-008-2570-2

22. American Joint Committee on Cancer. <https://www.facs.org/quality-programs/cancer/ajcc>. Accessed November 22, 2020.
23. Chen X-M, Splinter PL, O'Hara SP, LaRusso NF. A cellular micro-RNA, let-7i, regulates Toll-like receptor 4 expression and contributes to cholangiocyte immune responses against *Cryptosporidium parvum* infection. *J Biol Chem*. 2007;282(39):28929-28938. doi:10.1074/jbc.M702633200
24. Asaoka T, Hernandez D, Tryphonopoulos P, et al. Clinical significance of intragraft miR-122 and -155 expression after liver transplantation. *Hepatol Res*. 2015;45(8):898-905. doi:10.1111/hepr.12424
25. Jin Y, Wong YS, Goh BKP, et al. Circulating microRNAs as Potential Diagnostic and Prognostic Biomarkers in Hepatocellular Carcinoma. *Sci Rep*. 2019;9(1):1-12. doi:10.1038/s41598-019-46872-8
26. Miyaaki H, Takatsuki M, Ichikawa T, et al. Intrahepatic microRNA profile of liver transplant recipients with hepatitis C virus co-infected with human immunodeficiency virus. *Ann Transplant*. 2017;22:701-706. doi:10.12659/AOT.906236
27. Chang J, Nicolas E, Marks D, et al. miR-122, a mammalian liver-specific microRNA, is processed from hcr mRNA and may downregulate the high affinity cationic amino acid transporter CAT-1. *RNA Biol*. 2004;1(2):106-113. <http://www.ncbi.nlm.nih.gov/pubmed/17179747>. Accessed July 4, 2017.
28. Arora A, Simpson DA. Individual mRNA expression profiles reveal the effects of specific microRNAs. *Genome Biol*. 2008;9(5):R82. doi:10.1186/gb-2008-9-5-r82
29. Wienholds E, Kloosterman WP, Miska E, et al. MicroRNA Expression in Zebrafish Embryonic Development. *Science (80-)*. 2005;309(5732):310-311. doi:10.1126/science.1114519
30. Coulouarn C, Factor VM, Andersen JB, Durkin ME, Thorgeirsson SS. Loss of miR-122 expression in liver cancer correlates with suppression of the hepatic phenotype and gain of metastatic properties. *Oncogene*. 2009;28(40):3526-3536. doi:10.1038/onc.2009.211
31. Meng Z, Fu X, Chen X, et al. miR-194 is a marker of hepatic epithelial cells and suppresses metastasis of liver cancer cells in mice. *Hepatology*. 2010;52(6):2148-2157. doi:10.1002/hep.23915
32. Farid WRR, Pan Q, van der Meer AJP, et al. Hepatocyte-derived microRNAs as serum biomarkers of hepatic injury and rejection after liver transplantation. *Liver Transpl*. 2012;18(3):290-297. doi:10.1002/lt.22438
33. Raymond CK, Roberts BS, Garrett-Engle P, Lim LP, Johnson JM. Simple, quantitative primer-extension PCR assay for direct monitoring of microRNAs and short-interfering RNAs. *RNA*. 2005;11(11):1737-1744. doi:10.1261/rna.2148705
34. Venugopal SK, Jiang J, Kim T-H, et al. Liver fibrosis causes downregulation of miRNA-150 and miRNA-194 in hepatic stellate cells, and their overexpression causes decreased stellate cell activation. *Am J Physiol Gastrointest Liver Physiol*. 2010;298(1):G101-6. doi:10.1152/ajpgi.00220.2009
35. Jung HS, Seo Y-R, Yang YM, et al. Gα12gep oncogene inhibits FOXO1 in hepatocellular carcinoma as a consequence of miR-135b and miR-194 dysregulation. *Cell Signal*. 2014;26(7):1456-1465. doi:10.1016/j.cellsig.2014.02.022

36. Farid WRR, Pan Q, Van Der Meer AJP, et al. Hepatocyte-derived microRNAs as serum biomarkers of hepatic injury and rejection after liver transplantation. *Liver Transplant*. 2012;18(3):290-297. doi:10.1002/lt.22438
37. Zhao Y, Li F, Zhang X, et al. MicroRNA-194 acts as a prognostic marker and inhibits proliferation in hepatocellular carcinoma by targeting MAP4K4. *Int J Clin Exp Pathol*. 2015;8(10):12446-12454. <http://www.ncbi.nlm.nih.gov/pubmed/26722431>. Accessed July 4, 2017.
38. Yuan Z, Sun X, Liu H, Xie J. MicroRNA Genes Derived from Repetitive Elements and Expanded by Segmental Duplication Events in Mammalian Genomes. Tjwa M, ed. *PLoS One*. 2011;6(3):e17666. doi:10.1371/journal.pone.0017666
39. Piriyaopongsa J, Jordan IK. A family of human microRNA genes from miniature inverted-repeat transposable elements. Christoffels A, ed. *PLoS One*. 2007;2(2):e203. doi:10.1371/journal.pone.0000203
40. Liang T, Guo L, Liu C. Genome-Wide Analysis of mir-548 Gene Family Reveals Evolutionary and Functional Implications. *J Biomed Biotechnol*. 2012;2012:1-8. doi:10.1155/2012/679563
41. Hamdorf M, Kawakita S, Everly M. The Potential of MicroRNAs as Novel Biomarkers for Transplant Rejection. *J Immunol Res*. 2017;2017:1-12. doi:10.1155/2017/4072364
42. Zhao G, Wang T, Huang Q-K, et al. MicroRNA-548a-5p promotes proliferation and inhibits apoptosis in hepatocellular carcinoma cells by targeting Tg737. *World J Gastroenterol*. 2016;22(23):5364. doi:10.3748/wjg.v22.i23.5364
43. Guo L, Liang T, Gu W, Xu Y, Bai Y, Lu Z. Cross-mapping events in miRNAs reveal potential miRNA-Mimics and evolutionary implications. *PLoS One*. 2011;6(5). doi:10.1371/journal.pone.0020517
44. Koukos G, Polytarchou C, Kaplan JL, et al. A MicroRNA Signature in Pediatric Ulcerative Colitis. *Inflamm Bowel Dis*. 2015;21(5):996-1005. doi:10.1097/MIB.0000000000000339
45. Tamaddon G, Geramizadeh B, Karimi MH, Mowla SJ, Abroun S. miR-4284 and miR-4484 as Putative Biomarkers for Diffuse Large B-Cell Lymphoma. *Iran J Med Sci*. 2016;41(4):334-339. <http://www.ncbi.nlm.nih.gov/pubmed/27365556>. Accessed July 4, 2017.
46. Giannelli G, Villa E, Lahn M. Transforming growth factor- β as a therapeutic target in hepatocellular carcinoma. *Cancer Res*. 2014;74(7):1890-1894. doi:10.1158/0008-5472.CAN-14-0243
47. Lu J-W, Chang J-G, Yeh K-T, Chen R-M, Tsai JJP, Hu R-M. Decreased expression of p39 is associated with a poor prognosis in human hepatocellular carcinoma. *Med Oncol*. 2011;28(S1):239-245. doi:10.1007/s12032-010-9707-9
48. Shen X, Ye Y, Qi J, et al. Identification of a novel microRNA, miR-4449, as a potential blood based marker in multiple myeloma. *Clin Chem Lab Med*. 2017;55(5):748-754. doi:10.1515/cclm-2015-1108
49. Vos S, Vesuna F, Raman V, van Diest PJ, van der Groep P. miRNA expression patterns in normal breast tissue and invasive breast cancers of BRCA1 and BRCA2 germ-line mutation carriers. *Oncotarget*. 2015;6(31):32115-32137. doi:10.18632/oncotarget.5617
50. Ross MH, Kaye GI, Pawlina W. *Histology: A Text and Atlas with Cell and Molecular Biology*. Lippincott Williams & Wilkins; 2003. <https://books.google.com/books/about/Histology.html?>

Tables

Table 1: Descriptive statistics related to first group (test group) of patients

			Rejection condition		Total
			Rejected	Nonrejected	
Gender	Male	Count	3	6	9
		% within gender	33.3%	66.7%	100.0%
		% within rejection	60.0%	66.7%	64.3%
	Female	Count	2	3	5
		% within gender	40.0%	60.0%	100.0%
		% within rejection	40.0%	33.3%	35.7%
Total	Count	5	9	14	
	% within gender	35.7%	64.3%	100.0%	
	% within rejection	100.0%	100.0%	100.0%	

Table 2: The table shows the selected differentially expressed microRNA candidates, ranked according to the absolute value of the log fold change from the test group of samples.

Annotation	Average Hy3	Non-reject fold change	Reject fold change	Log FC
hsa-miR-122-3p	9.025	1.068	-1.013	-0.793
hsa-miR-194-5p	9.161	0.606	-1.051	-0.258
hsa-miR-548as-3p	8.661	0.015	-1.714	-1.326
hsa-miR-4284	13.779	0.893	-1.069	-0.759
hsa-miR-3158-5p	8.303	-1.651	0.745	0.873
hsa-miR-4449	7.259	-1.399	0.935	1.035
hsa-miR-4511	7.337	-1.656	0.775	1.204
hsa-miR-4633-5p	8.176	-1.704	0.658	0.973

Table 3: Sensitivity and specificity of down-regulated microRNAs between nonrejected and rejected groups of patients (validation group) after transplantation

miRNA	Area under the curve (AUC)	<i>p</i> value	95%CI
has-miR122-3p	0.7083	0.1228	0.4740-0.9427
has-miR4284	0.5228	0.8688	0.2506-0.7949
has-miR194-5p	0.7987	0.0372	0.5414-1.000
has-miR548as-3p	0.8250	0.0344	0.645-1.000

Table 4: Sensitivity and specificity of up-regulated microRNAs between nonrejected and rejected groups of patients (validation group) after transplantation

miRNA	Area under the curve (AUC)	<i>p</i> value	95%CI
has-miR4511	0.6000	0.5152	0.3134-0.8866
has-miR4633-5p	0.7424	0.1078	0.4985-0.9864
has-miR4449	0.8385	0.0122	0.6620-1.000
has-miR3158-5p	0.8561	0.0182	0.5946-1.000

Figures

Figure 1

The study sample division chart. The test group was composed of 14 liver transplanted patients and the validation group consisted of 30 liver transplanted patients.

Figure 2

A) Matrix PCA plot. Analysis was performed on all samples and the normalized log ratio values were used for the analysis. B) Each row represents a microRNA and each column represents a sample. The color scale illustrates the relative expression level of microRNAs. Red color represents an expression level below the reference, and green color represents expression higher than the reference genes. Pooled sample groups were used in order to reach more exact analyses and the results mentioned here is related to pooled samples. Here, PR means pool of reject samples while PNR means pool of nonrejected samples and R means one reject sample while NR means one nonrejected sample.

Figure 3

The expression level of selected miRNAs for down regulated miRNAs (A) and for up regulated ones (B) based on fold change. The fold changes are calculated by livak method ($2^{-\Delta\Delta Ct}$).

ROC curve: ROC of hsa-miR122-3p

ROC curve: ROC of hsa-miR4284

ROC curve: ROC of hsa-miR194-5p

ROC curve: ROC of hsa-miR548as-3p

Figure 4

Calculating the sensitivity and specificity of down-regulated microRNAs between nonrejected and rejected groups of patients after transplantation.

Figure 5

Calculating the sensitivity and specificity of up-regulated microRNAs between nonrejected and rejected groups of patients after transplantation.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [Additionalfile.docx](#)