



miR-515-5p and Notch1 as New Diagnostic Markers of Hepatocellular Carcinoma

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Abstract

Background: Modifications of miRNA expression have been related to various types of cancers including hepatocellular carcinoma (HCC). miRNAs directly act as repressors of gene expression, as they reside in fragile sites, as well as cancer-related genomic regions. Notch signaling is a conserved evolutionary pathway that controls cell functions. The dysregulation of this pathway leads to different diseases such as cancer.

Objectives: This study aimed to investigate the role of miR-515-5p and Notch1 as new diagnostic markers in HCC.

Methods: Forty formalin fixed paraffin embedded (FFPE) autopsy blocks and 40 FFPE normal liver tissues were selected from the archives of the pathology of Imam Reza hospital, Tabriz, Iran. Real-time polymerase chain reaction (PCR) was used for gene expression. Immune histochemistry method was used for detecting notch1 in normal and cancer FFPE tissues. Hematoxylin and eosin staining was also used for the diagnosis of normal and cancerous tissues.

Results: miR-515-5P showed higher expression in the cancer group compared to the normal group (4.7 fold). Hematoxylin and eosin staining of HCC tissues showed significant color intensity than that of normal tissues. Immune histochemistry results revealed significant Ag-Ab reaction in the cancer group. In this study, we analyzed miRNA gene expression and notch 1 level in HCC patients. miRNA dysregulation has been found in a large variety of HCCs. Hepatocarcinogenesis was associated with the expression level of miR-515-5p with carcinogenesis. Moreover, notch1 was a key protein in liver cell fate and a progressive molecule in HCC.

Conclusion: Our study demonstrated the main role of miR-515-5p in the pathogenesis of HCC. Likewise, it disclosed the expression of these genes could be utilized in HCC prognosis.

Keywords: Biomarker, Neoplasm, Notch1 protein, MicroRNA

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Background

Hepatocellular carcinoma (HCC) is a common form of liver cancer in Africa and Asia (1), as well as being a global health issue. Efforts should be made to improve treatment strategies and prognosis in HCC and recognize novel prognostic and predictive markers (2). HCC is a multifactorial and heterogeneous neoplasm with some genomic variations.

Potential curative treatment is required for 40% of patients (resection, transplantation, or local ablation) and chemotherapy for 20% (3,4).

Formalin fixed paraffin embedded (FFPE) materials afford a pathologically massive documentation for potential use in bimolecular studies and are available

universally (5). For a long-term storage, human tissue samples are fixed in formalin and inserted in paraffin for decades (6). Therefore, many institutes hold a vast number of books on paraffin-block that make it possible to preserve neoplasm samples such as those of sporadic tumors for a long time (7). Diagnosis of new biomarkers is required for HCC treatment. MicroRNAs (miRNAs) control essential functions especially gene expression and have a major effect on post-transcriptional regulation of gene expression (8). They control numerous cellular functions such as differentiation and apoptosis, as well as physiological and pathological procedures (9). MicroRNAs are connected with malignancies such as HCC. Alterations in microRNA expression have been associated with various types of

cancers. These miRNAs act as gene suppressors and are located at fragile sites and cancer-associated genomic regions. Abnormal expression of several miRNAs was discovered in human hepatocarcinogenesis (10,11). Various pathological features viz human malignancies, circulatory, and metabolic diseases have also been connected with miRNA dysregulation (12).

Notch signaling is an evolutionarily conserved pathway which controls numerous physiological phenomena including cell proliferation, differentiation, and apoptosis (13). Notch pathway includes elements like receptors, negative and positive modifiers, and translational factors (14). In mammals, this pathway plays crucial roles including regulation of metabolism, inflammation, liver regeneration, and repair (15).

Moreover, Notch signaling plays an important role in cancer. It is more critical than other conserved signaling pathways owing to its role in canonical and non-canonical signaling (16). The most traditional canonical pathway happens in cell-to-cell interactions (17,18). Notch has many carcinogenesis roles. In one study, the dysregulation of Notch signaling led to the overexpression of cyclin E, as an oncogenic protein, and development of cholangiocellular carcinoma (19). Inhibited notch activity caused tumor cell proliferation and apoptosis (20).

In this study, we aimed to investigate the role of miR-515-5p and Notch1 as new diagnostic markers in HCC.

Materials and Methods

FFPE materials are a valuable source for gene expression analyses. We randomly selected 40 FFPE autopsy blocks from all normal liver FFPE tissues and 40 HCC tissues from the Archives of the Pathology in Imam Reza hospital, Tabriz University of Medical Sciences (Table 1) (21).

We used 5 μ m histological sections from each block as mentioned in the protocol. Sterile conditions for each block was used to eliminate tissue contamination. For

Table 1. Demographic Information of the Study Population

	Control group (n=40)	Cancer group (n=40)	P-value
Sex (male: female)	32:8	30:10	0.392
Alcohol consumption (yes: no)	13:27	11:29	0.404
Cirrhosis (yes: no)	35:5	22:18	0.001
Hepatitis B Positive: negative)	6:34	21:19	0.001
Tumor size >5 cm: <5 cm	-	11:29	-
Metastasis stage (early: intermediate: advanced)	-	12:7:21	-

quantification of microRNAs by real-time polymerase chain reaction (PCR), we used RealQ Plus Ampliqon. Prime Script RT kit (Takara) was designed with random and oligo-dT primers for cDNA synthesis. Nano drop was used for measuring RNA concentration and RNA quality was evaluated using gel electrophoresis (Figure 1). Primer sequence was designed by miRBase program (Table 2). Furthermore, real-time PCR was performed in duplicate to evaluate gene expression in healthy and tumor samples and averaged from obtained threshold cycles. In all samples, RNU6 was used as the housekeeping gene. In this study, $2^{-\Delta\Delta C_t}$ method was used to show the extent of gene expression changes between the control and cancer groups due to the efficiency of PCR reactions, and results were calculated as fold change.

Statistical analyses were done using SPSS software version 19.0. We used Santa Cruz IHC (immunohistochemistry) kit for protein level evaluation. Results were quantified by ImageJ program.

Results

miR-515- 5p in cancer group showed high expression level than that in normal group (4.7 fold) ($P=0.001$, Figure 2). This gene is a member of c19 cluster which significantly increases in HCC patients. miR-515-5p gene has oncogenic properties and, in several malignancies, it is

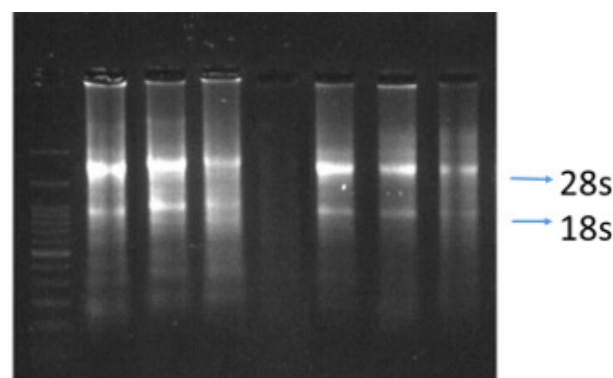


Figure 1. Integrity of Total RNA on Agarose Gel (1.5%).

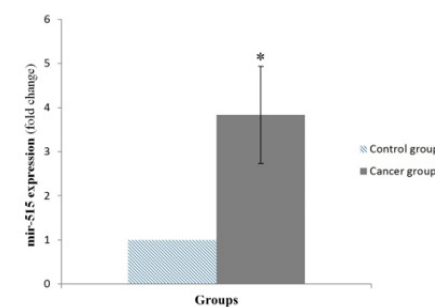


Figure 2. Fold Change of miR-515-5p. Data presented as mean \pm SD.

overexpressed (22).

For the evaluation of tumor marker, ROC curve was analyzed. Sensitivity and specificity of this gene was 95% and 98%, respectively (Figure 3).

In this study, cut off point refers to a critical point that distinguishes cases and controls and miR-515-5p cut point upper than this point refers to the control group.

For distinguishing cancer tissues and normal liver tissues, IHC and hematoxylin and eosin staining were done at first to distinguish cancer and non-cancer cases (Figure 4).

Based on hematoxylin and eosin staining results, the

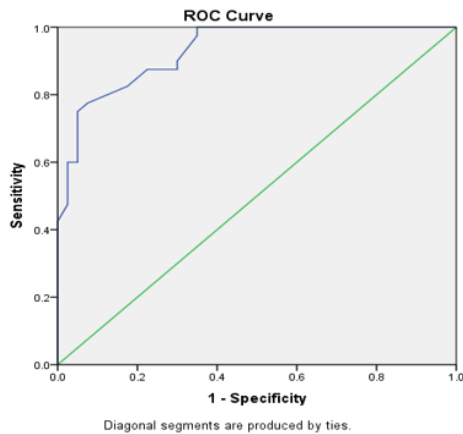


Figure 3. miR-515-5p ROC Curve.

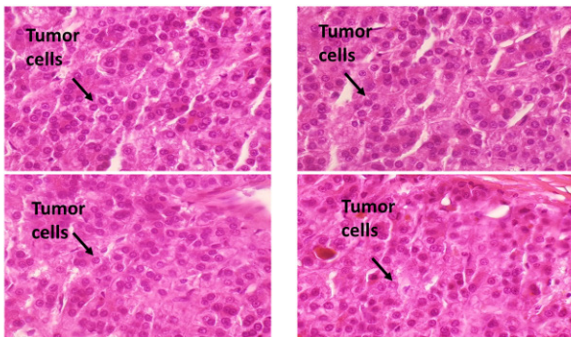


Figure 4a. Hematoxylin and Eosin Staining of Cancerous Liver. Note. Staining shows more circular nodules because of tumor cell proliferation.

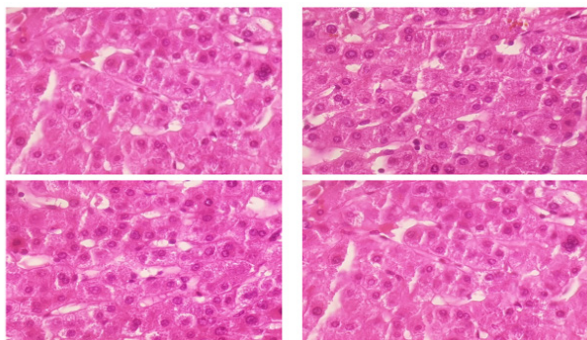


Figure 4b. Hematoxylin and Eosin Staining of Normal Liver Tissues.

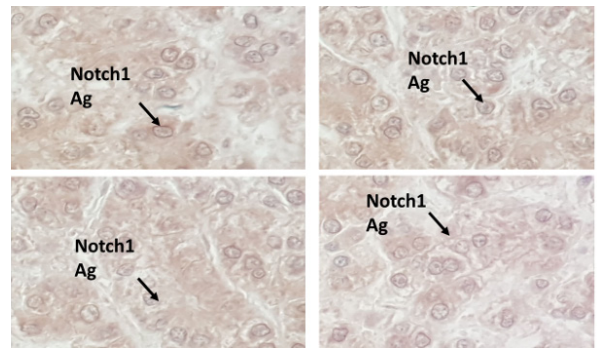


Figure 5a. IHC of HCC Liver Tissues. Note. Notch 1 as a membrane protein reacts with secondary Ab which is clear in all four figures.

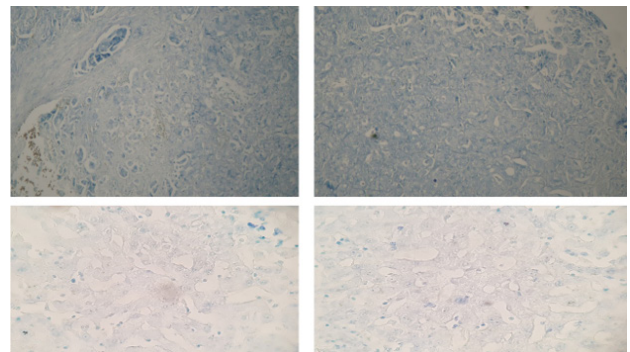


Figure 5b. IHC Normal Liver Tissue.

cancer group had numerous colored cells because of HCC cells compared to the non-cancer group.

Tissues were also assessed by IHC (Figure 5). Normal tissues showed no Ab-Ag reaction but cancer tissues showed circular membranous pigments that could be referred to the positive reaction between Notch1 and secondary Ab. Then positive and negative tissues were quantified by imageJ program (Table 3).

HCC tissues showed more positive Notch1 reaction compared to the normal liver with a *P* value of 0.001.

Discussion

For the assessment of RNA-based biomarkers, archived FFPE tissues are potential reservoirs based on studies, especially for diagnosis (23). FFPE archives are universally available and can be used for clinical cases due to chemical modifications and over-time degradation. RNA Extraction from such materials has been controversial.

In this study, we found that miR-515- 5p gene expression level was about four times higher in cancerous liver tissue compared to the normal tissue. In confirmation of our results, Augello et al reported that the level of cluster miRNAs on chromosome 19, which also includes miR-515-5p, was increased (24).

High expression of miR-515-5p in cancer cells which

Table 2. Primer Sequence of hsa-miR-515-5p

Primer	Primer Sequence
hsa-miR-515-5p	ACACCTGCCACCCAG GGTCCAGTTTTTTTTTTTTTCTTG

Table 3. Notch1 Score

Tissue	Notch1 level		Notch1 Score
	Positive No. (%)	Negative No. (%)	
Cancerous tissue	9 (22.5)	31 (77.5)	0.1± 2.6
Normal liver	34 (85)	6 (15)	0.03±1.0
<i>P</i> -value			0.001

have oncogenic properties, may interfere with the differentiation and proliferation of cells in the tumorigenic mechanism, and also induce metastasis by inducing cell invasion.

Li and colleagues also showed that it is possible to extract total RNA from FFPE blocks. Even this group has suggested that measuring the small amount of RNA in paraffin blocks can be a suitable method for molecular pathology studies, because of the less damage of small RNA molecules (such as miRNAs) to mRNAs (24).

miRNAs play critical roles in cancer, and their prognostic or diagnostic potency has been reported in numerous studies.

Augello et al reported that miRNAs could be used for cancer diagnosis; as for example, miR-515-5p is upregulated in metastatic HCC (25). Once a reliable miRNA is selected, it is expected that it yields an easy and precise alternative for cancer diagnosis. Importance of Notch in controlling mammalian cell fate has been diagnosed, along with its function which is crucial for development, particularly that Notch is a key regular of organ homeostasis (26).

Deregulation of Notch signaling is associated with liver malignancies, such as HCC. Dill et al reported Notch signaling pathway exhibits a carcinogenic role in HCC (27). Villanueva et al reported Notch expression deregulation in several malignancies such as cervical, endometrial, renal, lung, and gastric carcinomas (28). Our study showed that Notch 1 protein level in cancer tissues is higher than that in normal FFPE tissues. IHC results also showed significant Ab-Ag reaction in cancer tissues. Therefore, Notch signaling could be a target for therapeutic modulation of liver metabolism in diabetes and hepatosteatosis.

Conflict of Interests

The authors declare no potential conflict of interests relevant to this article.

Ethical issues

This study was approved by the Ethics Committee of Tabriz University of Medical Sciences (Code of Ethics: IR.TBZMED.REC.1396.941).

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