Evaluation of miR-625 and miR-302a Expression in Renal Cell Carcinoma

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ABSTRACT

BACKGROUND: Renal cell carcinoma (RCC) is the most common and malignant tumor of kidney tissue, with unknown molecular mechanism and pathogenesis. Recent studies have demonstrated that micro ribonucleic acids (miRNAs) may have potential role in initiation and progression of RCC. In this study we evaluated expression patterns of two RNA molecules, miR-625 and miR-302a in tumor and non-tumor portions of RCC samples.

METHODS: The relative expression levels of miR-625 and miR-302a were delineated by real-time PCR in 20 formalin-fixed paraffin-embedded (FFPE) RCC samples and compared with 20 non-tumor matched samples obtained from margins of the same samples.

RESULTS: We found approximately 15-fold increase in miR-625 expression in tumor samples compared to non-tumor samples of each patient, but it was not a significant difference (P Value: 0.097). On the other hand, miR-302a expression was similar between tumor and non-tumor sections of the sample (P Value > 0.05). Additionally, we did not find an association between age, gender, grade and stage of tumors, and expression level of either of the two miRNAs.

CONCLUSION: Our data demonstrated that there is no significant difference of miR-625 and miR-302a expression in tumor samples of RCC when compared to non-tumor samples. We further found no relationship between clinico-pathological characteristics and expression levels of miR-625 or miR-302a.

Keywords: Carcinoma; MicroRNAs; miR-625; Human; Gene Expression; Real-Time Polymerase Chain Reaction

INTRODUCTION

Renal cell carcinoma (RCC) and Wilms tumor are the most common types of kidney cancer among adults and children, respectively [1]. RCC is the seventh most common cancer in men and ninth in women [2]. Micro RNAs are a class of short non-coding RNA molecule with a single strand of 18-23 nucleotides that regulate expression of genes at post-transcriptional level. Recent evidence has demonstrated a role of micro RNAs in initiation and progression of different kinds of tumors [3-5]. Studies have found aberrant expression of miR-625 in several human malignancies including esophageal, gastric and breast cancers, acute lymphoblastic leukemia, and multiple myeloma [7-11]. The miR-625 molecules may regulate proliferation and invasion of cancer cells by targeting mRNA of SOX2 gene [12]. Additionally, miR-302a, an ESCs (embryonic stem cells) micro RNA, have expression patterns similar to OCT4 and SOX2. Transcription factors of SOX2/OCT4 bind to promoter region of miR-302a and induce its expression [13]. SOX2 and OCT4 may have a role in determining pluripotency and a potential role in oncogenesis [6]. Down-regulation of miR-302a has been reported in several human cancers including

prostate, breast and colorectal cancers; this data supports a functional role of miR-302a as a tumor suppressor [14-16]. While the roles of miR-302a and miR-625 have been examined in several malignancies, their role has not be examined in RCC. Therefore, we aimed to evaluate expression patterns of these two miRNAs in formalin-fixed paraffin-embedded (FFPE) tumors and their matched non-tumor sections in samples of RCC by real-time PCR technique.

METHODS

In silico analysis of target and expression profile of miR-625: We performed a bioinformatics analysis over Target scan database (*targetscan.org*) to evaluate the interaction between miR-625 and SOX2 mRNA. Based on mirwalk2 *(zmf.umm.uni-heidelberg.de/apps/zmf/mirwalk2),* validated miRNAs which target SOX2 gene was also considered. Additionally, miRmine Human miRNA Expression Database *(http://guanlab.ccmb.med.umich.edu/mirmie*) was explored to find expression profile of miR-625 in some human tissues.

Clinical RCC specimens: In this matched case-control study, 20 malignant and 20 adjacent non-malignant (health kidney tissue) FFPE tissue samples were obtained from specimen repository at our institution. These samples were collected between the years of 2010 to 2015. The grade and stage of tumor was assessed by an expert pathologist. World Health Organization (WHO) criteria and TNM classification were used for grade and stage classification. The Ethics Committees at our institution approved the experimental design. Paraffin block was cut at 0.5-micron thickness with a microtome. After fragmentation and deparaffinization by xylene solution and drying with alcohol, samples were treated with proteinase K.

Cell culture: The NTERA2 (NT2) cell line, embryonic-like cells, was obtained from Tarbiat Modares University (Iran, Tehran) to use as positive control. These cells share many characteristics with human ESCs, including similar patterns of gene expression and regulation [17,18]. The cells were cultured in DMEM (Gibco, USA) medium, supplemented with penicillin/streptomycin and 10% fetal bovine serum (FBS) at 37°C in a humidified atmosphere of 5% CO2. The cells were then lysed for RNA extraction, cDNA synthesis, and real-time PCR assay.

RNA extraction and cDNA synthesis: Total RNA was isolated from tissue specimens, using TRIzol reagent (Invitrogen, Carlsbad, CA), according to manufacturer's instruction. In order to remove any potential DNA contamination, the extracted RNA molecules were incubated with RNase-free DNaseI enzyme (Takara, Japan). The concentration and purity of the isolated RNA were delineated by a NanoDropND-100 spectrophotometer. The synthesis of cDNA was carried out by ParsGenome's miRNA amplification kit (ParsGenome Co., Iran) according to manufacturer's instruction. Briefly, the tubes were incubated for 60 minutes at 44°C followed by heat inactivation of the reverse transcriptase enzyme for 1 minute at 85°C.

Real-time PCR: Quantitative real-time PCR was performed using miR-625 and miR-302a primers obtained from ParsGenome Co. (Iran), as well as SYBR Green master mix (ParsGenomeCo., Iran) according to manufacturer's instruction. 5SrRNA gene was considered a housekeeping control gene. PCR reactions were performed at 95°C for 30 seconds followed by 40 cycles of 95°C for 5 seconds, and 60°C for 20 seconds in an ABI 7300 real-time quantitative PCR system (Applied Bio systems, USA). As mentioned above, NT2cells were considered as positive control of miR302a expression.

Statistical analysis: Due to non-normality of our data, we used non-parametric statistical tests to determine the difference between malignant and non-malignant section of the samples. The comparison of miR-625 expression levels between tumor and normal kidney tissue of patients was determined by Wilcoxon test (two related samples test). Briefly, to calculate the fold change expression of our candidate miRNAs, expression level in each sample was normalized to that of 5SrRNA, as a housekeeping control gene. Then, the expression level of each miRNA in tumor tissue samples was normalized to their matched non-tumor compartment. All real-time PCR data was analyzed with statistical program for social sciences (SPSS) software version 18 and MINITAB16, and $p<0.05$ was considered statistically significant. Data is presented as mean ± standard deviation (SD). Correlations between fold change and clinico-pathological characteristics such as grade, gender, and age were evaluated by Mann-Whitney test (2- independent samples test), and stage of tumor by Kruskal-

Wallis test (K independent samples). Correlations between miR-625 and miR-302a expressions were evaluated using Spearman correlation coefficient.

RESULTS

In silico characteristics of miR-625 target site and expression profile: Bioinformatics analysis over the Target scan database demonstrated that miR-625 has a binding site on 3'-UTR of SOX2 mRNA (Figure 1A). Validated miRNAs (according to original literature) which target SOX2 gene was evaluated over the Mirwalk2 database. The data revealed SOX2 mRNA is targeted by several miRNAs, in particular by miR-625 molecule (Figure 1B). Moreover, an exploration over the miRmine Human miRNA Expression Database (a collection of expression profiles from different publicly available miRNA-seq datasets) delineated expression profile signature of miR-625 in some human tissues such as bladder, blood and breast (Figure 1C).

Optimizing miRNAs extraction and amplification: We used 20 FFPE samples of patients with RCC in this study. Use of quality control ensured acceptable ratios for purity and concentration of RNA molecules isolated from the FFPE samples. The specificity of all primers was determined by examining the melting curves analysis. There was no amplification product in negative controls (also known as no RT) (Figure 2).

miR-625 in RCC tumors: We found higher fold-expression of miR-625 in tumor than in the control cells (Figure 3), however, this fold-difference did not reach statistical significance (fold change: 14.92, *P value: 0.097*). In contrast to miR-625, miR-302a expression was at a low levelin both tumor and non-tumor samples and failed to reveal a significant difference between the two matched groups. Furthermore, we failed to detect any significant and considerable correlation between expression levels of miR-625 and miR-302a in RCC cancer tissues by Spearman correlation coefficient (data not shown).

Association of miRNAs expression with patients' clinicopathological characteristics: A comparison between RCC tumors of different grade of malignancies including low grade (I-II) and high grade (III-IV) tumors failed to reveal a significant difference in miR-625 gene expression between the two groups. Furthermore, we could not detect any significant difference of miR-625 gene expression between RCC tumors at different stages of malignancies. Similarly, there was no difference in the expression levels by age or gender of patients.

Expression of miR-625 is compared between tumors vs. non–tumor ones, in high-grade vs. low-grade tumors, between different stages of RCC tumors, and between patients with the age of <50 years vs. >50 years, and by gender.

DISCUSSION

Dysregulation of miR-625 and miR-302a expressions have been reported in several kinds of cancers; however, we aimed to investigate the expression of miR-625 and miR-302a in FFPE RCC tumors. Our data demonstrated that expression levels of miR-302a and miR-625 were not significantly upregulated in RCC tumor samples compared to their non-tumor counterparts.

miRNAs are expressed in a tissue-specific manner and play an important role in cell proliferation, apoptosis, and differentiation. Recent studies have identified a link between aberrant expression of miRNAs and progression of human cancers [19,20]. Little is known about expression patterns and exact functional roles of miRNAs in initiation and progression of RCC cancer [10]. Molecular pathogenesis suggests that micro-RNAs in RCC can play an important role by suppressing tumor-related genes such as DIRC1 (Disrupted in Renal Cancer 1), ATP6V1B1 (Vacuolar ATPase, B1 subunit / ATPase H+ transporting V1 subunit B1) and VHL (Von Hippel-Lindau) [25]. Wang *et al*. reported down-regulation of miR-625 in esophageal Squamous cell carcinoma (ESCC) tumors. miR-625 expression was dramatically correlated with tumor depth, stage, and metastasis in esophageal cancer. It was suggested that miR-625 could regulate proliferation and invasion of esophageal cancer cells by regulating SOX2 mRNA [10]. Another study found down-regulation of miR-625 in esophageal cancer; low expression of miR-625 correlated with lymph node metastasis, distant metastasis, tumor differentiation, and advanced TNM stage. They found that distant metastasis, clinical stage, and miR-625 expression level were independent factors in predicting the overall survival of ESCC patients [12].

In line with the previous studies, Fang *et al*. found that miR-625 levels were frequently reduced in malignant melanoma tumors. They found that miR-625 inhibited proliferation, migration, and invasion -all the aspects which influence

Figure 1: In silico analysis of target and expression profile of miR-625. A Bioinformatics analysis over the Target scan database revealed the interaction between miR-625 and 3'-UTR of SOX2 mRNA (A). Several validated studies demonstrated that SOX2 gene was targeted with a number of miRNAs, in particular, miR-625 (B). Rely on RNA-Seq data, miRmine Human miRNA Expression Data base revealed expression profile of miR-625 in several human tissues (C).

A

	Predicted consequential pairing of target region (top) and miRNA (bottom)	Site type	Context++ score	Context++ score percentile	Weighted context++ score	Conserved branch length	P_{CT}
Position 934-940 of SOX2 3' UTR	5' ...UGAUAUUUUAAGGUUUUCCCCCC...	7mer-m8	-0.27	87	-0.27	0.223	N/A
hsa-miR-625	3' CCUGACAUCCUUGAAAGGGGGA						

B

Gene	EntrezID	miRNA	MIMATID	PMID
SOX2	6657	hsa-miR-122-5p	MIMAT0000421	17612493
SOX2	6657	hsa-miR-21-5p	MIMAT0000076	18591254
SOX2	6657	hsa-miR-145-5p	MIMAT0000437	19409607
SOX2	6657	hsa-miR-128-3p	MIMAT0000424	19409607
SOX2	6657	hsa-miR-126-3p	MIMAT0000445	21304604
SOX2	6657	hsa-miR-522-3p	MIMAT0002868	21304604
SOX2	6657	hsa-miR-34a-5p	MIMAT0000255	22020437
SOX2	6657	hsa-miR-34b-3p	MIMAT0004676	22020437
SOX2	6657	hsa-miR-34c-5p	MIMAT0000686	22020437
SOX2	6657	hsa-miR-140-5p	MIMAT0000431	23060440
SOX2	6657	hsa-miR-429	MIMAT0001536	23111103
SOX2	6657	hsa-miR-145-5p	MIMAT0000437	23541921
SOX2	6657	hsa-miR-145-5p	MIMAT0000437	23312222
SOX2	6657	hsa-miR-126-3p	MIMAT0000445	24384842
SOX2	6657	hsa-miR-21-5p	MIMAT0000076	22931209
SOX2	6657	hsa-miR-340-5p	MIMAT0004692	22797059
SOX2	6657	hsa-miR-625	MIMAT0003294	24508468
SOX2	6657	hsa-miR-638	MIMAT0003308	24886288
SOX2	6657	hsa-miR-1181	MIMAT0005826	25444909

C

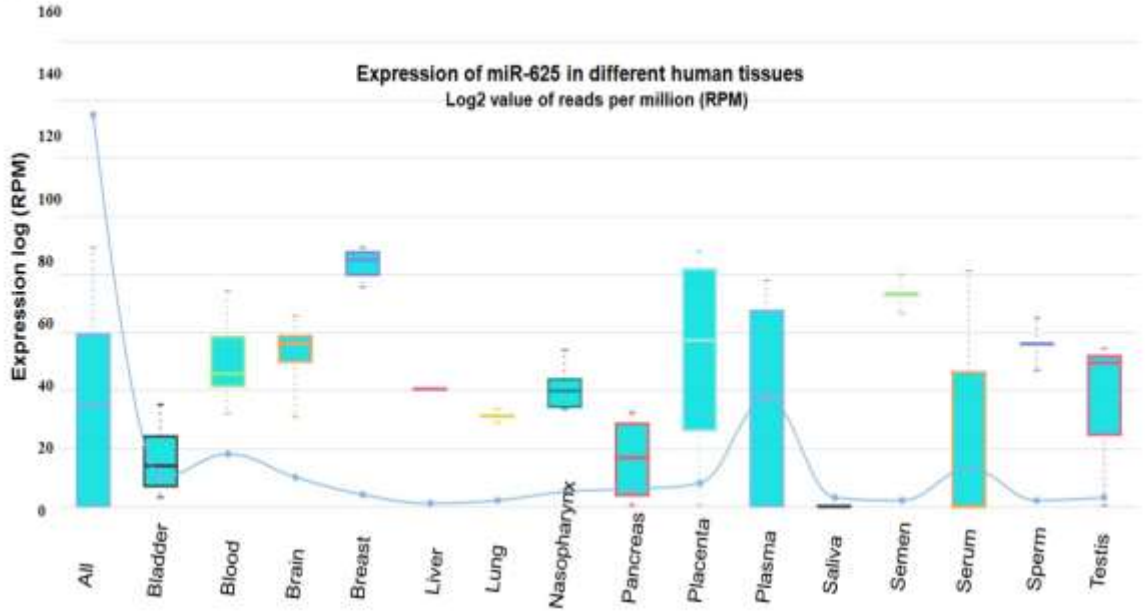


Figure 2: Representative amplification and melt curves**.** Representative amplification (A) melt curve graphs of real-time PCR products of miR-625 confirmed the authenticity of the amplified products (B)

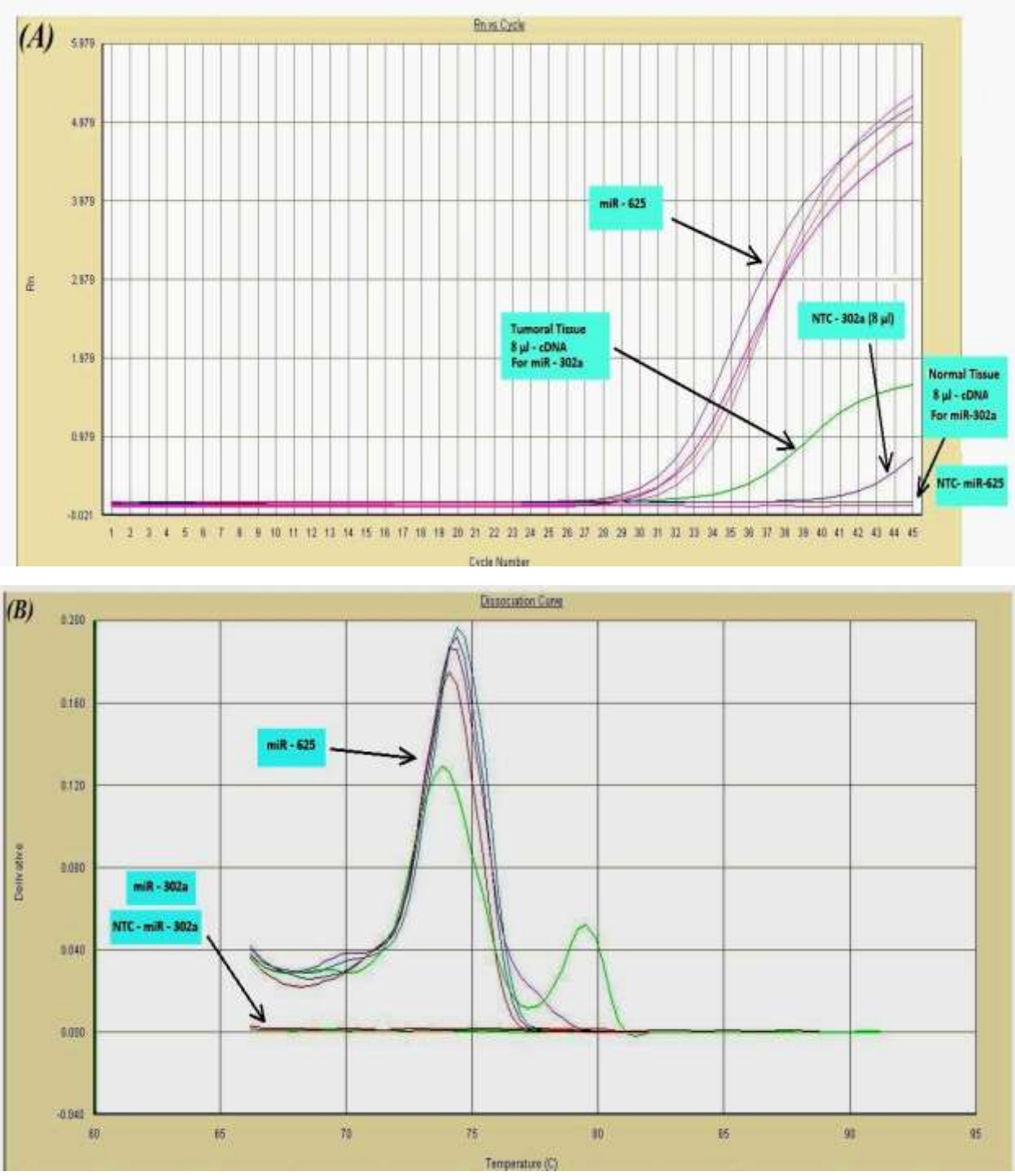
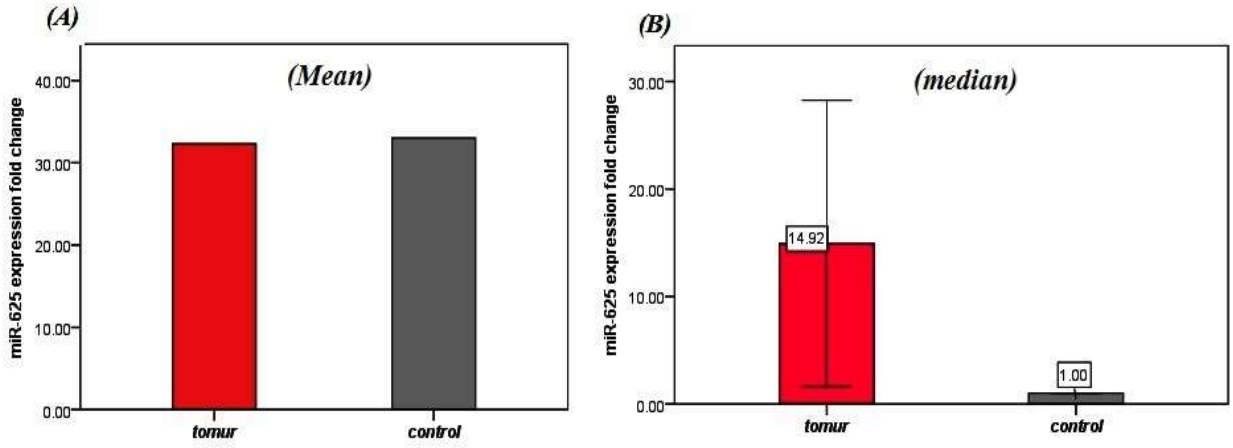


Figure 3: The real-time RT-PCR quantification of the expression levels of miR-625. (A-B). A Non-significant difference statistically between two groups, normal and control due to more distance among their fold change. (minimum fold: 0.001410087; maximum fold: 112.2055323)



tumorigenicity, by targeting SOX2 mRNA [21]. Other studies have found an aberrant expression of miR-625 in colon and endometrial cancers [1,22]. In contrast to non-significant expression of miR-625, our data revealed a low level of miR-302a expression in both tumor and non-tumor samples of RCC. Furthermore, we failed to detect any significant difference between the two groups. However, several studies demonstrated dysregulation miRNA-302a in human cancers. For instance, Zhang *et al.* demonstrated an inhibitory effect of miRNA-302a on cell proliferation in prostate cancer cells by inhibiting the transition from the G1 to S phase which is considered a key event during cell cycle progression [14]. It has been reported that over-expression of miR-302a inhibited proliferation and invasion of colorectal cancer cells by down-regulation of expression of proteins suppressing the MAPK and PI3K/Akt signaling pathways [23]. Guo *et al.* found that miR-302a plays a key role in the inhibition of ovarian cancer cells proliferation, and encourages cell's apoptosis [24].

CONCLUSION

We found no significant regulation of miR-625 molecule and dysregulation of mir-302a in RCC FFPE tumor tissues. Our small sample size may be the reason that we were unable to identify a statistically significant difference in miR-625 expression levels with tumor and non-tumor section of the samples. Future studies with larger sample size are needed to explore this relationship more thoroughly.

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