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MiR-21 Expression in Wilms' Tumor

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ABSTRACT

Background and Objective: Wilms' tumor (WT) is the most common genitourinary tract tumor in children. MicroRNAs (miRNAs) are small non-coding RNAs; their role in the pathogenesis of many types of human cancers has been identified. We aimed to evaluate the expression of miR-21, a well-known oncomir, in WT tissue samples which is a very common urinary tract malignancy in children.

Methods: We performed chromogenic in situ hybridization (CISH) to detect the sub-cellular localization of miR-21 in 25 formalin-fixed, paraffin-embedded (FFPE) samples of WT. We also evaluated miR-21 expression in 24 of these blocks and 6 normal kidneys as controls using quantitative real-time PCR technique.

Results: While our real-time PCR analysis showed miR-21 significant overexpression in 4 tumors compared to the normal kidney samples, we could not detect significant ISH signal in any of these samples.

Conclusion: Low expression of miR-21 in WT might pinpoint the weak involvement of this miRNA in the pathogenesis of this cancer.

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Introduction

Wilms' tumor (WT) is the most common neoplasm of the kidney in children. It is an

embryologic tumor that histologically mimics renal embryogenesis and is composed of a variable mixture of stromal, blastemal, and epithelial elements. It affects approximately one child per 10,000 worldwide before the age of 15 (1, 2) and accounts for 8% of pediatric cancers. Though the overall survival rate is less than 70% and up to 25% of the survivors suffer from serious chronic health conditions 25 years after diagnosis, it underscores its importance for exploring the molecular and cellular mechanisms of the tumor (3, 4).

The emergence of microRNAs (miRNAs) as the key regulators of fundamental cell processes such as proliferation, differentiation and apoptosis, has led to a striking tendency to introduce these small ribomolecules into clinic. In carcinogenesis and tumor progression, miRNAs play a pivotal role by post-transcriptional regulation of tumor suppressors or oncogenes (5, 6) mainly via binding to their target molecules by sequence complementation (7,8). The oncomir miR-21 is a well-known miRNA which has

been linked to apoptosis inhibition, cancer cell proliferation, tumor invasion and metastasis by targeting key molecules such as PDCD4, PTEN, RECK and TM1(9- 13). Recent studies demonstrated that miR-21 is dysregulated in kidney diseases, including both non-neoplastic (e.g. renal fibrosis) as well as neoplastic ones (14, 15). Due to the high incidence of WT in children, its aggressive nature, and its less-studied pathogenesis, we focused on the expression levels of miR-21 for its contribution to WT progression and potential targeting therapy.

Materials and Methods

Clinical sample collection

Totally, 25 formalin-fixed paraffin-embedded (FFPE) samples of WT and 5 normal FFPE tissues of kidney were selected from the archive of the Namazi Hospital (Shiraz University of Medical Sciences, Iran). Hematoxylin and Eosin (H&E)-stained slides were reviewed and proper corresponding paraffin blocks were selected for chromogenic in situ hybridization (CISH) study. The cases with extensive necrosis or inadequate tissue samples in paraffin blocks were excluded from the study. The sections of each block were used for ISH and qRT-PCR experiments in RNase-free conditions.

RNA purification procedure

Following deparaffinization of samples with xylol, Proteinase K (Fermentas, Lithuania) digestion was performed based on previously optimized conditions. RNA purification was performed using Trizol reagent (Invitrogen, USA) according to the manufacturer's instructions.

Real-time PCR procedure to quantify miR-21 expression

Following DNase I treatment (Fermentas, Lithuania), reverse transcription procedure was performed on 500 ng of total RNA using cDNA synthesis kit (Parsgenome, Iran). Real-time PCR was performed using specific primers for miR-21 (Exiqon; product no.:20423). The U6 snRNA (Exiqon; product no.: 203906) and 5S rRNA (Exiqon; product no.: 203907) were amplified as reference genes. The SYBR green master mix kit (Parsgenome, Iran) was used on an ABI 7500 real-time PCR machine. QRT-PCR data was analyzed using Mann-Whitney test and the miR-21 Cq values were normalized to the mean Cq values of 5S rRNA and U6 snRNA to overcome the variable expression of any of these reference genes in FFPE samples. The relative levels of miR-21 in tumor and normal adjacent kidney were calculated with the $2^{-\Delta\Delta CT}$ method.

To check any potential contamination of the samples with genomic DNA we used a no-reverse transcriptioncontrol.

In situ hybridization procedure

After deparaffinization by xylol and digestion with 15 µg/ml of Proteinase K (Exiqon, Denmark) for 15 min at 37°C, the slides were subjected to hybridization with double digoxigenin (DIG)-labeled miRCURY LNA probes for miR-21, U6 snRNA as positive internal control and scrambled probe as negative control (Exiqon, Denmark). The slides were sealed with Fixogum and incubated at 55°C in hybridizer (DAKO) for 1 hr.

Table 1 shows the probe sequences used in this study.

After removal of coverslip, the slides were placed in serial dilutions of SSC solutions (5x, 1x and 0.2x) at 51ºC for 5, 10 and 20 min, respectively, followed by placing in 0.2x SSC solution for 5 min at room temperature. At detection step, 300 µl of alkaline phosphatase-associated sheep anti-DIG (Roche, Germany) with 2% sheep serum was placed on each slide followed by 1 hr incubation at room temperature. Then, 300 µl of 4-nitro-blue-tetrazolium/ 5-bromo-4-cholor-3-indolyphosphate (Roche, Germany) was applied on the slides and incubated at 30°C for 2 hr. Following washing in buffer and deionized water, Nuclear Fast Red was applied on the slides as counterstain. The slides of esophageal SCC and U6 probe were used as positive controls whereas scrambled probe and non-tumoral kidney were used as negative controls of the procedure.

Table 1. Probe sequences used in ISH experiments

Target	Labeling	Sequence
miR-21	Double DIG-labeled	TCAACATCAGTCTGATAAGCTA
U6 snRNA	DIG-labeled	CACGAATTTGCGTGTCATCCTT
Scrambled (negative) control	Double DIG-labeled	GTGTAACACGTCTATAGCCCCA

Results

ISH detection of miR-21 on FFPE samples of WT

In each experiment we used esophageal squamous cell carcinoma tissues as positive control which previously had shown the cytoplasmic blue signals of miR-21 and had been confirmed by RT-PCR. While our positive control (esophageal SCC) showed the miR-21 signal (Figure 1a), and the U6 snRNA probe could detect the nuclear signals of U6 snRNA (Figure 1b), we could not detect the expected signal for miR-21 neither in WT FFPE samples nor in normal kidney tissues (Figure 1c).

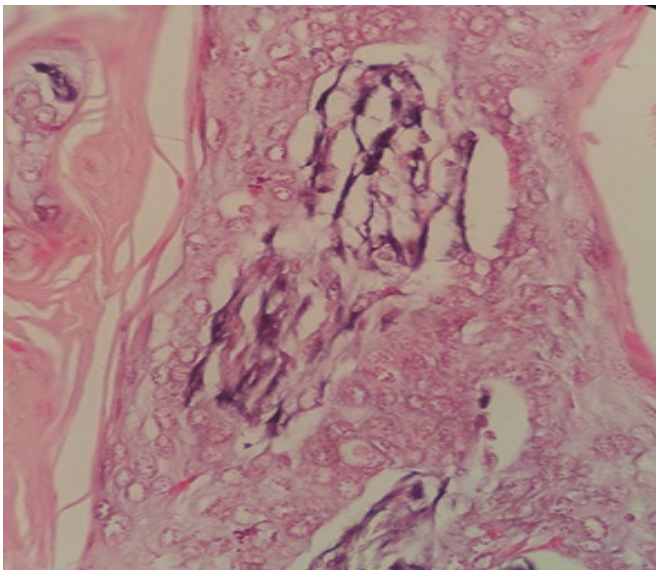

Figure 1a. Mir-21 positive control (esophageal SCC), stromal cells show miRNA expression (blue color)

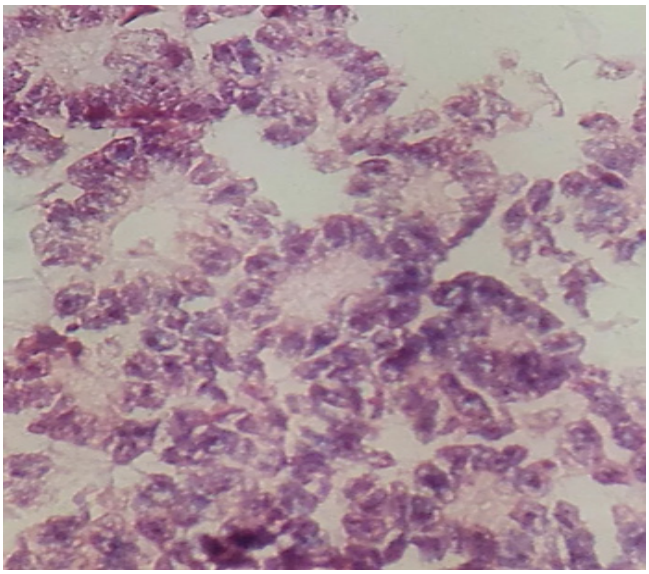

Figure 1b. Positive reaction for U6 snRNA probe as housekeeping internal control (Blue color)

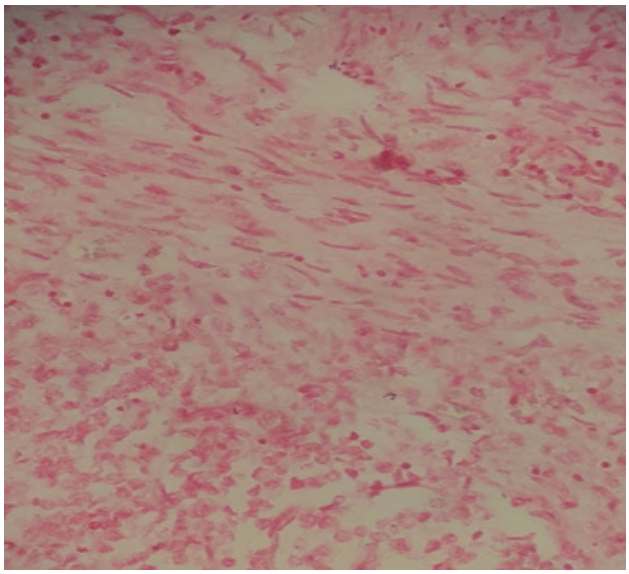

Figure 1c. Negative result of patient's sample

QRT-PCR of miR-21 expression

To evaluate the expression levels of miR-21, qRT-PCR was performed on 24 WT samples and 6 normal kidney tissues as control. Four tumors of these samples showed significant high expression (*P*=0.0021) and 3 tumors showed mild borderline expression compared to the normal kidney. The mirRNA 21 expression in the rest 17 samples were not different from the normal kidney tissues (Figure 2).

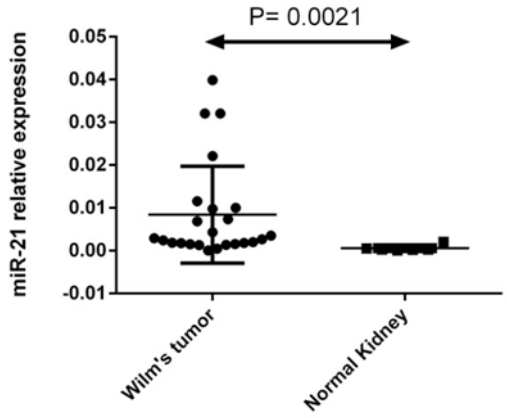

Figure 2. Mir-21 significant overexpression in Wilms' tumor in 4 samples compared to the normal kidney tissues (*P-value*=0.0021)

Discussion

Several previous studies have focused on the contribution of miR-21 in tumor progression and patients' poor survival (9,16-18). The ISH studies on lung, colon and breast cancers have shown miR-21 localization in the tumor stroma and particularly in the stromal fibroblast-like cells (19-22). We have previously shown that miR-21 expression is mostly confined to the fibroblasts in the tumor microenvironment (23). Here in this study, we used the same esophageal FFPE tissue blocks as positive control. While these positive controls showed the same pattern of cytoplasmic signals for miR-21, we could not detect the blue signals of miR-21 in the WT tissues. We concluded that this might be due to the weak miR-21 expression in the WT samples. In order to evaluate the miR-21 expression we performed qRT-PCR on FFPE samples of WT. Our real-time PCR data showed a significant upregulation of miR-21 in 4 WT tissues compared to the normal kidney samples. When we defined the tumor tissues as favorable (low grade) and unfavorable (high grade) groups, based on their histology, none of the samples with miR-21 overexpression were categorized to the unfavorable group. This might pinpoint the weak contribution of miR-21 in WT progression and anaplasia. The ISH for U6, as a housekeeping internal miRNA control for assessing the effectiveness of ISH, revealed acceptable reactivity in all samples. This finding precludes un-optimized situations of the experiment for ISH on our samples. Regarding our infelicitous data with ISH, we came to two different conclusions: a) ISH and real-time PCR techniques have different sensitivity for miRNA detection and ISH might be a less sensitive way of miRNA detection; b) miR-21 overexpression is not a common feature in WTs, thus, due to the low expression levels of this miRNA, we could not detect the signals in tumor tissues on the contrary to the real-time PCR technique. Overall, miR-21 might not be a prominent miRNA in WT anaplasia and we were unable to detect its overexpression in the unfavorable group of WT samples.

Cui et al. in a recent study analyzed the expression levels of miR-21 and the PTEN protein by qRT-PCR and Western blot in WT specimens. MirR-21 levels were significantly high (in 21 out of 41 patients) and the PTEN protein levels were significantly low (also in 21 out of 41 patients), compared to the adjacent non-tumorous renal tissues. These levels of miR-21 and PTEN were correlated with the age of the patients (> 24 months), unfavorable histopathology, late clinical stage and lymphatic metastasis (24). They also demonstrated that miR-21 affects biological behaviors of tumor cells, including cell proliferation and invasion promotion by inhibition of the apoptosis pathway, through direct targeting and negatively regulating PTEN at the post-transcriptional level in SK-NEP-1 WT cells. Discrepancy of our study results with those of Cui et al. study could be explained by variations in primer design and probably larger sample size (41 versus 25). Regarding the pathogenetic base of mir-21 oncogenesis, Wang et al. revealed that mir-21 inhibits apoptosis by modulating the activation of the phosphatidylinositol 3-kinase/Rac-α serine/threonine protein kinase (Akt) pathway (25). Also, Du et

al. demonstrated that the expression levels of Bcl-2, p-AKT, survivin and c-myc were significantly down-regulated in miR-21 inhibitor-transfected cells, whilst the expression levels of PDCD4, Bax and PTEN were significantly upregulated (26).

Other microRNAs may participate in tumorigenesis of WT. Kort et al. demonstrated that members of the oncomir-1 family, including miR-92, miR-17-5P and miR-20a, were upregulated in WT compared to the normal kidney tissue and other renal cancer types (27).

Watson et al. showed that downregulation of miR-27a and miR-193a-5P as well as upregulation of miR-628-5P, miR-590-5P, miR-483-5P, miR-302a and miR-367 were associated with high risk WT (11).

In conclusion, although our study revealed no altered expression of miR-21 in WT by CISH method, application of more sensitive method like qRT- PCR showed significant overexpression in 4 tumors compared to the normal kidney samples. Our finding could have been more valuable if we could examine larger sample size accompanied by long term follow up. However, increasing evidences have hinted at the pathogenic role of dysregulated microRNAs in WT, as they will probably participate in diagnosis, targeted therapy and determining the prognosis of the tumor in the future.

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Conflict of interest

The authors declare that there is no conflict of interest regarding the publication of this article.

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