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ARAŞTIRMA / RESEARCH

Role of miR-6814-5p for the formation of T5, a heparanase variant, in renal cell carcinoma

Renal hücreli karsinomda bir heparanaz varyantı olan T5 oluşumunda miR-6814-5p'nin rolü

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Abstract

Purpose: The aim of this study is to investigate whether the overexpression of human heparanase protein (HPSE) alternative variant protein called T5 is caused by increased expression of miR-6814-5p in human renal cell carcinoma (RCC) cases. In addition, the possible correlation between the clinical parameters of RCC cases and the expression levels of T5 and miR-6814-5p was evaluated.
Materials and Methods: *T5* and miR-6814-5p expression analysis was performed on ready-to-use RCC cDNA panel by qPCR method. This panel included 48 cDNA samples obtained from tumor tissues of 10 stage-1, 5 stage-2, 13 stage-3 and 11 stage-4 RCC patients and normal kidney tissues from 9 healthy individuals.
Results: There was no significant correlation between TNM stages, Fuhrman nuclear grade and histological type and miR-6814-5p and *T5* expressions. The expression level of miR-6814-5p in RCC tumor tissues was about 8-fold higher and the *T5* expression level about 5-fold higher than healthy controls. MiR-6814-5p and *T5* expression changes were statistically significantly correlated with neutrophil/lymphocyte ratio of RCC cases.
Conclusion: MiR-6814-5p may play a role in the formation mechanism of *T5* in RCC.
Keywords: Renal cell carcinoma, heparanase, gene expression regulation, miR-6814-5p

Öz

Amaç: Bu çalışmanın amacı insan renal hücre karsinomu (RHK) vakalarında insan heparanaz proteininin (HPSE) T5 adlı alternatif varyant proteininin aşırı ekspresyonunun nedeninin miR-6814-5p'nin artmış ekspresyonu olup olmadığını araştırmaktır. Ayrıca RHK vakalarının klinik parametreleri ile *T5* ve miR-6814-5p ekspresyon seviyelerinin olası korelasyonu değerlendirilmiştir.
Gereç ve Yöntem: *T5* ve miR-6814-5p ekspresyon analizi, kullanıma hazır RHK cDNA panelinde qPCR yöntemi ile gerçekleştirildi. Bu panel, 10 evre-1, 5 evre-2, 13 evre-3 ve 11 evre-4 RHK hastalarının tümör dokularından ve 9 sağlıklı bireyden alınan normal böbrek dokularından elde edilen 48 cDNA örneğini içeriyordu.
Bulgular: TNM evreleri, Fuhrman nükleer derece ve histolojik tip ile miR-6814-5p ve T5 ifadeleri arasında anlamlı bir ilişki bulunmadı. RHK tümör dokularında miR-6814-5p ekspresyon seviyesi, sağlıklı kontrollerden yaklaşık 8 kat daha yüksek ve T5 ekspresyon seviyesi yaklaşık 5 kat daha yüksekti. MiR-6814-5p ve T5 ekspresyon değişiklikleri, RHK vakalarının nötrofil / lenfosit oranı ile istatistiksel olarak anlamlı korelasyon gösterdi.
Sonuç: MiR-6814-5p, RHK'de T5 oluşum mekanizmasında rol oynayabilir.
Anahtar kelimeler: Renal hücreli karsinom; heparanaz; gen ifade düzenlenmesi; miR-6814-5p

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INTRODUCTION

Heparanase is a mammalian endo-β-D-glucuronidase cleaving heparan sulfate side chains in tumor cell proliferation. Alternative splicing rises coding potential of genome and enables various proteins to be produced from a single gene. Produced protein isoforms show distinct biological features playing a crucial function in tumor formation[1] Recently, a human *HPSE* (Heparanase) protein variant, *T5,* has been identified. *T5* has properties increasing independent growth, tumor development and cell proliferation. *T5* was observed to be overexpressed in 75% of human RCC biopsies and clinically associated with RCC. The genomic region of *T5* consists of the first four exons of *HPSE* gene, which consists of 14 exons, and 144 base pairs of exon 5.[2] But, the mechanism of *T5* formation hasn't yet been explained.

Also, miRNAs can bind to DNA and induce a transcriptional stop resulting in production of truncated proteins[3,4]. Based on these findings, we think that a miRNA binding to DNA immediately after 144 base pair segment of exon 5 may cause transcriptional arrest that will cause the formation of *T5* from *HPSE* gene. Therefore, we analyzed 30 base pair region just after boundry of *T5* genomic location using miRDB and STarMir databases and found that miR-6814-5p can target this region with the highest probability and arrest transcriptional activity (Fig. 1).[5-7]

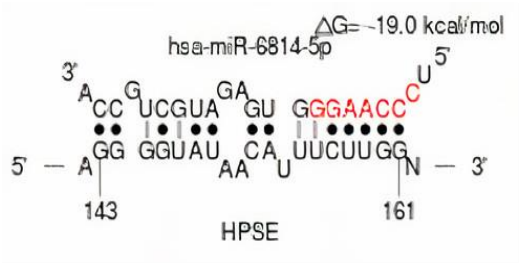

Figure 1. Binding of miR-6814-5p to HPSE

As the study experimental design, *T5* and miR-6814-5p expression analysis was performed on ready-to-use RCC cDNA panel by qPCR method. This panel included 48 cDNA samples obtained from tumor tissues of 10 stage-1, 5 stage-2, 13 stage-3 and 11 stage-4 RCC patients and normal kidney tissues from 9 healthy individuals. Statistical analysis was performed to measure the difference between study groups.

Our hypotheses were; over-expression of *T5* in RCC cases was due to upregulation of miR-6814-5p, and miR-6814-5p was associated with RCC subjects' pathophysiological parameters and was informative as an oncogenic marker. This study sheds light on miRNA-mediated epigenetic regulation of T5 overexpression specific to RCC pathogenesis for the first time in the literature.

MATERIALS AND METHODS

Study samples

In this study, Kidney Cancer cDNA Array (Origene, MD, USA), ready-to-use RCC cDNA panel, was used for miRNA expression analysis. [8] This panel included a total of 48 cDNA samples taken from tumor tissues of 10 stage-1, 5 stage-2, 13 stage-3 and 11 stage-4 RCC patients and normal kidney tissue from 9 healthy subjects. This study is designed as a cDNA panel-based, ethical committee approval is not required. In order to determine the sample size, power analysis was performed using the G*Power 3.1.9 program. Assuming that Student-t test will be used to compare two groups (patient-control) in the evaluation of the data to be obtained from the study, the minimum sample size required for 95% reliability, 80% power value and high-level effect size (d=0.8) was calculated as 48.

Expression analysis of miR-6814-5p and T5

Real-Time PCR method was applied for miR-6814-5p and *T5* expression analysis in this patient and control groups using RCC cDNA panel (Origene Technologies Inc., Rockville, MD, USA) and Rotor-Gene Q (Qiagen GmbH, Manheim, Germany) device was used. As a procedure, hsa-miR-6814-5p expression primer (ABM, Richmond, BC, Canada) and the primer pair we designed for *T5* gene (Forward: 5'-TTGCTAGATTGGTGCCCGA-3 'and Reverse: 5'-GCTACTCCGAGAACACTACCAG-3') were used.

As internal control for *T5* and miR-6814-5p, *GAPDH* and *SNORD48* were used, respectively. BrightGreen 2X qPCR MasterMix-No Dye and BrightGreen miRNA 2X qPCR MasterMix-No Dye (ABM, Richmond, BC, Canada) were used, respectively, in the premix prepared. Real-Time PCR reaction was performed according to kit protocol in

Rotor Gene Q instrument[9]. All Real-Time PCR experiments were realized in triplicate.

Statistical analysis

MiR-6814-5p and *T5* expression levels were normalized with *SNORD48* and *GAPDH*, respectively. Statistical analysis of the difference in miR-6814-5p expression between normal and tumor cDNA samples was performed and miR-6814-5p expression was statistically correlated with clinical parameters (Fuhrman grading, TNM staging, presence of metastasis) of the patients. [TNM staging is a system to describe the amount and spread of cancer in a patient's body, using TNM. T describes the size of the tumor and any spread of cancer into nearby tissue; N describes spread of cancer to nearby lymph nodes; and M describes metastasis (spread of cancer to other parts of the body)]. Analysis of relative gene and miRNA expression data was performed using $2^{-\Delta\Delta Ct}$ method [$(Ct_{target}-Ct_{reference})_{test}-(Ct_{target}-Ct_{reference})_{calibrator}$]. The test results were analyzed on SPSS 13.0.1 (SPSS Inc., Chicago, IL, USA). Comparisons of the groups were done by ANOVA and Kruskal Wallis tests for normal and non-normal distributions, respectively. Spearman correlation analysis was used to assess the associations among the parameters considering the skewness of data distribution. Statistical significance was accepted as $p<0.05$.

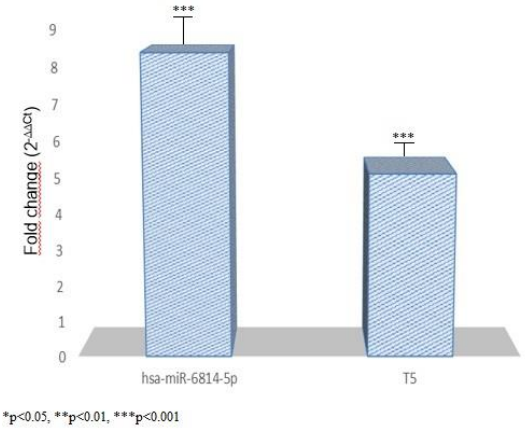

Figure 2. The fold changes of hsa-miR-6814-5p and T5 in tumor tissues of RCC patients when compared to healthy controls

RESULTS

The expression levels of miR-6814-5p and *T5* were compared in RCC tumor tissues and healthy kidney tissues, and potential associations between the clinical parameters of the study subjects and miR-6814-5p and *T5* expression levels were analyzed. Our study population included 24 (61.5%) male and 15 (38.5%) female RCC patients, and 5 (55%) male and 4 (45%) female healthy individuals (Table 1).

Table 1. Features of patients and control cases

		Patient (n=39, %)	Control (n=9,%)
Gender	Male	24 (61.5)	5 (55)
	Female	15 (38.5)	4 (45)
Age	35-44	3 (7.7)	0 (0)
	45-64	22 (56.4)	5 (55)
	>65	14 (35.9)	4 (45)
TNM stage	Stage I	10 (25.6)	
	Stage II	5 (12.8)	
	Stage III	13 (33.4)	
	Stage IV	11 (28.2)	
Fuhrman nuclear grade	Grade 2	6 (15.4)	
	Grade 3	21 (53.8)	
	Grade 4	12 (30.8)	
Histological type	Clear cell	26 (66.7)	
	Papillary	11 (28.2)	
	Chromophobe	2 (5.1)	

(TNM: Tumor Node Metastasis)

According to comparison between TNM stages, Fuhrman nuclear grade and histological type and miR-6814-5p and *T5* expression levels, no significant correlation was found (p> 0.05). MiR-6814-5p expression was 8-fold higher and *T5* expression was 5-fold higher in RCC tumor tissues than controls (p<0.001 for both of the fold changes) (Figure 2). The correlation of the expression levels of miR-6814-5p and *T5* was not found to be statistically significant [p=0.086, ϱ (rho): 0.573]. Finally, we compared miR-6814-5p and *T5* expression level changes with neutrophil / lymphocyte ratio, which is known to be associated with RCC, and found statistically significant correlations (p=0.023, ϱ (rho): 0.043 and p=0.001, ϱ (rho): 0.133, respectively).

DISCUSSION

T5 increases tumoral behaviour and was upregulated in RCC cases[2]. Although we didn't detect a statistically significant correlation between miR-6814-5p and T5 expression levels, we observed that miR-6814-5p was expressed 8-fold higher and *T5* was expressed 5-fold higher in RCC samples than control. This shows potential oncogenic role of miR-6814-5p for RCC, like *T5*. Sakir et al showed that miR-6814 mimic can induce cellular invasion on lung cancer cell line, H1299[10]. Also, Yonemori et al reported that miR-6814 was upregulated in pancreatic ductal adenocarcinoma[11]. These studies supports our findings giving potential oncogenic role to miR-6814-5p for RCC. Moreover, Shuai et al defined that a T-cell acute lymphoblastic leukemia (T-ALL) patient's B-cell lymphoma/leukemia 11B (BCL11B) gene was altered at position 2402 (T>C), including hsa-miR-6814-5p binding site, in the 3'UTR region. This change was thought to provide important data to elucidate the role of hsa-miR-6814-5p and BCL11B in T-ALL[12]. Furthermore, Sharma et al defined key cholesterol homeostasis genes (*HMGCR, SREBF2, NR1H3* and *NR1H2*) involved in colorectal cancer (CRC) by thinking cholesterol accumulation is an important pathway of tumor cell formation. Then, they identified microRNAs modulating these crucial genes in CRC and found that hsa-miR-6814-5p is a potential regulator of *NR1H2* in CRC[13]. Amazingly, Ye et al found via machine learning that miR-6814 had many features and couldn't be classified[14]. Our study provides valuable data to reduce this uncertainty about miR-6814.

According to comparison between TNM stages, Fuhrman nuclear grade and histological type and miR-6814-5p and *T5* expression levels, no significant correlation was found (p> 0.05). While there is such an increase (5-fold) in T5 expression specific to RCC samples, the inability to find a statistically significant association with RCC staging indicates that other factors together with T5 have additive contributions in RCC staging. Further studies in the future will allow us to explore these additive factors.

Also, we detected statistically significant correlations between miR-6814-5p and *T5* expression levels and neutrophil/lymphocyte ratio (NLR) (p=0.023 and p=0.001, respectively). Hu et al indicated that increased NLR showed poorer prognosis for RCC patients[15]. So, this finding supports clinical significance of miR-6814-5p for RCC, via NLR. All in all, our study showed that miR-6814 may be an important marker for RCC as a potential T5 formation trigger.

When we look at hsa-miR-6814-5p's targets other than *HPSE* gene, we confront very critical oncogenes according to targetscan database. For example, Chen et al found that *PKMYT1*, which is one of the targets of hsa-miR-6814-5p, was overexpressed in RCC and its presence shows poor prognosis[16]. So, this finding makes hsa-miR-6814-5p a probable therapeutic key player for RCC via being a potential inhibitor of *PKMYT1*.

Some limitations of this study should be noted. MiRNAs have normally many targets to bind and regulate their transcriptional activity. So, miR-6814-5p may target many other gene, other than T5, and contribute its pathological role in RCC. Even if, we focused on its effect on T5 by considering that it has the highest potential to bind and stop transcription according to our in silico analysis results.

All in all, miR-6814-5p may play a role in the formation mechanism of T5 in RCC. This study sheds light on miRNA-mediated epigenetic regulation of T5 overexpression specific to RCC pathogenesis for the first time in the literature. More comprehensive studies are required to figure out the functions of *T5* and miR-6814-5p in RCC pathophysiology.

Yazar Katkıları: Çalışma konsepti/Tasarımı: BÖ, SE; Veri toplama: BÖ, SE; Veri analizi ve yorumlama: BÖ, SE; Yazı taslağı: BÖ, SE; BÖ, SE; İçeriğin eleştirel incelenmesi: BÖ, SE; Son onay ve sorumluluk: BÖ, SE; Teknik ve malzeme desteği: BÖ, SE; Süpervizyon: BÖ, SE; Fon sağlama (mevcut ise): yok.
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Author Contributions: Concept/Design : BÖ, SE; Data acquisition: BÖ, SE; Data analysis and interpretation: BÖ, SE; Drafting manuscript: BÖ, SE; Critical revision of manuscript: BÖ, SE; Final approval and accountability: BÖ, SE; Technical or material support: BÖ, SE; Supervision: BÖ, SE; Securing funding (if available): n/a.
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