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ARAŞTIRMA/RESEARCH

Molecular genetics of renal cell carcinoma: polybromo 1 and set domain containing 2 genes

Renal hücreli karsinomun moleküler genetiği: polybromo 1 ve SET domain içeren 2 gen

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Abstract

Purpose: Renal cell carcinoma (RCC) is genetically characterized by the recurrent loss of the short arm of chromosome 3. Classically, Von Hippel Lindau (VHL) was the only frequently mutated gene in RCC. Recently, several novel frequent mutations of histone modifying and chromatin remodeling genes, including PBRM1 and SETD2, were identified. In the present study, we aimed to determine the possible relationship between PBRM1 and SETD2 genes, and renal cell carcinoma by molecular techniques.

Material and Methods: Screening possible mutation and determining mRNA expression level of PBRM1 and SETD2 genes in 20 pairs of tumor and normal samples of RCC patients were performed by nucleotide sequencer and reverse transcription- polymerase chain reaction (RT-PCR).

Results: The mRNA expression levels of both genes were significantly reduced in tumor samples when compared with the control samples. As a result of mutational analysis, a single insertion nucleotide polymorphism in exon 12 of SETD2 gene was detected in one patient.

Conclusion: Reduced mRNA expression level of PBRM1 and SETD2 might be risk factor for RCC development. Further analysis is warranted to investigate responsible genes rather than PBRM1 and SETD2 in RCC.

Key words: Renal cell carcinoma, PBRM1, SETD2, DNA sequencing, reverse transcriptase-PCR

Öz

Amaç: Renal hücreli karsinoma (RCC) 3. kromozomun kısa kolundaki tekrar kayıpları ile karakterizedir. Klasik olarak Von Hippel Lindau sendromunda sadece RCC gen mutasyonu sıklıkla görülmekteydi. Son zamanlarda PBRM1 ve SETD2 yi içeren histon modifikasyon ve kromatin remodeling genlerinde de mutasyonlar yoğunlukla tanımlanmıştır. Bu çalışmada moleküler teknikler ile PBRM1 ve SETD2 genleri ile RCC arasındaki muhtemel ilişkiyi belirlenmesi amaçlanmıştır.

Gereç ve Yöntem: RCC hastalarından alınan normal ve tümöral dokuda PBRM1 ve SETD2 genlerinin mRNA ekspresyon seviyeleri ve olası mutasyonları nükleotid sekanslama ve Reverse transkripsiyon polimeraz zincir reaksiyonu (RT- PCR) ile belirlendi.

Bulgular: Kontrol ile kıyaslandığında her iki gen için de mRNA ekspresyon değerleri tümöral dokuda anlamlı derecede azalmıştır. Mutasyon analiz sonuçlarına göre 1 hastanın SETD2 geninin 12 nolu exonunda tek insersiyon nükleotid polimorfizmi saptandı.

Sonuç: PBRM1 ve SETD2 mRNA ekspresyon seviyelerinin azalması RCC gelişmesi için risk faktörü olabilir. Gelecekte RCC de SETD2 ve PBRM1 in dışında farklı genlerinde araştırılması gerekmektedir.

Anahtar kelimeler: Renal hücreli karsinom, PBRM1, SETD2, DNA sekanslama, revers transkriptaz-PCR

INTRODUCTION

Renal cell carcinoma is the third most common urologic malignancy and the seventh most common cancer overall[1]. Kidney cancer generally refers to any cancer arising in the kidney or renal pelvis, but most of the tumors considered in the present study are renal cell carcinomas (RCCs), which arise from cells

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in the tubules of the filtration portion of the kidney1. RCCs include different subtypes (clear cell RCC, papillary RCC, chromophobe RCC and collecting duct RCC), and each of the RCC subtypes has a unique genetic abnormality and exhibits different biologic behavior[2,3,4]. The risk factors of RCC are not known clearly[5]. Epidemiologic evidence indicates that male gender, age beyond 50 years and end stage renal disease are risk factors for developing RCC[6]. Genetic and environment are two important risk factors of renal cancers[5,7]. Most RCCs are sporadic, the cell changes occur long after birth, possibly due to cancer-causing chemicals or other environment risk factors[8]. Hereditary forms of RCC represent less than 5% of the cases; none the less, they have played a pivotal role in understanding and characterizing the molecular pathway involved in sporadic RCC[9]. Molecular mechanisms involved in the pathogenesis of renal cancer have recently been identified leading to the development of targeted therapy with improved survival compared to cytokines and chemotherapeutic agent[10].

Classically, RCC is characterized by inactivation of the VHL tumor suppressor gene in the majority of cases[11-13]. Mutation of VHL alone is not sufficient to cause RCC cases[13]. Recently, large scale targeted and whole exome sequencing studies of RCC have identified other frequently mutated genes including polybromo 1 (PBRM1) and SET domain containing 2 (SETD2)[13]. The PBRM1 is a tumor suppressor gene, located on the short (p) arm of chromosome 3 at position 21[2]. The PBRM1 gene is comprised of six bromodomains involved in binding acetylated lysine residues on histone tails[2]. It functions in chromatin biology; encodes the polybromo 1 (BAF 180) protein, which is the chromatin targeting subunit of the polybromo complex SWI/SNF13. Knock-down of PBRM1 gene enhanced colony formation and migration of cancer cells, suggesting this gene acts as a negative regulator of cell proliferation[14,15]. SETD2 is a tumor suppressor gene; it is located on chromosome 3p21.31[15]. The SETD2 gene was found by the analysis of accumulated transcripts containing premature termination codons and encodes a histone methyltransferase, which is responsible for trimethylation of the lysine residue at position 36 of histone H3 and may play a role in suppressing tumor development[1,2]. The importance of PBRM1 and SETD2 genes in RCC is reinforced by their position close to the VHL gene on chromosome 3p[16]. Thus deletion of this region simultaneously removes one copy of each gene[16].

In the present study, in order to investigate the possible role of PBRM1 and SETD2 genes in RCC, we aimed to investigate the probable mutation and mRNA expression level of PBRM1 and SETD2 genes in RCC patients by monitoring nucleotide sequencing and RT- PCR techniques.

MATERIALS AND METHODS

Patients

In the present study 20 pairs of tumor and normal samples of renal cell carcinoma patients (9 pairs clear cell, 7 pairs papillary, 4 pairs chromophobe) were obtained by primary surgery at the Rizgary hospital in Erbil, Iraq. The obtained tissues were placed into liquid nitrogen directly after the biopsy and kept at -80°C until DNA and RNA extraction. The study was approved by the local ethics committee and was conducted in accordance with the guidelines of the declaration of Helsinki.

DNA sequencing and mutation analysis

DNA samples from renal biopsied tissues were extracted using a commercial extraction kit (Bioneer, AccuPrep Genomic DNA Extraction Kit, Korea) according to the manufacture's instruction. Quantification and qualification of DNA concentration was performed using NanoDrop (ND- 1000, USA). Mutation analysis of exon 4 in PBRM1 and exon 12 in SETD2 were performed using DNA sequencing analysis. Primers were designed by employing primer- BLAST (Table 1).

PCR amplification was conducted using a gradient thermal cycler device (Eppendorf, Germany), 50 µL reaction mixture was prepared in PCR tubes containing 1.5 µL DNA template, 25 µL OnePCRTM master mix (GeneDirex, Korea), 1 µL forward primer, 1 µL reverse primer and 21.5 µL ddH2O. The cycling conditions comprised of initial denaturation at 95°C for 4 min, 35 cycles of denaturation at 95°C for 30 sec, annealing temperatures in Table 1 for 30 sec and extension at 72°C for 30 sec, and final extension at 72°C for 4 min. The PCR products of PBRM1 and SETD2 genes were analyzed on a 2.0% agarose gel and stained with ethidum bromide.

In order to monitor the probable mutations of the selected regions of PBRM1 and SETD2 genes, the 15 DNA fragments (5 normal samples and 10 tumor samples) were excised from the agarose gel and analyzed by ABI 3130 nucleotide sequencer.

RT-PCR and mRNA expression analysis

RNA samples from renal biopsy tissue were obtained using the extraction kit (Bioneer, ExiPrepTM Tissue total RNA kit, Korea) according to the manufacture's instruction. Quantification and qualification of total RNA concentration was performed using NanoDrop (ND- 1000, USA). In the present study, complementary DNA (cDNA) was synthesized using the AccuPower Cyclescript RT PreMix dNs kit (Bioneer, Korea). cDNAs were amplified by semi- quantitative reverse transcriptase- PCR and utilized the expression primers (Table 1). glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene was used as a housekeeping gene for the normalization of PBRM1 and SETD2 gene expressions data[4]. PCR reaction and condition was performed using MJ Research, AB Applied Biosystem thermal cycler. 50 µL reaction mixture was prepared in PCR tubes containing 2 µL cDNA template, 25 µL OnePCRTM master mix (GeneDirex, Korea), 1 µL forward primer, 1 µL reverse primer and 22 µL ddH2O. The cycling conditions comprised of initial denaturation at 95˚C for 4 min, 35 cycles of denaturation at 95˚C for 30 sec, annealing temperatures in Table 1 for 30 sec and extension at 72˚C for 30 sec, and final extension at 72˚C for 4 min. Expression alterations were demonstrated by agarose gel electrophoresis (2% W/V) in the presence of ethidum bromid. The mRNA expression level was quantified by ImageJ software program (V1.46r) (15). The statistical analysis of gene expression was carried out using Wilcoxon signed rank test, significance was assumed at value of $p \leq 0.05$. The statistical tests were made by employing SPSS software (V.16).

Table 1. The primers utilized for PCR amplification.

Primer	Primer sequence	Expected size of PCR product (bp)	Annealing temperature (˚C)
F-PBRM1 exon 4	CAGATTCTCCTGAATATAAAGCCGC	143	55.2
R-PBRM1 exon 4	TCCTTCAGTCACTGTGCCCT		
F-SETD2 exon 12	TATAAAGACTTTGGAACACTTGCCC	660	55.1
R-SETD2 exon 12	TTTAGGTCTTTCCAACTGTCCAGG		
F-PBRM1ex	CGGAGGAGCAATAGCAGCAG	454	55
R-PBRM1ex	CTGGAAGTCAGCAGTGAGCA		
F-SETD2ex	AAATGTTTCTGCGGATCAGCCAA	250	55.8
R-SETD2ex	CTGTATGAGTTCCAGACAGGTAAGT		
F-GAPDHex	GGTCCACCACCCTGTTGCTGT	456	59.4
R-GAPDHex	AGACCACAGTCGATGCCATCAC		

RESULTS

Mutation screening

The DNA sequence of PBRM1 and SETD2 genes were obtained from the NCBI website, to compare the resulting DNA sequence of patient and normal samples (Query Sequence) with the reference sequence (Subject Sequence). only one sample was detected in the study with a single nucleotide polymorphism in the SETD2 gene in a sample of clear cell RCC. Figure 1 indicates and reveals the mutated sequence. However, no sequence variation was found in the PBRM1 gene.

Gene expression

The mRNA sequences of exon 1, 2, 3 and 4 for PBRM1, and the mRNA sequences of exon 9 and 10 for SETD2 gene were separated by 2% agarose gel electrophoresis. Figure 2 shows and reveals the expression alteration result. Expression level of mRNA of PBRM1 and SETD2 were obtained from 20 pairs of tumor and normal samples. Quantity of mRNA expression of PBRM1 (Figure 3, A) and SETD2 (Figure 3, B) tumor samples were

decreased when compared to mRNA expression of normal samples. Quantity of mRNA expression of PBRM1/GAPDH and SETD2/GAPDH (Figure 3) tumor samples were significantly down- regulated when compared to mRNA expression of normal samples. (PBRM1/GAPDH: p= 0.001/ SETD2/GAPDH: p= 0.001). N=20, p<0.05. When the specimens were subdivided according to the subtypes of RCC; the clear cell, Papillary and Chromophobe types of RCC displayed the significantly low expression of PBRM1 and SETD2 (Table 2).

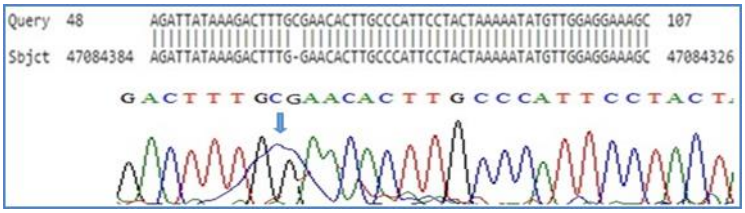

Figure 1. DNA sequencing results showing a frame-shift mutation in a clear cell RCC patient as a result of cytosine insertion (84610< C) in exon 12 of SETD2 gene.

Figure 2. The mRNA expression results of 2% agarose gel electrophoresis staining with ethidium bromide: A) PBRM1 and GAPDH genes expression in normal controls and tumor samples in RCC: B) SETD2 and GAPDH genes expression in normal controls and tumor samples in RCC. N= normal sample: T= tumor sample.

DISCUSSION

PBRM1 and SETD2 are two of the chromatin remodeling and histone modifying genes in human. We aimed to determine probable mutations and mRNA expression level of both genes in renal cell carcinoma patients. Many expression profiling researchers have reported the potential of gene expression models to distinguish between histologic subtypes of RCC such as conventional clear cell RCCs, oncocytomas, chromophobe carcinomas, papillary type 1 and 2 carcinomas of the renal pelvis[15]. Recent mRNA expression analyses have showed PBRM1 and SETD2 genes as a diagnostic marker for RCC[13].

In our study, the mRNA expression levels of PBRM1 and SETD2 genes were significantly decreased (down-regulated) as shown in Figure 2,3 and 4. Similarly, Khailany et al. reported the down-regulation of VHL, PBRM1 and SETD2 expression. Loss of the PBRM1 gene expression was seen in several ccRCC cell lines as well as in 70% of clear cell RCC patient samples[17]. This finding confirms recently published data that most, if not all, truncating mutations found in ccRCC affect PBRM1 production[17].

A global view of the mRNA expression patterns and deregulated pathways may provide a more accurate picture of renal cancer including its clinical behavior[9]. However, the function and role of most of these genes in tumor development are unknown, and some may even be by standard genes that play no role in tumorigenesis[9]. Nevertheless, these signatures may serve as effective biomarkers because of their unique differential expression patterns[9]. RCC, with different histopathological features, genetic expression and clinical behavior, is a heterogeneous disease with many subtypes[4].

The most common subtypes of RCC are clear cell (70 to 80%), papillary (14 to 17%), and chromophobe (4 to 8%). Expression level of all RCC subtypes of PBRM1 and SETD2 genes are significantly decreased among patients (Table 2). Depending on our data, reduced gene expression of PBRM1 and SETD2 are useful markers for

diagnosis in clear cell, Papillary and Chromophobe subtypes of RCC patients.

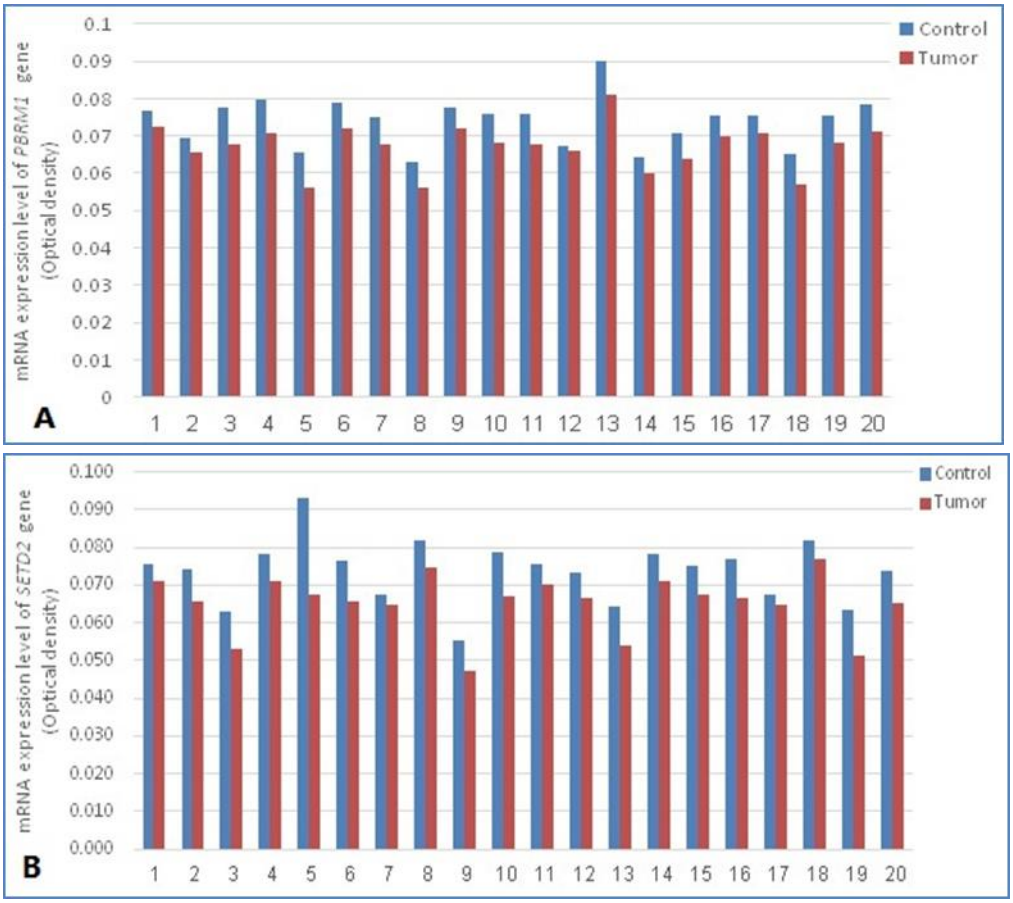

Figure 3. A. The mRNA expression level of normal control and tumor samples according to PBRM1/GAPDH gene: B) The mRNA expression level of normal control and tumor samples according to SETD2/GAPDH gene.

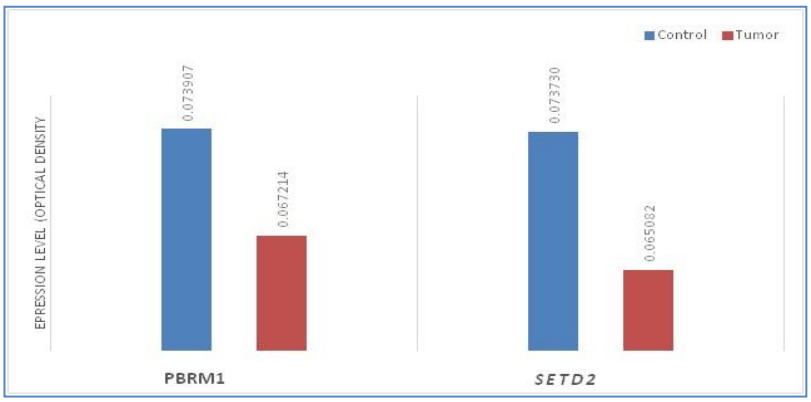

Figure 4. Statistical results of the mRNA expression level of PBRM1/GAPDH, and SETD2/GAPDH genes in both normal and tumor samples

Table 2. The statistical results of renal cell carcinoma subtypes.

Subtype	Total number	p value
PBRM1 gene		
Clear cell RCC	9	0.000
Papillary RCC	7	0.000
Chromophobe RCC	4	0.020
SETD2 gene		
Clear cell RCC	9	0.000
Papillary RCC	7	0.001
Chromophobe RCC	4	0.002

Recently, several frequently mutated genes in RCC including PBRM1 and SETD2 were identified [11,13]. Hence the mutation status of VHL alone is quite unlikely to be useful as a biomarker for disease aggressiveness [18]. However, a new phylogenetic assessment of tumor heterogeneity in RCC suggested that mutations of chromatin modulators are secondary events and contribute to invasive and metastatic phenotypes [18].

In the present study, we detected a point insertion mutation of exon 12 (84610< C) in SETD2 gene (Figure 1), the clinicopathological features of the mutated case includes; 57 years old, female gender, left kidney and papillary RCC subtype. However, it was not statistically significant ($p > 0.05$). There is no significant relationship between RCC pathogenesis and nucleotide sequence of SETD2 suggesting that somatic mutation may not be a cause of SETD2 inactivation. This finding suggests that further investigation of SETD2 gene alterations as possible risk factors for ccRCC would be valuable.

Epigenetic alterations are the hallmarks of cancer cells and their roles in renal tumor development and progression[2]. According to the last 10 years report, epigenetic alterations also play an important role in renal tumorigenesis[2]. This is of special interest in RCC because large-scale sequencing analysis revealed that candidate tumor suppressor genes are mutated in < 10% of tumors with exclusion of the PBRM1 and VHL genes[2]. Aberrant DNA methylation, altered chromatin remodeling/histone onco-modifications and deregulated microRNA expression not only contribute to the emergence and progression of RCTs, owing to their ubiquity, but they also constitute a promising class of biomarkers tailored for disease detection, diagnosis, assessment of prognosis, and prediction of response to therapy[2].

In conclusion, our findings indicate the interaction between expression alterations of PBRM1 and SETD2 genes and RCC. The mRNA expression level of PBRM1 and SETD2 genes were significantly down-regulated. In order to understand the investigation between RCC and molecular biomarkers; further analysis is necessary.

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