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VHL, PBRM1 and SETD2 Genes in Kidney Cancer: A Molecular Investigation

Rozhgar A. Khailany, Mehri Igci, Emine Bayraktar, Sakip Erturhan, Metin Karakok, Ahmet Arslan

***Abstract*—**Kidney cancer is the most lethal urological cancer accounting for 3% of adult malignancies. *VHL*, a tumor-suppressor gene, is best known to be associated with renal cell carcinoma (RCC). The *VHL* functions as negative regulator of hypoxia inducible factors. Recent sequencing efforts have identified several novel frequent mutations of histone modifying and chromatin remodeling genes in ccRCC (clear cell RCC) including *PBRM1* and *SETD2*. The *PBRM1* gene encodes the BAF180 protein, which involved in transcriptional activation and repression of selected genes. *SETD2* encodes a histone methyltransferase, which may play a role in suppressing tumor development. In this study, RNAs of 30 paired tumor and normal samples that were grouped according to the types of kidney cancer and clinical characteristics of patients, including gender and average age were examined by RT-PCR, SSCP and sequencing techniques. *VHL, PBRM1* and *SETD2* expressions were relatively down-regulated. However, statistically no significance was found (Wilcoxon signed rank test, $p>0.05$). Interestingly, no mutation was observed on the contrary of previous studies. Understanding the molecular mechanisms involved in the pathogenesis of RCC has aided the development of molecular-targeted drugs for kidney cancer. Further analysis is required to identify the responsible genes rather than *VHL, PBRM1* and *SETD2* in kidney cancer.

***Keywords*—**Kidney cancer, molecular biomarker, expression analysis, mutation screening.

I. Introduction

THE term kidney cancer generally refers to any cancer arising in the kidney or renal pelvis, but most of the tumors are renal cell carcinomas (RCCs), which arise from cells in the tubules of the filtration portion of the kidney [1]. Kidney cancer is the third most common urologic malignancy and the seventh most common cancer [1]. Incidence worldwide is about 209 000 new cases per year with 102 000 deaths per year [2]. Kidney cancer affects both men and women, but 1.5 times more common in men [1], [3]. The majority of kidney tumors in humans are RCC (80-85%), originating from the renal parenchyma [4]. The remaining (15-20%) are mainly transitional cell carcinoma of the renal pelvis, while other neoplasms, e.g. angiomyolipoma or nephroblastoma (Wilms' Tumor), are rare [4], [5]. In children, the most common form of kidney cancer is Wilms' tumor, which also exhibits unique genetic abnormalities and biologic behavior [6]. Many studies have reflected that kidney cancer is associated with a number of risk factors; gender, age, tobacco use, obesity, family history, hypertension, diet and work related exposures to certain substance, such as leather dyes [7], [8]. Patients who have kidney cancer often present with few signs and symptoms, when discovered in its early stages, the disease is curable, but metastatic kidney cancer is usually fatal [1], [9].

RCC is a collective term applied to a set of cancers arising in the epithelium of the renal tubules composed of four main histopathological entities can be distinguished based on morphologic and genetic characteristics; clear cell RCC, papillary RCC, chromophobe RCC and renal oncocytoma [2], [5]. Most cases of RCC are sporadic; up to 4% of patients have an inherited predisposition to this disease, including families with von Hippel-Lindau (VHL) disease, hereditary papillary renal cancer (HPRC), hereditary leiomyomatosis and renal cancer cell (HLRCC), and Birt-Hogg-Dubé (BHD) syndrome [10]. The identification of the genes predisposing to inherited syndromes of RCC has supplied much of our knowledge of the molecular basis of sporadic RCC [2].

The gene best known to be associated with RCC is the von Hippel-Lindau (*VHL*) gene, [11]. This gene was established by positional cloning from the locus associated with the VHL disease, a familial syndrome accompanying cancer in the kidney, brain, spinal cord, eye, pancreas and adrenal glands [11]. *VHL* gene, a tumor suppressor, located on the short arm of chromosome 3 (3p25-26). The gene spans 10 kb, is composed of three exons and encodes two proteins (pVHL19 and pVHL30) [13]. The complete VHL protein consists of 214 amino acids and has two structural domains: the α-domain and the β-domain [13]. VHL functions as a negative regulator of hypoxia inducible factors (HIFs), a family of transcription factors that regulate genes involved in the cellular response to hypoxia [14]. The best characterized function of pVHLs is its role as a substrate recognition component of the E3 ubiquitin protein ligase complex comprising pVHL, Elongin C, Elongin B, Cullin 2 and Rbx1 [13]. In this complex, pVHL targets the α-subunit of hypoxia inducible factor 1 (HIF-1α) and hypoxia inducible factor 2 (HIF-2α, also known as EPAS1) transcription factors for ubiquitin-mediated proteolysis [13].

Recent DNA sequencing studies have identified a number of novel frequent mutations of histone modifying and chromatin remodeling genes in ccRCC including *PBRM1* and *SETD2* [15]. *PBRM1* and *SETD2* genes are located in close proximity to *VHL* within a commonly lost (approximately

Rozhgar A. Khailany is with the medical biology department, health science institute, Gaziantep university, Turkey (009647504632673; e-mail: rozhgarbio@yahoo.com).

Mehri Igci is with medical biology department, health science institute, Gaziantep University, Turkey (e-mail: mehriigci@gmail.com).

Emine Bayraktar and Ahmet Arslan are with the medical biology department, health science institute, Gaziantep University, Turkey.

Sakip Erturhan is with the urology department, health science institute, Gaziantep University, Turkey.

Metin Karakok is with the pathology department, health science institute, Gaziantep University, Turkey.

90%) 3p locus [15]. *PBRM1* gene, a tumor- suppressor, which is located to chromosome 3p21 and encodes the BAF180 protein, a chromatin targeting subunit of a SWI/SNF chromatin remodeling complex, which is involved in transcriptional activation and repression of selected genes [12], [16]. *PBRM1* acts as a negative regulator of cell proliferation [16]. *PBRM1* mutations are more common in patients with advanced stages and loss of *PBRM1* protein expression has been associated with advanced tumor stage, low differentiation grade and worse patient outcome. *PBRM1* mutations and/or loss of protein expression have been suggested in a large portion of clear cell subtype of RCC. Therefore, *PBRM1* is the second most common mutated gene after *VHL* [15].

The *SETD2* gene is located on the short (p) arm of chromosome 3 (3p21-31), 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 [12], [17], [18]. *SETD2* gene was recently implicated in association with RCC, by means of advanced technologies such as the next-generation sequencing, a microarray based analysis and a mouse transgene analysis [12].

Since *VHL*, *PBRM1* and *SETD2* are mostly mutated genes leading to kidney cancer development and located in the same region of chromosome 3, we aimed to determine mRNA expression levels and probable mutations of *VHL*, *PBRM1* and *SETD2* genes among kidney cancer patients.

II. Materials and Methods

A. Sample Collection

The samples were collected from the Urology department at Gaziantep University. A total of 60 samples were analyzed. The study included 30 paired normal and tumor fresh samples of patients that were grouped according to the types of kidney cancer and the clinical characteristics of the patients, including gender and average of age (Table I). The tissue samples of the kidney were stored at -80˚C until further analysis. The study was approved by the local Ethics Committee (approval number: 02.04.2013\ 137) and was conducted in accordance with the guidelines of the Declaration of Helsinki.

TABLE I
Total Number of Patient Samples According to Type of Kidney Cancer and Gender

Cancer types	Male	Female	Total
Clear cell RCC	10	8	18
Papillary RCC	4	1	5
Chromophobe RCC	3	3	6
Oncocytoma	0	1	1

B. RNA Extraction

RNA from kidney tissue was extracted with RNeasy Total RNA Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Sample with (A260 – A320) / (A280 – A320) ratios less than 1.7 and/or yields less than 0,5 µg total RNA were excluded from subsequent analysis.

C. Complementary DNA Synthesis

In this study ProtoScript First Strand cDNA Synthesis Kit (BioLabs, England) was utilized. A control reaction without reverse transcriptase was prepared to examine the DNA contamination in the samples.

D. RT-PCR Amplification and Gel Discrimination

Semi-quantitative reverse transcriptase-PCR technique was used in this research to amplify the VHL, PBRM1 and SETD2 mRNAs. Three pairs of primers were designed by SDSC workbench online primer design program (Table II) and a pair primer of GAPDH (housekeeping gene) were designed. PCR reaction was performed by using MJ Research, AB Applied Biosystem thermal cyclers. Agarose gel electrophoresis (2% w/v) is employed to check the efficiency of PCR reactions and stained with ethidium bromide (EtBr) to make the DNA visible under UV light.

TABLE II
Primer Sequences, PCR Product Size of Three Targets Region of *VHL*, *PBRM1* and *SETD2* Genes and Optimal Annealing Temperature

Primer name	Sequence 5, to 3,	Optimal Annealing Temperature	PCR product size
	VHL isoform 1		
FW Primer	AGATGCAGGGACACACGATG	53 °C	273 bp
RV primer	ATCCGTTGATGTGCAATGCG		
	PBRM domain1		
FW Primer	TCCAACTGTAGATCCTATTGCCG	55,4°C	394 bp
RV primer	CATCTGCTTCTCCTTTCTGAACA		
	SETD2		
FW Primer	ACGTCATGGGCTGTCTTTGT	54,1°C	300 bp
RV primer	TTGGGAGTGTCTGTGTCAGC		

E. Measurement of Expression Analysis

The denaturing polyacrylamide gel was utilized to separate and measure mRNA expression level of VHL, PBRM1 and SETD2 genes. Also GAPDH (housekeeping gene) was measured by urea denaturation. In this study, the expression level of mRNA was quantitated by ImageJ software program (version 1.46r, downloaded from http://imagej.nih.gov/ij) by measuring the integrated densities of the fragments [24]. The statistical analysis of mRNA expression was carried out using Wilcoxon signed rank test. Significance was assumed for values $p \leq 0.05$.

F. PCR-Single-Strand Conformation Polymorphism (SSCP) and Sequence Analysis

In order to monitor the probable variations of the selected regions of VHL, PBRM1 and SETD2 genes, SSCP analysis was applied. The fragments which display different electrical mobility were assessed via nucleotide sequence analysis. In this study ABI 3130X nucleotide sequence analyzer (Singapore) was used. The PCR fragments of the VHL, PBRM1 and SETD2 were excised from the polyacrylamide gel and used as a source of DNA template for sequence-specific PCR amplification.

III. Results

A. Gene Expression Results

In this study the amplified regions of VHL, PBRM1 and SETD2 mRNAs were separated by denaturing 7% polyacrylamide gel electrophoresis with urea and visualized by silver nitrate staining. The GAPDH (housekeeping gene) was treated in the same way, as well.

The quantitative mRNA expression level of VHL, PBRM1 and SETD2 tumor samples were decreased comparing to expression of normal samples (VHL; p = 0,312/ PBRM1; p = 0,168/ SETD2; p = 0,226). However, the results were statistically insignificant (Wilcoxon signed rank test). The mRNA expression level for both normal controls and tumors are shown in Fig 1.

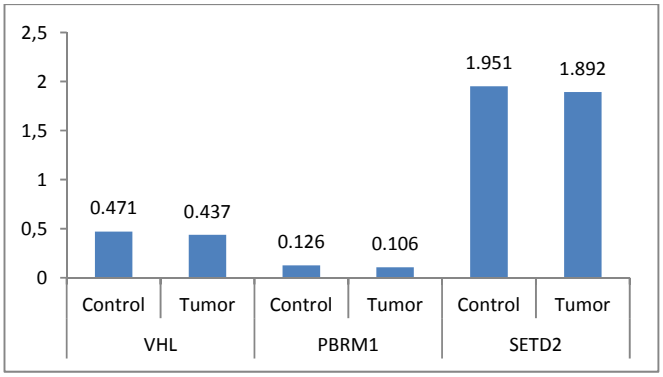

Fig. 1 The mRNA expression level of VHL/GAPDH, PBRM1/GAPDH and SETD2/GAPDH genes for both normal and tumor samples

B. SSCP Results

The three target regions of mRNA sequences of VHL, PBRM1 and SETD2 genes were screened. By the study it was tried to find different bands (different genotypes). All PCR products of the VHL isoform 1, Polybromo domain 1 and a random region of SETD2 were electrophoresed on a 7% polyacrylamide gel. However no different fragment was observed.

C. Sequence Results

The SSCP technique by polyacrylamide gel electrophoresis was employed for the detection of probable gene mutations. However, no different band was found in samples. After that, in order to support SSCP results sequence analysis was undertaken and conducted.

The reference DNA sequences of VHL, PBRM1 and SETD2 genes were obtained from the NCBI website, in order to compare the resulting DNA sequences of patient samples (Query Sequence). However, no variation was found in the sequence of PCR template for three regions after comparing with the reference sequence (Fig. 2).

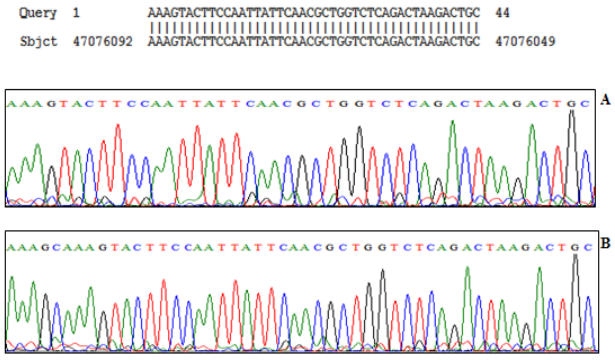

Fig. 2 The sequencing result of SETD2 gene. A) The normal sample. B) The tumor sample. No variation was observed

IV. Discussion

Lots of expression profiling researches have suggested the potential of gene expression models to distinguish between histologic subtypes of RCC, such as papillary type 1 and type 2 carcinomas, conventional ccRCCs, oncocytomas, chromophobe carcinomas and urothelial carcinoma of the renal pelvis [2]. Gene expression analysis has showed many specific diagnostic markers [2]. Examples are a-methylacyl-CoA racemase in pRCC, the S100A1 gene as a marker to discriminate between renal oncocytoma and chromophobe RCC, and glutathione S-transferase highly expressed in ccRCC [2], [11].

Previous work has demonstrated that VHL loss alone is not enough for ccRCC tumorigenesis arguing the need for additional genetic events and has extra reported the being of a 3p21 “gatekeeper” ccRCC mutation based on LOH researches [18]. Multiple cancers have apparently concomitant VHL, PBRM1 and SETD2 mutations, with all of three genes mapping to chromosome 3p, suggesting that the mutations are non-redundant functionally [18].

Varela et al. (2011) suggested that inactivation of PBRM1 comprises this second major mutation in ccRCC development. Nearly all (36/38) PBRM1 mutant cases fell into the hypoxia signature group. Understanding the contribution of PBRM1 mutation to clinical disease progression and consequence as well the potential for exploiting SWI/SNF complex abrogation therapeutically are more important areas of renal cancer study [18].

Xie et al. (2008) demonstrated that SETD2 downregulated hdm2 expression by targeting its P2 promoter and then enhanced p53 protein stability. Collectively, these findings suggest that the histone methyltransferase SETD2 could selectively control the transcription of subset genes via cooperation with the transcription factor p53 [19].

In our study, the mRNA expression levels of VHL, PBRM1 and SETD2 genes were decreased (downregulated) as shown in Fig. 1. However, these were not statistically significant by Wilcoxon signed rank test; p > 0,05. Similarly, Yao et al (2002) also reported the downregulation of VHL expression [20].

Functional inactivation of PBRM1 in the context of pVHL

loss of function may represent a key event in facilitating the development of key aspects of an aggressive tumor behavior [19]. Given the role of PBRM1 in chromatin modification, the gene expression pathways disrupted by the inactivation of this protein may lead to new treatment strategies for ccRCC [19].

Epigenetic studies, epigenetic alterations are the hallmarks of cancer cells and their roles in renal tumor development and progression [21]. According to the last 10 years report, epigenetic alterations also play an important role in renal tumorigenesis [2], [21]. 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 [21]. A microRNA expression study; involved in tumor development and tumor progression including metastasis [2]. Junker et al. (2013) analyzed distant metastases with primary tumors and found a distinct miRNA signature at metastases. Some of the primary tumor samples clustered together with the distant metastasis, suggesting that these primary tumors have a metastasis-specific signature [2].

Many previous studies have demonstrated that VHL includes some types of mutations. Gillian et al. (2010) identified 2 point mutations in three samples VHL genes. Cherkasova et al (2011) reported 9 of 14 ccRCC cell lines had mutations or deletions in one of three VHL exons resulting in amino acid substitutions or the creation of stop codons. Varela et al. (2011) identified three insertion of frame shift, two in-frame deletion, six missense and nonsense mutations [22], [23].

Hakimi et al. (2012) suggested terms of SETD2, they identified 11 loss-of-function and 3 low impact missense mutations. Among the 11 tumors bearing SETD2 loss-of-function mutations, 10 occurred in stage 3, of which 2 presented with metastatic disease and 2 subsequently developed metastatic diseases. In total, the overall metastatic rate of patients with SETD2 mutated primary tumors is 36% (4/11), implicating a functional connection between SETD2 and cancer metastasis [15].

In this study, not all but mostly mutated regions of VHL (isoform 1), PBRM1 (domain 1) and a random region of SETD2 genes were analyzed by SSCP and sequence analysis. However, no mutation was detected in these regions on the contrary of previous studies.

In conclusion, the mRNA expression level of VHL, PBRM1 and SETD2 genes were insignificantly down-regulated. Understanding the molecular mechanisms involved in the pathogenesis of RCC has aided the development of molecular-targeted drugs for kidney cancer. In order to understand the relationship between the kidney tumors and biomarkers; further analysis is warranted.

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