European Review for Medical and Pharmacological Sciences 2019; 23: 9313-9320

Transcriptomic expression levels of the VHL, TIMP-3, and RASSF1A genes in renal tumors

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Abstract. – OBJECTIVE: In this study, we aimed to investigate the relation between the mRNA expression levels of VHL, TIMP-3 and RASSF1A genes, and the histopathological and clinical characteristics of patients with renal tumors.

PATIENTS AND METHODS: Radical nephrectomy specimens of cases presented without neoadjuvant treatment were confirmed to be cancerous, non-cancerous, benign, and healthy after removal from separate localizations. A total of 69 patients with kidney tumors (138 tissue samples) were included in the study group. RNA isolation, reverse transcriptase PCR (RT-PCR), and quantitative real time PCR (qPCR) were performed, and the GAPDH gene was used to normalize mRNA levels.

RESULTS: In the RCC cancerous tissue, TIMP-3 levels increased 1.3 times and RASSF1A levels increased 1.4 times compared to the corresponding levels in non-cancerous tissues, and there was no statistically significant difference in these values. On the other hand, VHL gene expression levels in cancerous tissue were 2.8 times higher than in matched adjacent non-cancerous tissues ($p < 0.05$). In the case of oncocytomas, TIMP-3 levels were found to be 3.2 times higher, RASSF1A levels 3.8 times higher, and VHL levels 2.2 times lower than the corresponding levels in healthy tissues ($p < 0.05$).

CONCLUSIONS: The roles of VHL, TIMP-3, and RASSF1A mRNA expression in contributing to the development of renal tumors could not be clearly established. Further studies are therefore required to elucidate the mechanisms underlying renal tumors.

Key Words:
Renal cell carcinoma, qPCR, mRNA expression.

Introduction

Renal cell carcinoma (RCC) is a common oncological disorder that accounts for about 3% of all malignancies in adults and 85% of all malignant tumors in the kidney. Over the last thirty years, the incidence rates in Europe and America have increased each year[1]. RCC is usually an asymptomatic type of cancer, and reliable diagnostic and prognostic tumor markers have not yet been identified. Thus, the identification of molecular profiles that are based on global analyses of gene and protein expression could help to identify a new marker specific to RCC[2].

The disruption of the function of the Von Hippel-Lindau (VHL) gene results in the formation of clear cell carcinoma, the most common renal cancer in adults. The VHL gene product is a component of the ubiquitin-ligase complex and is responsible for the control of multiple gene expression[3]. The best-defined function of VHL protein (pVHL) is the control of the cellular response to oxygen. More specifically, pVHL activity leads to hypoxia-induced factor (HIF) inactivation in the presence of oxygen[4]. In normal cells, HIF generally helps to coordinate the changes observed in gene expression in response to oxygen[5]. Generally, the activation of the HIF pathways is low in non-cancerous tissues *in vivo* and standard cell culture conditions. When the amount of oxygen decreases, VHL becomes inactive, and HIF stability increases; the transcription of a large number of target genes is subsequently induced following activation of the pathways[6]. Therefore, alterations in the VHL gene have an important role in the development and progression of RCC[7].

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Matrix metalloproteinases (MMPs) are enzymes that enable the cleavage of various extracellular matrix (ECM) components and are responsible for remodeling the ECM. However, the irregularity of MMPs has been observed in several disorders, including autoimmune diseases and cancers[8]. In fact, the increase in expression of MMPs is important for tumor invasion and metastasis in many cancers[9]. Tissue metalloproteinase inhibitor (TIMP) family members inhibit the proteolytic activities of active MMPs by forming inhibitor complexes with enzymes. There are four members of the TIMP family: TIMP-1, -2, -3, and -4[10]. TIMP-1 possesses the potential to inhibit the activity of many MMPs other than MMP-2, whereas TIMP-2 is a potential inhibitor of many MMPs other than MMP-9. TIMP-3 binds to MMP-1, -2, -3, -9 and -13, while TIMP-4 binds to MMP-1, -3, -7, and -9[11].

The Ras-association domain family 1 isoform (RASSF1A) is a promoter in the 3p21.3 region that directly binds to the Ras gene and induces GTP-dependent apoptosis. At the same time, Ras regulates cell proliferation in the molecular pathway and binds to microtubules to induce tumor suppression[12]. RASSF1A is methylated in lung, breast, ovarian, kidney, prostate, and thyroid cancers. Re-expression of RASSF1A stops the growth of human cancer cells, and this feature supports the identification of RASSF1A as a tumor-suppressing gene[13]. RASSF1A has seven different isoforms due to its alternative cutting regions and is translated into mRNA from two different promoters composed of CpG islets[14,15]. During RCC development, the relation between the reduction of RASSF1A expression and the early stage of tumor development has been determined[16].

The aim of this study was to determine whether there is a relation between clinical, histological, and pathological differences of patients with renal tumors and the mRNA expression level of VHL, TIMP-3, and RASSF1A genes. If such a relation exists, the role of the gene products with respect to the development of the disease, the general molecular basis of the disease, and the most effective forms of treatment can be determined.

Patients and Methods

Histopathologically, the cancerous and benign tumor tissues in the radical nephrectomy specimens of 79 cases presented without neoadjuvant treatment were confirmed to be benign and cancerous after they were extracted from separate localizations. A total of 69 pairs of tissues (61 patients with RCC and eight patients with benign renal tumors) and their matched adjacent non-cancerous or healthy renal tissues obtained from patients who underwent surgery at the Department of Urology, Faculty of Medicine, Gazi University, were histopathologically confirmed.

The Gazi University Medical Faculty Ethics Committee for Medical Research approved the investigation. A consent form was completed by all patients so that patient samples could be used. Tissue specimens were obtained, and transport was secured through nitrogen vapor and storage at –80°C until RNA isolation. In the study, the isolation of RNA from the tissue, reverse transcriptase PCR (RT-PCR), and quantitative real time PCR (qPCR) analysis were performed.

Determination of the Expression of VHL, TIMP-3, and RASSF1A Genes in mRNA Levels

With respect to RNA isolation, an approximately 0.1 g tissue sample from each kidney tumor was sectioned using a homogenizer (IKA, Wilmington, NC, USA). RNA was extracted using TRIzol reagent (peqGOLD TriFast™, peqlab, Erlangen, Germany) according to the method previously described by Konac et al[17].

For the reverse transcriptase PCR (RT-PCR) method, complementary DNA (cDNA) was synthesized from 1 µg of total RNA with random hexamer primers using a Transcriptor First Strand cDNA Synthesis Kit (RocheDiagnostics GmbH, Mannheim, Germany) according to the manufacturer's instructions.

The quantitative real time PCR (qPCR) method was used for the quantitative analysis of expression at the relevant genomic mRNA level. For the identification of VHL, TIMP-3, and RASSF1A gene expressions, PCR primers [exon–exon junction to allow discrimination between cDNA and genomic DNA (gDNA)] and Universal Probe Library (UPL) probes were used (UPL; Roche Diagnostics, Germany). The primers and UPL probe numbers for this study are provided in Table I.

All PCR reactions were performed with the LightCycler® 480 instrument (Roche Diagnostics, GmbH, Mannheim, Germany) using the following program conditions: 50 cycles at 95°C for 15 s and 60°C for 20 s, with the samples ulti-

Table I. The gene-specific primer sequences and probe numbers.

Gene	Forward Primer	Reverse Primer	UPL probe no.
GAPDH	5′-AGCCACATCGCTCAGACAC-3′	5′-GCCCAATACGACCAAATCC-3′	60
VHL	5′-CCGTTACAACGGCCTACG-3	5′-CGAGTCGACCTCCGTAGTCTT-3	75
TIMP-3	5′-TGCAACTCCGACATCGTG-3′	5′-AAGGGCCCCTCCTTTACC-3′	60
RASSF1A	5′-GACTCTGGGGAGGTGAACTG-3′	5′-GGAGTACTTCTGCAGGATCTGG-3	52

mately being cooled to 40°C. The mRNA level of the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene was used to normalize the mRNA expression levels for the genes of interest.

Statistical Analysis

Pearson's χ^2 and Fisher's precision tests were used to compare patients' demographics. $p < 0.05$ was considered statistically significant. VHL, TIMP-3, and RASSF1A mRNA expressions in renal tumors and non-cancerous tissues were evaluated by comparison with the REST 2009 V2.0.13 (Qiagen, Hilden, Germany) program.

Results

The mean age of the patients was 55.9 (14-78) years. Of the 69 patients, 47 (68.1%) were male and 22 (31.9%) were female. The mean age of patients with RCC was 58.1 (30-78). Of the 61 patients, 43 were male and 18 were female. In these patients, the level of expression of TIMP-3 was found to be 1.3 times higher in the tumor, and the corresponding tumor expression level of RASSF1A was 1.4 times higher than the level in the matched adjacent non-cancerous tissues. Yet, the upregulation of the mRNA levels of TIMP-3 and RASSF1A in RCC cancerous tissue was not statistically different compared with the level in the matched adjacent non-cancerous tissues. On the other hand, the level of VHL gene expression in RCC cancerous tissues was found to be 2.8 times higher than that of the matched non-cancerous tissues. This increase was statistically significant ($p = 0.04$). Notably, there was no significant difference in the expression levels of TIMP-3, RASSF1A and VHL genes between male and female patients when RCC cancerous tissues and their non-cancerous tissue counterparts were compared ($p = 0.123$, $p = 0.099$, and $p = 0.162$, respectively). When RCC patients were grouped according to pathological stages, no significant differences in TIMP-3, RASSF1A, and VHL gene expressions between RCC cancerous tissues and its paired non-cancerous tissues were found ($p = 0.131$, 0.454, and 0.090, respectively). When RCC patients were clustered according to histopathologic type (clear cell, papillary, chromophobe, and sarcomatoid renal cell carcinoma), no differences were found in terms of TIMP-3, RASSF1A and VHL gene expression changes ($p = 0.079$, 0.112, and 0.056, respectively). The expression changes in the genes of RCC patients are summarized in Table II.

The mean age of benign renal tumor patients was 48.7 (14-56). Of the eight patients, four were male and four were female. When these patients were categorized by gender, there were no differences in the expression of TIMP-3, RASSF1A, and VHL genes between benign tumors and their healthy tissue counterparts. Similarly, when patients were classified according to histopathological types, there were no differences in the TIMP-3, RASSF1A, and VHL gene expressions between benign tumors and their paired healthy tissues in the groups with oncocytoma, angiomyolipoma, and metanephric adenomas ($p = 0.642$, 0.732, and 0.454, respectively). However, in the case of oncocytomas, TIMP-3 levels increased 3.2 times, RASSF1A levels increased 3.8 times, and VHL levels decreased 2.2 times in the benign tumor tissues in comparison to the corresponding levels in healthy tissues ($p < 0.01$, $p < 0.01$, and $p = 0.02$, respectively). The clinicopathologic and statistical data of patients with benign tumors are summarized in Table III.

Discussion

Inactivation of the pVHL is a frequent cause of clear cell renal carcinoma (ccRCC), the most common form of RCC. It has been established that pVHL fulfills a number of functions including serving as a substrate recognition module for a ubiquitin ligase complex, targeting the alpha subunits of the heterodimeric HIF transcription factor for proteasomal degradation[18]. There have been many studies[19-21] conducted on the frequent

Table II. Differences (fold) in mRNA expressions of the relevant genes in cancerous tissues relative to non-cancerous tissues of the RCC patients (n = 61).

	n (%)	TIMP-3	RASSF1A	VHL
Gender				
Male	43 (70.5%)	1.4	1.3	3.2
Female	18 (29.5%)	1.2	1.3	2.1
p		0.123	0.099	0.162
T Stage				
T1	3 (4.9%)	1.5	1.2	3.1
T2	20 (32.7%)	1.2	1	2.4
T3	25 (41%)	1.4	1.1	2.9
T4	13 (21.3%)	1.3	1.7	2.2
p		0.131	0.454	0.090
Histology				
Clear Cell	48 (78.7%)	1.3	1.4	2.8*
Papillary	7 (11.5%)	1.3	1.6	2.1
Chromophobe	5 (8.2%)	1.1	0.9	2.8
Sarkomatoid	1 (1.6%)	1.9	1	3.4
p		0.079	0.112	0.056

*Difference is statistically significant ($p < 0.05$).

occurrence of mutations in the VHL gene in patients with RCC. Gossage et al[22] noted that, of the 61 ccRCC patients, inactivation of VHL in cancerous tissues (coding mutation or promoter methylation) was detected in 75% of ccRCCs. It has been shown that somatic non-coding VHL alterations were identified in 29% of these RCCs and may be associated with improved overall survival rates[22]. In another recent study by Huang et al[23], the mRNA and protein expression levels of VHL, HIF-1, BCL2 interacting protein 3 (BNIP-3) and vascular endothelial growth factor (VEGF) were measured in 30 ccRCCs and their adjacent non-cancerous tissues[23]. They found that the expression levels of BNIP3 and VHL were lower in the ccRCC tissues than they were in the pericarcinous tissues. Also, there was no significant correlation between the BNIP-3 mRNA and their protein levels and the expressions of VHL, HIF-1alpha, and VEGF. Xiao-Fen et al[24] showed that the expression levels of VHL and a tumor suppressor gene Jade-1 were analyzed in RCC tissues. Researchers found a downregulation in the expression of the VHL gene in 62.7% of the 75 RCC tissue samples compared to the matched adjacent non-cancerous tissues[24] .

However, our findings present a number of differences from the previously discussed studies. We showed that the RCC cancerous tissues exhibited significantly higher expression of VHL, compared with the matched non-cancerous tissues. We also detected a diminished expression level

Table III. Differences (fold) in mRNA expressions of the genes in benign tumor tissues relative to healthy tissues of the patients (n= 8).

	n (%)	TIMP-3	RASSF1A	VHL
Gender				
Male	4 (50%)	3.1	2.7	0.7
Female	4 (50%)	3.3	2.4	0.9
p		0.123	0.099	0.162
Histology				
Oncocytoma	4 (50%)	3.2*	3.8*	↓2.2*
Angiomyolipoma	3 (37.5%)	2.7	2.2	1
Metanephric adenoma	1 (12.5%)	3.9	2.1	0.8
p		0.642	0.732	0.454

*Difference is statistically significant ($p < 0.05$); ↓: downregulation.

of VHL in benign tumors, when compared to the matched healthy tissues. Yet, this decrease was not statistically significant. The increased VHL expression seen in RCC tumors and not in benign tumors suggests that the HIF pathways may in some cases use VHL-independent mechanisms. Another reason why the level of VHL mRNA in RCCs was shown to be significantly higher in cancerous tissues than in benign tumor tissues may be that we have not shown whether there was a mutation in the VHL gene. In other words, although there exists an expression of mRNA, we do not know whether or not there is a functional protein present. This can be regarded as a limitation of our study. A report which supports the importance of functional VHL protein was published in 2011 by Cherkasova et al[25]. In this study, mutations in the VHL gene were detected in non-RCC tumors even though there was no VHL promoter methylation. Since the VHL protein performs tumor suppressor function, mutations that would prevent active protein formation is more important than promoter methylation status or mRNA expression changes. A study conducted by Banks et al[26] emphasizes the importance of mutation in this regard. In this case, the researchers found promoter methylation in only 19 of 93 (20.4%) sporadic ccRCC samples examined, in one of three transitional cell carcinoma (TCC) samples, in four of eight papillary RCC samples, and in one of two unclassified RCC samples.

Several studies have shown that an increase in the expression of the RASSF1A gene induces apoptosis and cell cycle arrest. Lack of RASSF1A leads to elevated mitotic activity and chromosomal defect[27]. Indeed, the reduction in RASSF1A expression is usually due to promoter hypermethylation. In many studies[28-30], it has been shown that RASSF1A methylation can be used as a prognostic marker in small cell lung cancer, breast cancer, and ccRCC. In many studies, it has been determined that the expression of the RASSF1A gene in RCC is reduced at the mRNA level. As the tumor progresses, this expression appears to decrease[31-33]. In contrast to some previous studies, Pronina et al[34] found an increase in RASSF1A expression levels in renal, breast, ovarian, and colorectal cancers. They noted that RASSF1A expression changes occurred due to tumor specificity and may indicate unstable RASSF1A functions in tumors, that is, RASSF1A may function as a tumor suppressor gene or a proto-oncogene. In our study, we found no significant differences in the mRNA expression levels of RASSF1A between the cancerous tissues and their non-cancerous tissue counterparts regardless of the histopathological type.

One of the most important factors contributing to the development and especially the progression of RCC is the pro-angiogenic shift of the pro-angiogenic and anti-angiogenic balance. The majority of these activators are kinase receptor ligands such as VEGF. The only known physiological antagonist of VEGFR2, one of the VEGF receptors, is TIMP-3[35]. Hagemann et al[36] examined the expression patterns of TIMP 1, 2, 3, and 4 in the cancerous tissues of patients with RCC and found that expression of these genes was reduced in all types; however, TIMP-4 increased in the cancerous tissue of the papillary cell type[37]. This study shows that both the TIMP subtypes and the histopathological type of RCC may differ in their gene expression levels. In our study, there was no significant reduction in the expression of TIMP-3 in the cancerous tissue, which may indicate that it may not be accurate to explain the metastatic potential of RCC cancer cells through only the TIMP-3 mRNA level. It should also be noted that Masson et al[37] have found that the expression of TIMP-3 in the cancerous tissue is reduced, especially in Grade 4 tumors, compared to corresponding levels in non-cancerous tissues. We have seen such an increase in our own work, but this increase was statistically insignificant. As in the case of the VHL gene, mutations and mutation-related dysfunctional protein theory may also apply here. Another important finding has shown that as the stage progressed from T1 to T4, the expression of the VHL gene mRNA decreased[38]. In parallel, we have observed that the expression of mRNA of the VHL gene in cancerous tissues was the highest at T1 stage.

There are not many studies in the literature on benign renal cortical tumors that reveal the relation between the expression levels analyzed in our study. Brauch et al[38] have investigated the expression of VHL genes in the histopathological subtypes of renal epithelial tumors and have shown that this gene's methylation or mutation increases in all subtypes except for chromophobe renal cell cancer and oncocytoma. In a study aiming to establish a genomic algorithm to determine the histopathologic type of renal cortical tumors, the absence of the VHL gene mutation appears in the first leg of the algorithmic stratum advancing to oncocytoma[39]. In our study, it was observed that there was a significant decrease in the expression of the VHL gene in

oncocytoma cases. Within various human renal cancer subtypes, weak VHL immunohistochemical expression predominates in descending order: Chromophobe RCC > ccRCC > papillary RCC > unclassified RCC > oncocytoma[40]. Patients with weak VHL expression tended to show a shorter overall survival, indicating loss of VHL protein as a possible negative factor for patient survival. This situation contradicts the limited knowledge in the literature, but it shows that further investigations are needed in this regard.

Conclusions

We showed that the absence of any currently valid markers for the diagnosis of RCC generally made it difficult to detect this asymptomatic type of cancer. For this reason, many genomic products that can be used as a diagnostic marker for RCC are being investigated today. The expression levels of some of these genes, including RASSF1A, TIMP-3, and VHL, have been examined in our study, but no evidence has been found to support the use of these gene constructs as markers for RCC. If RCC pathogenesis is thought to be based on multifactorial genetic factors, it is evident that more in-depth studies are needed to clarify this issue.

Conflict of Interest

The Authors declare that they have no conflict of interests.

Ethical Approval

24.06.2009 – No: 392 Gazi University Faculty of Medicine Clinical Research Ethics Committee.

Acknowledgements

This study was conducted with financial support from the Gazi University Research Fund with the project code number 01/2010-25.

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