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SHORT COMMUNICATION

VHL and HIF-1α: gene variations and prognosis in early-stage clear cell renal cell carcinoma

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Abstract Von Hipple–Lindau gene (*VHL*) inactivation represents the most frequent abnormality in clear cell renal cell carcinoma (ccRCC). Hypoxia-inducible factor-1α (HIF-1α) expression is regulated by O_2 level. In normal O_2 conditions, VHL binds HIF-1α and allows HIF-1α proteasomal degradation. A single-nucleotide polymorphism (SNP) has been found located in the oxygen-dependent degradation domain at codon 582 (C1772T, rs11549465, Pro582Ser). In hypoxia, VHL/HIF-1α interaction is abolished and HIF-1α activates target genes in the nucleus. This study analyzes the impact of genetic alterations and protein expression of *VHL* and the C1772T SNP of HIF-1α gene (*HIF-1α*) on prognosis in early-stage ccRCC (pT1a, pT1b, and pT2). Mutational analysis of the entire *VHL* sequence and the genotyping of *HIF-1α* C1772T SNP were performed together with *VHL* promoter methylation analysis and loss of heterozygosis (LOH) analysis at (3p25) locus. Data obtained were correlated with VHL and HIF-1α protein expression and with tumor-specific survival (TSS). *VHL* mutations, methylation status, and LOH were detected in 51, 11, and 12 % of cases, respectively. Our results support the association between biallelic alterations and/or *VHL* silencing with a worse TSS. Moreover, we found a significant association between the *HIF-1α* C1772C genotype and a worse TSS. The same association was found when testing the presence of HIF-1α protein in the nucleus. Our results highlight the role of VHL/HIF-1α pathway in RCC and support the molecular heterogeneity of early-stage ccRCC. More important, we show the involvement of *HIF-1α* C1772T SNP in ccRCC progression.

Keywords Renal carcinoma · VHL · HIF-1α · ccRCC

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Introduction

Renal cell carcinoma (RCC) is the most common malignant tumor in the adult kidney, accounting for about 3 % of all human malignancies with the clear cell subtype (ccRCCs) represented 80 % of all RCCs [1].

One of the most common alterations in sporadic ccRCC is the biallelic inactivation of Von Hipple–Lindau gene (*VHL*) due to mutation, promoter hypermethylation, or loss of heterozygosity (LOH) of chromosome 3p [2, 3].

Von Hipple–Lindau gene is a tumor suppressor gene (TSG) mapping at 3p25–26 and containing three exons [1]. Under normal conditions, the protein encoded by *VHL*, pVHL, localizes preferentially in the cytoplasm maintaining, however, the ability to shuttle between cytoplasm and

nucleus [4]. pVHL exists in two different isoforms: pVHL30 and pVHL19 [4]. pVHL19 has been shown to contribute to the formation of an ubiquitin ligase (E3) complex (VEC), which controls the proteasomal degradation of several target genes including the hypoxia-inducible factor-1α (HIF-1α) [5]. The ability of pVHL and HIF-1α to bind each other depends upon the cellular oxygen level. Under normal oxygen conditions, HIF-1α is hydroxylated in the nucleus on conserved prolyl residues (Pro 402 and Pro 564) within the oxygen-dependent degradation (ODD) domain [6]. Hydroxylated HIF-1α is able to bind pVHL and such binding allows its polyubiquitination and its consequent degradation [6]. On the contrary, under hypoxic conditions, the non-hydroxylated HIF-1α cannot bind pVHL and is free to translocate into the nucleus. Once in the nucleus, HIF-1α dimerizes with the HIF-1β subunit and *trans*-activate its target genes [6], which are mainly involved in the resistance to hypoxia, in the glucose uptake and metabolism (Glut1), regulation of extracellular pH (CAIX), angiogenesis (VEGF), erythropoiesis (EPO), and mitogenesis (PDGF-B) [5].

Von Hipple–Lindau gene alterations suppress the ability of pVHL proteins to bind HIF-1α causing the constitutive expression of HIF-1 and its inducible genes [7]. Of note, HIF-1α expression has been shown to be high in ccRCC [7].

Von Hipple–Lindau gene inactivation can occur through mutation (approximately 50 %) [2, 7, 8], hypermethylation (10–20 %) [2, 9], and/or LOH (84–98 %) at locus 3p25 [10].

In our previous study, we showed that the cytoplasmic-nuclear trafficking of pVHL is blocked in ccRCC and the absence of pVHL concurrently associates with a strong HIF-1α expression [11]. A relatively recent study from Kim et al. [12] showed that a SNP mapping in the *HIF-1α* ODD domain (C1772T, rs11549465) associates with HIF-1α protein expression in breast cancer. This SNP leads to a proline to serine substitution at codon 582 [13, 14]. Although C1772T has been shown not to affect the hydroxylation process [15], it might confer susceptibility to RCC and its presence has been demonstrated to correlate with the development of metastases [14]. Here, we evaluated the prognostic significance of such SNP in ccRCC patients. It should be noted that although a growing number of evidence on the molecular alterations underlying ccRCC pathogenesis is currently being accumulated [16], the prognostic relevance of such alterations still lacks of an appropriate investigation. Moreover, contrarily to the majority of the studies already published in the literature, which pool together samples from RCC patients at different stages, we specifically selected a cohort of 136 early-stage ccRCC patients (pT1a, pT1b, and pT2). By simultaneously analyzing clinical samples through direct sequencing, DNA promoter methylation, immunohistochemistry and survival analyses, we show here the ccRCC molecular heterogeneity and evaluate the prognostic significance of *VHL* and *HIF-1α* molecular alterations in this specific subset of early-stage ccRCC patients.

Materials and methods

Tumor samples

Formalin-fixed paraffin embedded (FFPE) tissues were collected from a total of 136 intracapsular early-stage ccRCC patients (pT1a, pT1b, and pT2) (mean age 62 year, range 28–85), who underwent radical nephrectomy from 1991 to 2001. Clinical characteristics of this series are reported elsewhere [17]. The evaluation of pathological staging, histotype, nuclear grading, and DNA content was performed as previously described [17].

Tissue microarrays

Tissue microarrays (TMAs) were established in order to avoid inter-analysis variability during IHC. Core tissue biopsies (1 mm in diameter) were taken from morphologically representative, non-necrotic regions of individual RCC tumor samples and arrayed into new paraffin blocks (45 × 20 mm^2) using Chemicon ATA-100 Tissue Arrayer (Chemicon International Inc., Temecula, CA, USA). Sections of the TMA blocks (5-mm thick) were transferred to glass slides. Each TMA contained 50 tissue cylinders: ten from each of the three primary tumor areas and ten from each of the two paired normal tissues. TMA sections were then deparaffinized and rehydrated.

Immunohistochemistry

Two commercially available monoclonal pVHL antibodies, clone Ig32 (BD-Bioscience, San Jose, CA, USA) and clone Ig33 (Lab Vision, Kalamazoo, MI, USA), were used at 1:50 and 1:200 dilution, respectively, with clone 32 recognizing both pVHL30 and pVHL19 isoforms and clone 33 specific for pVHL30. A polyclonal anti-HIF-1a antibody (Zymed, Las Condes, Santiago, Chile) was used at 1:50 dilution. The reactions were performed in an automated system (BenchMark XT Ventana, Oro Valley, AZ, USA) and the avidin–biotin peroxidase method was used for visualization. Each IHC analysis was done in duplicate.

VHL sequencing analysis

Von Hipple–Lindau gene sequence was screened for mutations by direct sequencing. Genomic DNA was isolated from FFPE samples, using QIAmp DNA Mini Kit (Qiagen, Germantown, MD, USA), following the manufacturer's instructions. Primers were designed using

Table 1 Primer sequences

VHL1a forward	5′-GGTCTGGATCGCGGAGGGA-3′
VHL1a reverse	5′-GCCCGGCCTCCATCTCCT-3′
VHL1b forward	5′-GCGGAGAACTGGGACGAG-3′
VHL1b reverse	5′-GCGATTGCAGAAGATGACCT-3′
VHL1c forward	5′-GCCGAGGAGGAGATGGAG-3′
VHL1c reverse	5′-GCTTCAGACCGTGCTATCGT-3′
VHL2 forward	5′-AACCTTTGCTTGTCCCGATA-3′
VHL2 reverse	5′-CAGGCAAAAATTGAGAACTGG-3′
VHL3 forward	5′-GATTTGGTTTTTGCCCTTCC-3′
VHL3 reverse	5′-CCATCAAAAGCTGAGATGAAA-3′
C1772T forward	5′-GCTCCCTATATCCCAATGGA-3′
C1772T reverse	5′-CAGTGGTGGCAGTGGTAGTG-3′
D3S1597 forward	5′-CACACAAATGTCTCTCCCTGTG-3′ (FAM)
D3S1597 reverse	5′-TCGTTCATTGCTCAAACTCTTT-3′
D3S1038 forward	5′-CACGGGAGTCTACAGCTTGG-3′ (FAM)
D3S1038 reverse	5′-TCTGCTCAACTCCCTCCAGT-3′

Primer3 software (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) (Table 1).

PCR was performed using 9700 GeneAmp PCR System (Applied Biosystems, Foster City, CA, USA) following standard procedure: initial denaturation at 95 °C for 7 min; 40 cycles at 94° for 45 s, specific annealing temperature for 45 s, 72 °C for 1 min; final step at 72 °C for 10 min. PCR products were purified and labeled using Big Dye terminator kit version 3.1 (Applied Biosystems, Foster City, CA, USA). Sequencing was then performed using Biosystems 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA) and analyzed by sequencing analysis software 3.4 version.

In order to avoid artifacts arising from technical issues, we repeated all the molecular analyses (included the ones described below) 3 times for each samples.

LOH analysis at locus 3p

Loss of heterozygosis was assessed for the D3S1597 and D3S1038 microsatellites located at 3pter–3p24.2 and 3p26.1–3p25.2, respectively.

Primers are reported in Table 1. PCR was performed as described above. PCR products (163 bp length for D3S1597 and 174 bp length for D3S1038) were loaded on ABIPRISM 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA) and analyzed by GeneScan software.

VHL promoter methylation-specific PCR

One μg of genomic DNA was bisulfite-converted using EZ DNA Methylation Kit (Zymo Research Irvine, CA, USA). A positive control was created using human blood treated with CpG Methylase (SssI) (Roche, Basel, Switzerland). PCR amplification was then performed using primers specific for methylated and unmethylated DNA as described by Kuroki et al. [18]. PCR was carried out as described above. PCR products were finally run on a 3 % agarose gel with appropriate size markers.

Table 2 List of VHL mutations detected in this study

Sample	Mutation	Description	Hetero/homo
1	c. 224_225del	Deletion	Heterozygous
2	c. 484T>C	Transition	Heterozygous
3	c. 220_240dup20	Duplication	Heterozygous
4	c. 294del	Deletion	Heterozygous
5	c. 452T>C	Transition	Heterozygous
6	c. 416del	Deletion	Heterozygous
7	c. 497T>A	Transversion	Heterozygous
8	c. 509del	Deletion	Heterozygous
9	c. 164del	Deletion	Heterozygous
10	c. 266 T>A	Transversion	Heterozygous
11	c. 160_174del	Deletion	Heterozygous
12	c. 494T>A	Transversion	Heterozygous
13	c. 242 C>T	Transition	Homozygous
14	c. 259G>A	Transition	Homozygous
14	c. 343C>T	Transition	Homozygous
14	c. 559G>A	Transition	Homozygous
15	c. 434del	Deletion	Heterozygous
16	c. 388G>A	Transition	Homozygous
16	c. 463G>A	Transition	Homozygous
17	c. 221T>A	Transversion	Homozygous
18	c. 461C>T	Transition	Homozygous
18	c. 625C>T	Transition	Heterozygous
19	c. 424G>A	Transition	Homozygous
20	c. 213_233dup20	Duplication	Heterozygous
21	c. 263_270del	Deletion	Heterozygous
22	c. 287del	Deletion	Heterozygous
23	c. 263G>A	Transition	Homozygous
24	c. 238A>T	Transversion	Heterozygous
24	c. 239G>T	Transversion	Heterozygous
25	c. 309del	Deletion	Heterozygous
26	c. 217_225dup8	Duplication	Heterozygous
27	c. 343C>A	Transversion	Heterozygous
28	c. 368G>A	Transition	Homozygous

HIF-1α C1772T SNP sequencing

A 178-bp-length PCR region including C1772T SNP was amplified as described above using the primers reported in Table 1. The PCR product was then purified, labeled, and sequenced as described above.

Statistical analysis

Two-tailed chi-square test was used to assess differences in pVHL and HIF-1α molecular status and protein expression. Survival probability was estimated by Kaplan–Meier method. All the statistical analyses were performed using MedCalc (MedCalc Software, Ostend, Belgium) and Instat3 software (GraphPad Software, Inc. La Jolla, CA, USA).

Results

VHL mutational analysis

Von Hipple–Lindau gene mutational analysis was performed in 55 cases out of the 136 samples available. *VHL* mutations were detected in 28/55 (51 %) ccRCCs. Four additional patients presented multiple mutations. The mutations were located in exon 1 (17/33, 51 %), in exon 2 (10/33, 30 %), and in exon 3 (6/33, 18 %). Among the 13/33 (39 %) truncating mutations, 11 were frameshift and 2 were non-sense mutations. All known mutations detected in this study (Table 2) have been reported previously in the Human Gene Mutation Database (Institute of Medical Genetics in Cardiff: The Human Gene Mutation Database; www.hgmd.cf.ac.uk/ac/) and in the Catalogue of Somatic Mutations in Cancer (COSMIC; www.sanger.ac.uk/genetics/CGP/cosmic/).

LOH analysis at VHL locus 3p

Loss of heterozygosis was assessed in 79 and 75 cases at the marker loci D3S1038 (centromeric to *VHL* locus) and D3S1597 (telomeric to *VHL* locus), respectively. Sixty-one cases were informative for D3S1038 and 64 cases were informative for D3S1597. LOH was found in 15/79 (19 %) and 21/75 (28 %) for D3S1038 and D3S1597, respectively. Forty-one cases have been analyzed for both microsatellites. 6/49 (12 %) cases presented LOH in both the markers and 12/49 (24 %) and 19/49 (39 %) cases had D3S1038 and D3S1597 LOH, respectively.

VHL promoter methylation-specific PCR

Von Hipple–Lindau gene promoter methylation was analyzed in 53 cases. *VHL* promoter was found methylated in 6/53 (11 %) cases. No case presented promoter methylation in both alleles.

HIF-1α C1772T SNP

HIF-1α C1772T SNP was genotyped in 117 cases. The 1772T allele was present in 35/117 (30 %) cases. The variant allele (T) was present in heterozygosis (C/T) in 30/35 (86 %) and in homozygosis (T/T) in 5/35 (14 %).

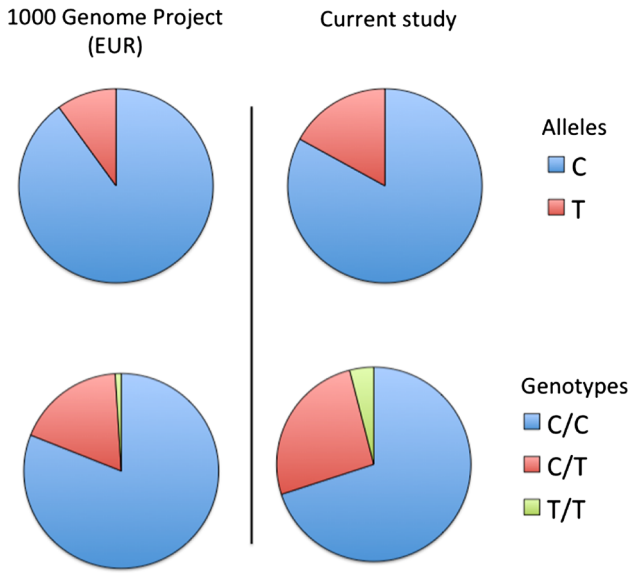

Fig. 1 Pie charts of the allele and genotype frequencies of HIF-1α C1772T SNP in the EUR populations of the 1000 Genome Project and our cohorts of early ccRCC, respectively

In order to investigate whether the genotype and allele frequencies of this SNP in our cohort of ccRCC patients differed from the ones reported for healthy individuals in the 1000 Genome Project, we selected the European populations from the 1000 Genome Project dataset (EUR) and compared the genotype and allele frequencies. Concordant to the literature [19], we found a higher T allele and TT genotype frequency in our cohort of ccRCC patients compared with the European healthy subjects from the 1000 Genome Project (Fig. 1), supporting the cancer-predisposing role of the T allele. Although we acknowledge that a control group should have been recruited from the same population of the ccRCC patients, we believe that the comparison with the 1000 Genome project data provides indication of the potential role of the C1772T SNP in predisposing for ccRCC.

VHL gene variation and protein expression

No significant association was found between *VHL* mutation, microsatellite, or methylation analysis (taken as singular molecular events) and VHL or HIF-1α protein expression. Thus, we decided to pool together samples with two alterations (biallelic).

Out of 50 cases analyzed for *VHL* mutation, LOH and methylation:

- 16/50 (32 %) cases presented biallelic alterations meant as mutation in homozygosis or as presence at the same

Table 3 Correlation between biallelic VHL alterations and pVHL expression

	pVHL+	pVHL−	Total
Non-biallelic alterations	28	3	31
Biallelic alterations	13	2	15
Total	41	5	46

$\chi^2 = 0.14$, $p = 0.7$

Table 4 Correlation between HIF-1α C1772T SNP and the presence of HIF-1α in the nucleus

	C/T + T/T	C/C	Total
HIF-1α nucleus+	11	50	61
HIF-1α nucleus−	15	23	38
Total	26	73	99

$p = 0.021$

time of LOH and mutation, LOH and methylation or methylation and mutation;

- 15/50 (30 %) cases had only one alteration (mutation, LOH and methylation);
- 19/50 (38 %) cases carried no alteration.

The association between pVHL expression and the biallelic alteration of *VHL* was assessed in a total of 46 samples for which all the molecular data were available without reaching statistical significance (Table 3).

HIF-1α C1772C genotype associates with HIF-*1α* protein expression

When assessing the association between the HIF-1α C1772C genotype and HIF-1α protein expression, we found that the HIF-1α C1772C homozygous genotype (absence of the pathological variant) significantly associates with a higher HIF-1α protein nuclear expression ($p = 0.021$) (Table 4).

VHL biallelic variations and tumor-specific survival (TSS)

We then grouped together the normal samples and those with the monoallelic alteration; we found that overall samples carrying biallelic alterations had a worse TSS as compared to samples with no molecular alterations. However, such association did not reach statistical significance (Fig. 2a).

HIF-1α C1772T SNP and TSS

We further analyzed the correlation between TSS and *HIF1α* C1772T SNP. Overall, samples carrying the T/T genotype tended to have a better prognosis as compared to samples carrying the C/C and C/T genotype (Fig. 2b).

Discussion

Renal cell carcinoma has been described as made up of several cancers, having different histology and characterized by a different clinical course and response to therapy [20]. The ccRCC subtype accounts for 80 % of sporadic RCCs and can often be cured, if diagnosed on time, and treated when still localized to the kidney (intracapsular) [1]. Overall, intracapsular ccRCCs are known to have a good prognosis. However, 15–20 % of patients might develop recurrence and eventually die for the disease [21].

The inactivation of the VHL tumor suppressor protein and the deregulation of VHL/HIF-1α axis are common events in ccRCC [5]. The role of pVHL has been extensively studied [5]. pVHL is a multifunctional protein whose function has been clearly linked to kidney carcinogenesis due to its role in the polyubiquitination process. The expression of pVHL is often found altered in ccRCC due to mutations, promoter methylation and LOH of the *VHL*.

When assessing the *VHL* mutational status in our cohort, we found that *VHL* coding sequence was mutated in 51 % of cases, with exon 1 being the most mutated, as already shown in a previous study [8]. A wide discrepancy exists in literature about the *VHL* mutation frequency in ccRCC, ranging between 21 and 66 % [22, 23], which may be explained by the different ethnic origin of the populations recruited in the different studies or, more importantly, by the differential efficiency of the technical approaches applied (for example Sanger sequencing is more sensitive than SSCP).

The frequency of *VHL* LOH at 3p25–26 detected in our study was 11 %, which resulted lower than the one reported in the literature [24, 25]. This could be explained by the fact that we analyzed exclusively early-stage intracapsular ccRCCs while other studies grouped together RCC samples at different stages of progression. Thus, it is likely that LOH may be a molecular event, which gradually accumulates during tumor progression.

Von Hipple–Lindau gene promoter was found methylated in 11 % of cases, concordantly to the findings of other studies, which have reported *VHL* promoter hypermethylation in 7–11 %, cases examined [2, 9].

The prognostic relevance of *VHL* molecular alterations in patients with sporadic ccRCCs remains to be established. Few studies have addressed the issue of clinical impacts of somatic *VHL* alterations in sporadic RCCs reporting, however, conflicting results [2, 15, 26]. Brauch et al. [2] have showed a significant association between *VHL* alterations (mutation and methylation) and advanced RCC

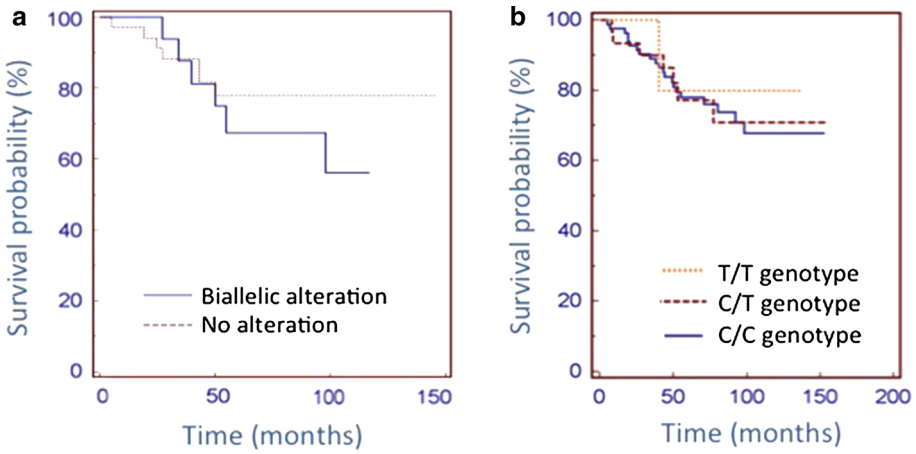

Fig. 2 Kaplan–Meier curves of samples carrying no and biallelic alterations (**a**) and carrying T1772T, C1772T, and C1772C genotypes

tumor stage. Similarly, Schraml et al. [27] showed that ccRCC with *VHL* open reading frame mutations, which potentially lead to pVHL loss of function, are associated with a worse prognosis. Kondo et al. [26], in another study, were not able to find any significant association between *VHL* alteration and disease prognosis.

When assessing here the clinical impact of *VHL* molecular alterations on the prognosis, we found that overall patients with biallelic *VHL* alterations had a shorter TSS, supporting the relevance of *VHL* in RCC carcinogenesis. However, this association did not reach statistical significance, maybe because the casuistic recruited was not large enough to allow the detection of statistical significance. Larger sample size studies are warranted to exploit this issue.

No association between *VHL* mutation, methylation and LOH analysis taken as a singular molecular event and TSS was found in this study, supporting the *VHL* function as TSG. Indeed, TSGs generally require multiple inactivation events to loose their function, according to the two-hits Knudson hypothesis.

Another important player in RCC development is *HIF-1α*. *HIF-1α* resides on chromosome 14q, whose homozygous deletion leads to absent protein production [28]. In some cases, alternative mRNA splicing around deleted *HIF-1α* exonic sequence leads to the production of aberrant HIF-1α isoforms [28]. Although rare, intragenic *HIF-1α* mutations, including missense mutations, have been described in clear renal carcinoma tumors [28]. Besides mutational events, inherited polymorphisms might also play a role in RCC carcinogenesis. A common SNP mapping in the ODD domain in exon 12 of *HIF-1α* (C1772T, Pro582Ser) has been described in the literature [13, 29]. Although not involved in the process of hydroxylation [14], this SNP may confer susceptibility to renal cancerogenesis and progression. Supporting this hypothesis, Kim et al. [8] showed that the Pro582Ser change contributes to the development of metastases. When comparing the distribution of this SNP in our cohort with the 1000 Genome Project data, we found that the T allele frequency was higher in the ccRCC patients compared with the healthy subjects. Moreover, when correlating the different C1772T genotypes with the HIF-1α protein nuclear expression, we found the C/C genotype to be associated with a higher nuclear protein expression ($p = 0.021$). Conversely, samples carrying the T/T genotype did not express HIF-1α in the nucleus. Not surprisingly, heterozygous samples had 50 % presence and 50 % absence of the nuclear HIF-1α protein expression. These data suggest that the C1772T SNP may influence the HIF-1α nuclear expression or, likely, the recruitment of transcriptional cofactors may be enhanced or suppressed depending on the specific allele through conformational changes caused by amino acid substitution. Studies aiming at assessing the functional role of this SNP on the protein nuclear localization are required to exploit this intriguing association.

When assessing the genotype association with TSS, quite surprisingly, we found that the T/T genotype overall associated with a better TSS (Fig. 2b), suggesting that carrying the T allele in homozygosis may determine an advantage in terms of prognosis. The mechanisms behind this phenomenon are not known. We are currently undertaking studies to exploit this association.

Conclusions

In conclusion, our study provides insights about the molecular alteration of VHL/HIF-1α axis in early-stage ccRCC and show the potential role of *HIF-1α* C1772T SNP in affecting HIF-1α protein nuclear expression. Additional functional studies are, however, required to unmask the mechanisms underlying such association.

Conflict of interest None.

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