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Key words: VHL gene, renal cell carcinoma, paraffin, VEGF, angiogenesis

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Word count: 2680 words Abstract: 250 words

Abstract

Introduction: Literature controversies exist regarding the prognostic value of *VHL* mutations. The objective was to compare paraffin-embedded and frozen section specimens for *VHL* mutations detection and to evaluate the reliability of DNA analysis in formalin-fixed tissues.

Methods: 76 patients with clear cell RCCs previously assessed for VHL status from frozen samples were included. 73 tumour samples were known to be mutated for *VHL*. DNA was extracted and an electrophoresis was performed to determine DNA quality. The whole coding sequence was synthesized by double PCR amplification followed by sequencing. Sequencing results were compared to those previously determined from frozen samples.

Results: DNA could be extracted from the 76 paraffin samples. DNA quality was highly degraded and significantly less amplified by PCR in 34.2%, resulting in no sequence available for analysis in 57.7% and discordance with frozen samples in 42.3% of the cases respectively. *VHL* mutations were found in 52.1% of the whole paraffin samples whereas 98% were mutated. 72% could be sequenced, resulting in 69.1% of *VHL* mutations in this subset. Only half of observed mutations were fully consistent with frozen analysis in the 3 exons. Neomutations were found in 10.5% and 28.9% of known mutations in frozen samples were not detected in paraffin blocks. Only DNA quality significantly influenced PCR amplification and sequencing.

Conclusion: Tumoral DNA extraction and *VHL* mutation analysis can be performed from FFPE tissue in RCC. But mutations identified tissues are not strictly concordant with those from frozen analysis and therefore results obtained from FFPE samples should be interpreted with care.

Introduction

Renal cell carcinoma (RCC) is one of the most lethal urological cancers. About 30% of patients do have metastases at presentation and 40% will subsequently develop distant tumor spreading[1]. Obviously, the occurrence of solid tumors results from accumulation of genetic changes and von Hippel-Lindau (VHL) inactivation is the most frequent genetic event in sporadic RCC[2].

The *VHL* gene is located on chromosomal region 3p25-26, and is composed of 3 exons[3]. Inactivation of the *VHL* tumor suppressor gene plays an important role in hereditary and sporadic clear cell RCC[4]. The main consequence of *VHL* gene inactivation is over expression of a transcription factor called hypoxia inducible factor (HIF), that activates genes involved in chronic or acute hypoxia, such as vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF), or transforming growth factor (TGF)[5]. Significant progress in understanding molecular pathways involved in RCC recently led to the development of novel therapies targeting VHL downstream products, with unprecedented response rates, improved progression free and overall survival in metastatic RCC[6]. However, there is an increasing need for identifying new predictors for drug efficacy in the context of anti-angiogenic treatment in metastatic disease. Because *VHL* gene alteration is usually considered as an early event in RCC carcinogenesis, its determination could also be of interest for predicting outcome in localized disease.

Controversies exist in the literature regarding relationship between *VHL* alterations and renal cancer aggressiveness. Some authors consider *VHL* mutations as carrying out a favourable prognosis[7,8], while others found no association or even demonstrate a poorer VHL altered associated prognostic [9-11]. These studies are based on heterogeneous populations and methods , reporting mutation rates ranging from 29% in paraffin-embedded tissues[12] to 71%

in frozen specimens[13]. There is no study available so far comparing the performance of determining *VHL* status in sporadic RCC in frozen and in formalin-fixed paraffin-embedded (FFPE) tissue respectively.

Since 2003, we prospectively determined *VHL* status in clear cell RCC tumors operated at our institution based on frozen samples analysis[14]. We therefore decided in the present study to compare paraffin-embedded and frozen section specimens for VHL mutations detection and furthermore to evaluate the reliability of DNA analysis in FFPE tissues.

Materials and methods

Data collection

This retrospective study included 76 patients operated for a sporadic clear cell RCC at the department of Urology of the Rennes University Hospital between 2002 and 2005, and for whom *VHL* mutations had been characterised prospectively on frozen samples[14]. Among these patients, 3 were free of mutation and considered as a control group. The study protocol was approved by the institutional ethics committee and informed consent for participating in this study was obtained in each case. Clinical parameters such as age, sex and type of surgery and information on DNA concentration and quality, general aspect of the paraffin-embedded block, proportion of tumor present on the block, length of formalin fixation, and type of mutation were collected in all cases.

Pathological analysis

FFPE sections were stained with hematoxylin and eosin-safran for light microscopy. The slides were reviewed by one pathologist (NRL). Only conventional clear cell carcinomas were considered for analysis. Macroscopic and histologic parameters which were analysed included tumor size (cm) and nuclear Fuhrman grade. Tumor stage was defined according to the 2002 TNM classification[15].

DNA extraction

For each patient, the best PFFE block was selected by a single uropathologist (NRL) using the following criteria: predominance of tumor present on the block, tumor homogeneity, zones of high Fuhrman grade, absence of tumor necrosis, cystic zone or normal renal tissue.
Genomic DNA was extracted from eight 10 µm-slices from each sample and prepared as follow: paraffin was removed with xylene according to standard procedures, followed by a

proteic digestion with a proteinase K solution. DNA was then extracted using a specific kit and according to the manufacturer's instructions (RecoverAll™ Total Nucleic Acid Isolation – cat 1975, AMBION®). The DNA concentration was measured at 260 nm.

DNA quality was determined by electrophoresis on a 1% agarose gel running with 0.5X TBE buffer at room temperature for 30 min at 100V. Each sample was compared to a ladder (Smartladder®, Eurogentec) and classified according to their smear as "highly degraded" if only small DNA fragments were present, or "slightly degraded" if large DNA fragments were found (Figure 1).

VHL mutational analysis

DNA fragments encompassing the 3 exons of the *VHL* gene were amplified. In exon 1, the sequence located between codon 1 to 54 was not amplified because no mutation has ever been found either in our series, or in the literature. We used specific primers, as previously described for frozen samples analysis[14,16]. A double amplification was performed using 100 ng of the extracted DNA which was subjected to 35 cycles of PCR after an initial denaturation of 9 min at 95°C including: denaturing during 1 min at 95°C, annealing during 45 s at 58°C for exon 1 and 2, and 45 s at 57°C for exon 3, then extension during 45 s at 72°C.

Exon amplification was performed in 30µl containing: 500 mM KCl, 10 mM Tris-HCl (pH 8.3), 1.5 mM $MgCl_2$, 250 µM dNTPs, 10 pmol of each primer and 1 U AmpliTaq Gold® polymerase (Applied Biosystems). For exon 1 and 3, 3% DMSO was used because of their high content of guanine and cytosine. Electrophoresis was carried out on a 2% agarose gel in the same conditions than previously described to control the success of the amplification procedure.

For sequence analysis, purification of the amplified products was performed with ExoSAP-IT® (USB), according to the manufacturer's instructions, to filter out small fragments. Amplification products detected on SSCP were purified using chromatography columns (Sephadex G50®, Amershan Bioscience). Sequencing used BigDye® Terminator mix V3. 1 (Applied Biosystems) and samples were re-amplified during 30 cycles of PCR: 30 s at 96°C, 15 s at 55°C at Tm, and 4 min at 60°C. Finally, the sequenced products were subjected to automated sequence analysis on an ABI PRISM® 3130 Genetic Analyser (Applied Biosystems), and compared with the VHL sequence accession number AF010238[3]. Mutations were identified by visual aspect of sequences by 2 persons and called when unequivocally present on the sense and/or the antisense strand.

Statistical analyses

χ^2 analysis was used for assessing differences in clinicopathologic parameters distribution. Associations between variables were assessed by χ^2 analysis. All analyses were conducted with SPSS 13.0.1 software (SPSS Inc, Chicago, Il, USA), and *p*-value significance was fixed at 0.05.

Results

Patients and tumors

The population was composed of 46 men (60.5%) and 30 women (39.5%) with a median age of 65 years [40-83]. Tumors were organ confined in 46 cases (60.5%). Median tumor size was 6.7 cm [1.5 – 18]. Nodal invasion and distant metastases were present in 5 (6.6%) and 19 (25%) cases, respectively. Tumors were of high nuclear Furhman grade (grade 3 and 4) in 47 cases (61.8%). At the end of follow-up 15 patients (19.7%) died from renal cancer (Table 1).

DNA characteristics

Median DNA concentration was 222 ng/µl [30 – 693]. Even though the best quality block was selected by a single pathologist in all cases, imperfections potentially influencing DNA extraction were noticed in half of the FFPE blocks: presence of necrosis, fat, oedema and fibrosis, haemorrhagic or cystic zones. Most of the samples were formalin-fixed during 24h (52.6%). DNA was considered as highly degraded on electrophoresis in 26 cases (34.2%) (Table 2). In this setting, no sequencing was possible in 57.7% of the cases (n= 15), and in 11 cases (42.3%) a sequencing discordance was found compared to frozen samples analysis ("extinction" of the mutation, other mutation, or multiple mutations present on the same exon or in different exons). Among the 76 samples, 72% could be sequenced, resulting in 69.1% *VHL* mutation rate in this subset. Multiple mutations were found in 5 specimens. In frozen analysis, 98% of the specimens were *VHL* mutated while a 52% mutation rate was identified in FFPE specimens. Among paraffin-embedded identified mutations, only 40.8% were fully consistent with frozen analysis in the 3 exons. Mutations not pre-existing in frozen samples were found in 10.5%, whereas 28.9% disappeared.

Regarding DNA quality and sequencing analysis, 26 samples presented a "highly degraded" DNA. Only 11 samples have been sequenced (42.3%). In this subset, no mutations was found

in 2 cases, mutations not pre-existing in frozen samples were found in 2 cases, and mutations had disappeared in 4 cases. Nevertheless, no mutations was fully consistent with frozen samples.

In the 50 "slightly degraded" DNA subgroup sample, the 3 exons of the *VHL* gene have been sequenced for 44 samples (88%). Thirty (68.2%) were strictly consistent with frozen samples. Mutations not pre-existing in frozen samples were found in 4 cases, and mutations had disappeared in 2 cases.

Parameters influencing sequencing analysis

We therefore tried to identify parameters influencing PCR amplification and sequencing analysis in FFPE tissue (Tables 3 & 4). Only quality of extracted DNA appeared as a statistically significant parameter (p= 0.0001). DNA concentration, presence of more than 70% of tumor on the block, imperfections on the block, duration of formalin-fixation or nuclear Furhman grade did not influence significantly sequencing analysis and occurrence of discordance.

Similarly, we tried to identify parameters influencing quality of extracted DNA. Neither duration of formalin-fixation, imperfection on the block, anteriority of the sample, nor percentage of tumor influenced the quality of extracted DNA.

For making sure that such discordances were not due to block selection or presence of different mutated clones, we identified 10 samples with highly degraded DNA for which PCR amplification or sequencing analyses had not been possible, or where new mutations had appeared or disappeared during sequencing analysis. We subsequently repeated analysis in both new matched paraffin blocks and in frozen specimens. The same mutation was always identified in frozen specimen. In FFPE tissues, PCR amplification rates were comparable but

70% could not be sequenced as compared with 30% in initial samples. Only one result was retrieved within the 2 subsets: no mutation found whereas a mutation was identified in frozen analysis. In 2 cases, the mutation was not found whereas it had been initially identified.

Discussion

VHL is considered as an early "gatekeeper" tumor suppressor gene, involved in cell cycle regulation, regulation of hypoxia inducible genes and proper fibronectin assembly in extracellular matrix[2]. It is presumed that further genetic alterations are needed for progression of preneoplastic lesions. Nevertheless, restoration of pVHL function in *VHL*-deficient renal carcinoma cells can suppress tumor growth both in vivo and in vitro[17]. However, in 10 to 20% sporadic ccRCCs, no alteration in the *VHL* alleles is detected, suggesting that other genes or pathways are involved in renal carcinogenesis[18].

Gene abnormalities in hereditary Von Hippel-Lindau disease have been extensively analyzed. However few studies have focused on *VHL* alterations in sporadic ccRCC. These studies have been performed on frozen section tissues or on paraffin-embedded material and conflicting results have been reported. For example, 42 to 71% mutation rates have been described in frozen samples [7,9,13,19], while results seem to be inferior in FFPE tissues ranging from 20 to 61%[10,12,20,21]. Currently, frozen section tissue is considered as the benchmark for DNA analysis[22]. Therefore, the objective of our study was to analyze the reliability of paraffin derived technique, compared to results obtained from frozen samples in matched specimens. Indeed, no study had previously addressed the accuracy of *VHL* gene analysis on FFPE material compared to frozen tissue even though the issue of predicting anti-angiogenic drug response through *VHL* gene analysis has gained a recent interest[20,23].

Overall, in terms of *VHL* mutation location our results are consistent with the current literature (Table 5). Similarly to us, the majority of the studies reported exon 1 as being the predominant mutation site. A group identified exon 2 as the more frequent mutation site but the methodology used in this small series is subject to criticisms since reverse sequencing was performed only when any abnormality was detected[24]. Only 22% *VHL* mutations were

identified on frozen section tissue, by amplifying and sequencing only one DNA strand. Another group reported VHL mutation predominance in exon 3 through a limited series of 67 tumors, and including only 24 ccRCCs[25].

Our main finding was that out of the *VHL* mutated tumor population, only 52.1% mutations remained in FFPE tissue. New mutations not identified in frozen samples were found in 10.5%, whereas 28.9% disappeared. In other words, only half of the true *VHL* abnormalities were identified when using FFPE tissue. Furthermore, only half of these mutations were strictly concordant within the 3 exons.

When looking more accurately at the published data issued from the 2 types of tissue analyses, it appears that many discrepancies exist. First, similarly to what we obtained in our frozen series[14], no mutation has ever been identified before codon 54 in frozen samples[9,13,19,26]. This region is known to interact with fibronectin and has been recently described as an important mediator for tumor invasion[27]. However, in FFPE tissues, similarly to the present series, many authors reported mutations in this region[9,10,21,25]. Interestingly, as others[9,10,21], we found multiples mutations in some specimens, whereas it has never been described in any frozen series. Silent mutations have also been identified in FFPE samples in 11 to 13% of the cases[11,12,21], whereas it has never been noticed in frozen samples. It is likely that these uncommon mutations have been created by the technique. These artifacts can be ascribed to postmortem deamination of cytosine and adenine, resulting in uracil or hypoxanthine residues respectively[28]. Several authors have described this phenomenon in different tissues [29]. Williams et al reported up to 1 mutation artifact per 500 bases recorded[30]. It could also explain why some investigators found *VHL* mutations in papillary [21], undifferentiated or chromophobe carcinomas [11,25], and even in benign tumors like oncocytomas [11], while *VHL* abnormalities are highly specific for clear cell histological subtype [31]. No study on frozen section tissues has ever identified *VHL* mutations in non clear cell RCC. Finally, the high percentage of errors

along with the observation of mutational heterogeneity raises the question whether paraffin material is suitable for VHL analysis. Both from our study and from a comprehensive analysis of the literature, it appears that paraffin derived technique is inferior to frozen analysis for *VHL* mutation analysis, resulting in quantitative and qualitative quality losses. In a retrospective study of brain tissue, Ferrer *et al.* analyzed the effects of formalin fixation and time storage on DNA preservation, and compared with frozen specimens [32]. Acceptable results were obtained if DNA extraction was performed after a short time of fixation. Suboptimal and bad results (degraded DNA not allowing sequence analysis) occurred in FFPE tissues stored longer than 6 months. Moreover, the chance to obtain positive results was almost null in tissues that have been stored for years. Similar results were obtained with colorectal tissues. Though different artifacts have been used to optimize DNA extraction performance like higher-temperature heating under an alkaline condition [33] or longer rehydratation step during DNA extraction [34]. All this data taken together strongly suggests that paraffin derived technique should not be considered as a standard for *VHL* gene analysis whether this evaluation should become important for predicting outcome following RCC treatment.

Beyond *VHL* gene analysis issues, this study suggests that results for DNA extraction and sequencing analysis from paraffin materials are less reliable than those taken from frozen samples. Therefore, caution is required when analyzing results of series using this material. However, when frozen tissue is not available, a very stringent selection of paraffin blocks with limited necrosis, fat, oedema, fibrosis, cystic or hemorrhagic zones is required. Additionally, selection of a homogeneous tumoral zone, exhibiting high nuclear grade and without any normal renal tissue is also of utmost importance. Ultimately, quality of the extracted DNA appears to be the major criterion for limiting errors associated with FFPE tissue analysis.

Conclusion

Tumoral DNA extraction and *VHL* mutation analysis can be performed from FFPE tissue in RCC. But mutations identified from FFPE tissues are not strictly concordant with those from frozen analysis. If available, frozen tissue analysis should be considered the gold standard. Otherwise paraffin-embedded tissue remains a great opportunity for DNA analysis in long term-follow-up series. Our study opens the gate for a critical analysis of the literature in this field.

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Variables	n (%)
Median age	65 years old [40-83]
Sex ratio (M/F)	46/30 (60.5%)
T stage :	
T1	33 (43.4%)
T2	13 (17.1%)
T3	27 (35.5%)
T4	4 (3.9%)
N stage:	
N0	71 (93.4%)
N1-2	5 (6.6%)
M stage :	
M0	57 (75%)
M1	19 (25%)
Furhman grade:	
Grade 1	0 (0%)
Grade 2	29 (38.2%)
Grade 3	34 (44.7%)
Grade 4	13 (17.1%)
Median tumor size (cm)	6.7 cm [1.5-18]
Type of surgery	66 Total nephrectomies (86.8%) 10 Nephron sparing surgeries (13.2%)
Death	19 (25%)
Death from cancer	15 (19.7%)

Table 1: General characteristics of the studied population (n= 76).

Variables	n (%)
Median DNA concentration	**222 ng/µl [30 – 693]**
Median tumor % on the FFPE block	**70% [30-100]**
Samples sequenced in the 3 exons	**55/76 (72.4%)**
DNA quality on the smear:	
- Highly degraded	**26/76 (34.2%)**
- slightly degraded	**50/76 (65.8%)**
General aspect of the FFPE block :	
- Excellent quality	**38/76 (50%)**
- Necrosis	**25/76 (32.9%)**
- Fat	**5/76 (6.6%)**
- Œdemea	**1/76 (1.3%)**
- Hemorragia	**1/76 (1.3%)**
- Fibrosis	**3/76 (3.9%)**
- Cystic	**3/76 (3.9%)**
Formalin-fixed duration:	
24h	**40/76 (52.6%)**
48h	**13/76 (17.8%)**
72h	**20/76 (26.3%)**
96h	**3/76 (4.1%)**
Mutations identified in FFPE tissue	**38/73 (52%)**
Mutations errors	**29/76 (38.2%)**

Table 2: Biological and pathological characteristics of the 76 samples.

	No sequence available (n= 21)	Sequence available (n= 55)	*p*
Mean DNA concentration (ng/µl)	206.1	260.9	**0.08**
≥70% of tumor present on the block	16/21 (76.2%)	35/55 (63.6%)	**0.8**
Presence of imperfections on the block	12/21 (57.1%)	27/55 (49.1%)	**0.6**
Time of formalin fixation ≤24h	14/21 (66.7%)	26/55 (47.3%)	**0.2**
SMEAR **DNA highly degraded**	15/21 (71.4%)	11/55 (20%)	**0.0001**
Furhman grade : **Grade 2** **Grade 3** **Grade 4**	**5/21 (23.8%)** **10/21 (47.6%)** **6/21 (28.6%)**	**24/55 (43.6%)** **24/55 (43.6%)** **7/55 (12.7%)**	**0.2**

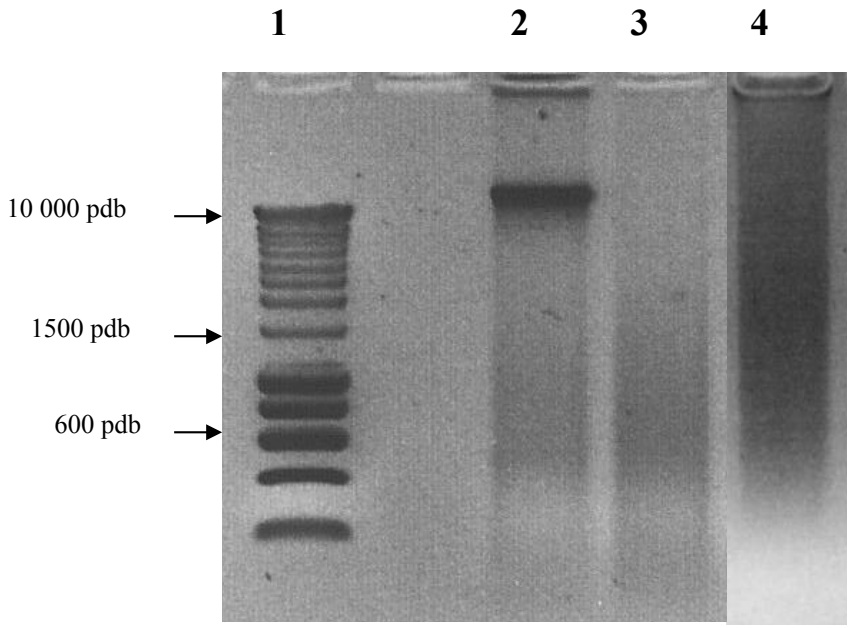
Table 3: Parameters influencing sequencing analysis.

	Discordance (n= 28)	Samples fully consistent with frozen samples (n= 30)	*p*
Median DNA concentration (ng/µl)	266.1	248.1	**0.5**
≥70% of tumor present on the block	20/28 (71.4%)	17/30 (56.7%)	**0.4**
Presence of imperfections on the block	13/28 (46.4%)	17/30 (56.7%)	**0.5**
Time of formalin fixation ≤24h	16/28 (57.1%)	14/30 (46.7%)	**0.2**
SMEAR **DNA highly degraded**	14/28 (50%)	0/30 (0%)	**0.0001**
Furhman grade : **Grade 2** **Grade 3** **Grade 4**	**10/28 (35.7%)** **14/28 (50%)** **4/28 (14.3%)**	**14/30 (46.7%)** **11/30 (36.7%)** **5/30 (16.7%)**	**0.8**

Table 4: Parameters influencing sequencing errors.

	ccRCC (n)	Mutation rate	Predominant mutation site	Predominant type of mutation
Ma et al (2001)	81	54%	Exon 1	**FRAMESHIFT**
Schraml et al (2002)	113	34%	Exon 1	**FRAMESHIFT**
Barnabas et al (2002)	24	50%	Exon 3	**FRAMESHIFT**
Kim et al (2005)	56	20%	Exon 1	**MISSENSE**
Van Houwelingen et al (2005)	187	61%	Exon 1	**FRAMESHIFT**
Rini et al (2006)	43	58%	Exon 1	**FRAMESHIFT**

Table 5: *VHL* mutational analysis in FFPE histologic specimens, results from the literature.



<u>Figure 1:</u> DNA quality determined by electrophoresis on a 1% agarose gel. Each sample was compared to a ladder (1) and classified according to their smear as "highly degraded" if only small DNA fragments were present (3), or "slightly degraded" if large DNA fragments were found (4). N°2 represents a frozen sample.