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The von Hippel–Lindau tumour suppressor gene: uncovering the expression of the pVHL172 isoform

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Background: The von Hippel–Lindau (*VHL*) gene encodes two mRNA variants. Variant 1 encodes two protein isoforms, $pVHL_{213}$ and $pVHL_{160}$, that have been extensively documented in the literature. Variant 2 is produced by alternative splicing of exon 2 and encodes a pVHL isoform of 172 amino acids with a theoretical molecular weight of 19 kDa ($pVHL_{172}$), the expression of which has never been demonstrated so far due to the absence of suitable antibodies.

Methods: We have generated an anti-pVHL monoclonal antibody (JD-1956) using pVHL172 recombinant protein. We tested the antibody against exogenous or endogenous expressed proteins in different cell lines. We identified the pVHL172 using a silencing RNA strategy. The epitope of the antibody was mapped using a peptide array.

Results: We efficiently detected the three different isoforms of pVHL in cell lines and tumorigenic tissues by western blotting and immunohistochemistry and confirmed for the first time the endogenous expression of pVHL172.

Conclusions: The endogenous expression of the three isoforms and particularly the pVHL172 has never been shown before due to a lack of a highly specific antibody since none of the available commercial antibodies distinguish the three isoforms of pVHL in cells or in both normal and cancerous human tissues. Evidence of pVHL172 expression emphasises the need to further study its implication in renal tumorigenesis and VHL disease.

The von Hippel–Lindau (*VHL*) gene was isolated in 1993 (Latif *et al*, 1993) and was then characterised as a tumour suppressor gene (Iliopoulos *et al*, 1995; Clark and Cookson, 2008). Germline mutations in the *VHL* gene cause the *VHL* disease, an autosomal dominant familial cancer syndrome that predisposes to the development of retinal angioma, cerebellar and spinal haemangioblastoma, clear-cell renal cell carcinoma (ccRCC) and pheochromocytoma, as well as pancreatic disease. Somatic *VHL* mutations have also been found in patients with sporadic renal cell carcinoma, particularly ccRCC, which is the most common type of kidney cancer (Gnarra *et al*, 1994). These mutations are often detected in malignant and frequently metastatic ccRCCs, suggesting that *VHL* has a key role in the development/progression of kidney cancer.

The *VHL* gene encodes pVHL, a substrate-binding component of an E3 ubiquitin ligase complex. This complex targets HIF-α, a hypoxia-inducible transcription factor, for proteasomal degradation in a pVHL-dependent manner. pVHL has also other functions that are considered to be independent from its role in the E3 ubiquitin ligase complex. These include, for instance, regulation of the extracellular matrix, microtubule stability and primary cilium maintenance, regulation of E-cadherin and stabilisation of *p53* and Jade-1 (genes involved in apoptosis and epithelium differentiation, respectively) (Bader and Hsu, 2012; Hsu, 2012). Failure of pVHL to

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control these functions may contribute to tumour progression and metastasis formation.

The complexity of pVHL functions is not only due to its involvement in multiple cellular processes, but also to the fact that the *VHL* gene, which is located on the short arm of human chromosome 3 (3p25.3), produces two different VHL mRNAs (Gnarra *et al*, 1994; Richards *et al*, 1996): variant 1 (V1; NM_000551) includes exons 1, 2 and 3, while variant 2 (V2; NM_198156) lacks exon 2 (Richards *et al*, 1996).

V1 encodes a protein of 213 amino acids with apparent molecular weight of ~30 kDa ($pVHL_{213}$) and a smaller 160 amino-acid long isoform of 19 kDa ($pVHL_{160}$) (Schoenfeld *et al*, 1998) the translation of which initiates from an internal translation start site (codon 54) within the *VHL* open reading frame (Figure 1). Although they are both ubiquitously expressed, high expression levels are observed specifically in the urogenital system, brain, spinal cord, sensory ganglia, eyes and bronchial epithelium (Richards *et al*, 1996). Moreover, while $pVHL_{213}$ is primarily found in the cytoplasm with minor pools in the nuclear and membrane compartments, $pVHL_{160}$ is equally distributed in the nucleus and cytoplasm (Iliopoulos *et al*, 1998). When over-expressed in mammalian cells, the subcellular localisation of $pVHL_{213}$ appears to vary in a cell density-dependent manner (Lee *et al*, 1996). Both $pVHL_{213}$ and $pVHL_{160}$ are functional tumour suppressors. Indeed, reintroduction of either isoform in *VHL*-defective ccRCC cells suppresses their ability to form tumours when xenografted in nude mice (Schoenfeld *et al*, 1998).

The second mRNA variant (V2) is produced by alternative splicing of exon 2 and should generate a protein composed of 172 amino acids with a theoretical molecular weight of 19 kDa ($pVHL_{172}$) (Figure 1). In healthy adult kidney or in cultured renal proximal tubule cells, the expression of V2 mRNA can be barely detected by quantitative RT–PCR analysis and is very low compared with V1 (Herman *et al*, 1994; Gnarra *et al*, 1994; Richards *et al*, 1996). As a consequence, functional studies have mainly focused on the $pVHL_{213/160}$ isoforms. However, there are at least two contexts in which high V2 expression has been reported: (i) during human embryogenesis (8–10 gestational weeks) in kidney, brain, spinal cord, eyes, testis and lung; and (ii) in sporadic ccRCC with specific *VHL* point mutations in exon–intron boundaries or in the coding region (Martella *et al*, 2006; Taylor *et al*, 2012). In some cases, V2 is the only *VHL* transcript detected in ccRCC samples or cell lines (Herman *et al*, 1994; Gnarra *et al*, 1994), suggesting that the protein encoded by this alternatively spliced transcript is defective in tumour suppressor activity (Gnarra *et al*, 1994; Shuin *et al*, 1994; Whaley *et al*, 1994) and/or participates in tumour initiation or progression.

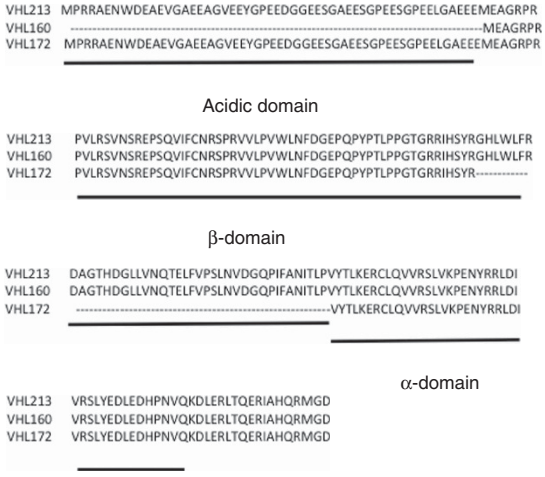


Figure 1. Structural domains and amino acid sequence comparison of the three different pVHL isoforms. Structural organization of the acidic domains and amino acid sequence alignment of the three pVHL isoforms.

So far, $pVHL_{172}$ expression has not been rigourously demonstrated due to the lack of adequate tools and thus it has been impossible to compare the expression profile of all three protein isoforms to study their respective roles in ccRCC development and/or progression. Indeed, based on their primary sequence similarity, $pVHL_{213}$ and $pVHL_{160}$ are expected to share many functions (Robinson and Ohh, 2014). Conversely, the absence of part of the β-domain (aa 114–154) in the $pVHL_{172}$ isoform modifies the number of beta sheets in its structure, which is likely to be involved in altered protein folding with structural and functional consequences on its activity and protein interaction network.

We have therefore generated a monoclonal antibody that efficiently discriminates between closely related pVHL isoforms. Using highly purified recombinant human $pVHL_{172}$ as the antigen, this new antibody recognises the full spectrum of endogenous pVHL isoforms, including the particular $pVHL_{172}$ isoform, which has been a subject of debate within the scientific community. This new development enables us to perform detailed studies on the expression of the different pVHL isoforms with particular focus on $pVHL_{172}$. We report for the first time the use of an unambiguous technology for the *in vitro* and *in vivo* study of isoform-specific pVHL expression in various cell lines and tumour tissues.

MATERIALS AND METHODS

Real-time PCR analysis. Real-time PCR was performed on total RNA extracted from cells using the QIAamp total RNA kit (Qiagen, Courtaboeuf, France). Five micrograms of total RNA were reverse-transcribed using oligo-dT primers and M-MLV reverse transcriptase. The resulting cDNAs were then PCR amplified using the following primers designed from human cDNA sequences: 5′-CCCGTATGGCTCAACTTCG-3′ (forward) and 5′-TCAGGTCGCTCTACGAAGATCT-3′ (reverse) for VHL variant 1 (308 bp); 5′-CCCGTATGGCTCAACTTCG-3′ (forward) and 5′-TCAGGTCGCTCTACGAAGATCT-3′ (reverse) for VHL variant 2 (185 bp) (Martin *et al*, 2013). Assays were performed in triplicate, using the RotorGene 3000 instrument (Corbett Research, Biolabo, Archamps, France) with SYBR Green I master mix (Roche Diagnostics, Mannheim, Germany). For each sample, the relative amounts of VHL and GAPDH transcripts were calculated from these standard curves using the RotorGene software.

Expression and purification of pVHL isoforms. There is conflicting nomenclature used by different investigators to assign pVHL isoforms: $pVHL_{213}$ is also known as $pVHL_{30}$ or $pVHL_{25}$, while $pVHL_{160}$ is also referred as $pVHL_{19}$ or $pVHL_{21}$. In this study, to prevent this confusion, each isoform will be named according to the number of amino acids of the molecule, that is, $pVHL_{213}$, $pVHL_{160}$ and $pVHL_{172}$.

All recombinant proteins were prepared using the *E.coli* strain BL21(DE3)pLysS transformed with pET21a-hVHL213, hVHL172 or hVHL160 and induced to express the proteins $VHL_{213}(His)_6$, $VHL_{172}(His)_6$ and $VHL_{160}(His)_6$ with IPTG. Proteins were prepared and purified by Talon affinity chromatography following the manufacturer's instructions (Clontech, Mountain View, CA, USA) as described by Martin *et al* (2013). The purity of the eluted fractions was assessed by 12.5% SDS–PAGE and silver staining.

Antibodies. The rabbit polyclonal antibody against human VHL (#6030) (Martin *et al*, 2013) and the mouse monoclonal antibody JD-1956 (Patent No. 14305925.1-1402-2014) against human VHL were produced in our laboratory (CNRS-EFS). The commercial

antibody against human VHL was from Santa Cruz Biotechnology (sc-5575; Heidelberg, Germany). The actin antibody was from Sigma-Aldrich (Saint-Quentin Fallavier, France). The horseradish peroxidase-conjugated secondary antibodies were from Jackson Immuno-Research Laboratories (Baltimore, MD, USA).

Production of anti-pVHL antibody. Anti-pVHL monoclonal antibodies were raised by immunising six mice with recombinant human $pVHL_{172}$ $(His)_6$ subcutaneously. After 3 months, blood was collected from each mouse and the immunoreactivity of the different blood samples was tested. Mouse n°28 was killed and splenocytes were collected. A first selection of fused cells was done using an ELISA assay with recombinant $pVHL_{213}(His)_6$, $pVHL_{172}(His)_6$ and $Aurora(His)_6$ to identify cells producing antibodies against the $(His)_6$ tag. Ten to 100 ng of each recombinant protein ($pVHL_{213}(His)_6$, $pVHL_{172}(His)_6$ and Aurora-A) were loaded on polyacrylamide gels and transferred onto nitrocellulose membranes to test the clones. We found 25 clones that were immunoreactive against the recombinant VHL proteins. The immunoreactivity of the positive clones was then tested using total protein extracts from cells expressing exogenous VHL proteins.

Western blot analysis. Frozen tissues or pelleted cells were lysed in RIPA buffer (50 mM Tris-HCl, pH 7.4, 1% NP-40, 0.5% sodium deoxycholate, 150 mM sodium chloride, 1 mM EDTA, 1 mM sodium fluoride, 1 mM AEBSF, 10 μg/ml aprotinin, 10 μg/ml leupeptin and 1 mM sodium orthovanadate) and centrifuged at 9000 **g** for 10 min. Fifty micrograms of total proteins from each extract were separated by SDS–PAGE on 12.5% polyacrylamide gels in denaturing conditions and transferred onto nitrocellulose membranes. Membranes washed with TBST were incubated with primary antibodies in 2.5% low-fat milk in TBST at 4 °C overnight, followed by incubation with horseradish peroxidase-conjugated anti-rabbit or anti-mouse IgG antibodies (1 : 15 000 in TBST/2.5% BSA at RT for 1 h). Sample loading was controlled with a polyclonal anti-tubulin antibody (1 : 500). Western blots were revealed by chemiluminescence using the Super Signal ECL reagent kit (Pierce, Rockford, IL, USA).

Tissue samples. Tumour and matched normal tissue samples were obtained from patients with ccRCC who underwent partial or total nephrectomy between 2002 and 2005. The Ethics Committee of Rennes University Medical School and Hospital approved this prospective study and all patients signed the informed consent. Tissue samples were immediately frozen in liquid nitrogen and stored at −80 °C in the Centre de Ressources Biologiques (CRB, Rennes, France) and formalin-fixed and paraffin-embedded. To determine the *VHL* status of the collected samples, denaturing high-performance liquid chromatography was carried out on a WAVE Nucleic Acid Fragment Analysis system (Transgenomic, Glasgow, UK) with a DNAsep column (Patard *et al*, 2009). Aberrant peaks were further analysed by direct sequencing using standard procedures. All mutations were confirmed by a second PCR and sequencing reaction. Multiplex ligation-dependent probe amplification was used for VHL deletion analysis.

Cell culture. Human tumour cell lines (HeLa, HEK-293T and RCC4) were maintained in DMEM (Life Technologies, St Aubin, France) supplemented with 10% foetal calf serum and antibiotics at 37 °C in a humidified 5% CO_2 atmosphere. The two primary cell lines (R-180 and R-305) derived from two human ccRCCs and the 786-0 were cultured in RPMI-1640 (Life Technologies) supplemented with 10% foetal calf serum (Sigma-Aldrich), 1% penicillin/streptomycin (Sigma-Aldrich) and 10 mM HEPES buffer (Dugay *et al*, 2014). HeLa cells were transfected using JetPRIME as recommended by the manufacturer (PolyPlus, Ozyme, Montigny-le-Bretonneux, France). To overexpress pVHL in the various cell lines, we used the pCDNA-VHL_{213} and pCDNA-VHL_{160} plasmids (subcloned from pCMV-FlagHA-VHL213/160 that were a generous gift from Dr A Buchberger-Max Planck Institute of Biochemistry, Department of Molecular Cell Biology, Martinsried, Germany) and the pcDNA-FlagHA$pVHL_{172}$ plasmid.

Immunohistochemistry. Five-micron sections of formalin-fixed paraffin-embedded tissues were transferred to glass slides and incubated in TBST in the presence of 5% BSA. Reactivity to JD-1956 (1 : 100) was revealed with the biotin–streptavidin detection system (Dako, Glostrup, Denmark) using diaminobenzidine as chromogen (Sigma-Aldrich). Photographs were taken using a Leica DMRXA microscope equipped with a CoolSnapsHQ camera (Photometrics, Tucson, AZ, USA). The images were processed with ImageJ 1.4 software (the National Institute of Mental Health, Bethesda, MD, USA).

Epitope mapping. The 213 amino acids sequence of $pVHL_{213}$ (Uniprot accession code P40337) was used to generate an overlapping peptide library. The library is composed of 20-mer peptides with 5 amino acid off-set (40 peptides). Peptides were synthesised on cellulose membrane by SPOT technology (Frank, 2002) using an automated multiple synthesiser (MultiPep RS, Intavis, Köln, Germany). The membrane was rinsed with a small volume of methanol for 5 min to avoid precipitation of hydrophobic peptides during the subsequent procedure. The membrane was processed as described in western blot analysis, then incubated with the antibody JD-1956 (dilution 1 : 200) in 2.5% low-fat milk in TBST at 4 °C overnight, followed by incubation with horseradish peroxidase-conjugated anti-mouse IgG antibodies (1 : 15 000 in TBST/2.5% BSA at room temperature for 1 h). The blot was revealed by chemiluminescence using the Super Signal ECL reagent kit (Pierce).

RESULTS

Immunodetection of separate pVHL isoforms. As most of the available antibodies against pVHL are unspecific and cannot efficiently distinguish or detect the different pVHL isoforms (Supplementary Figure S1 and Supplementary Table S1), we produced a new monoclonal antibody against VHL (JD-1956) using recombinant human $pVHL_{172}$ as antigen. This antibody has been developed with the intention of specifically recognising the $pVHL_{172}$ isoform. However, given the high sequence similarity of the three isoforms (Figure 1), it was expected to target also the isoforms encoded by V1 ($pVHL_{213}$ and $pVHL_{160}$). The antibody specificity was first assessed by immunoblot analysis using T7- and His_6-tagged recombinant $pVHL_{213}$, $pVHL_{172}$ and $pVHL_{160}$. The amount of loaded proteins was controlled by silver staining (Figure 2A, left panel) and immunodetection with the anti-T7 antibody (Figure 2A, right panel). The membrane was then immunoblotted with different antibodies against pVHL: (i) the polyclonal rabbit antibody VHL-6030 that preferentially recognises the NH2-terminal part of full-length pVHL (Martin *et al*, 2013), (ii) the polyclonal antibody sc-5575 from Santa Cruz Biotechnology raised against full-length pVHL and that was one of the most efficient among the tested commercial antibodies (Supplementary Table S1) and (iii) our new monoclonal antibody JD-1956. All three antibodies could detect recombinant $pVHL_{213}$ (Figure 2B). The $pVHL_{172}$ isoform was clearly detected by the antibodies VHL-6030 and JD-1956, but not by the commercial antibody (only a very faint signal). Finally, $pVHL_{160}$ was mainly revealed by the JD-1956 antibody. Thus, the JD-1956 antibody recognised efficiently all three pVHL isoforms. It should be noted that the apparent molecular weight of $pVHL_{172}$ (23 kDa) differed significantly from the expected theoretical molecular weight (19 kDa), whereas in the case of $pVHL_{160}$ the difference was minor (19 kDa instead of 18 kDa).

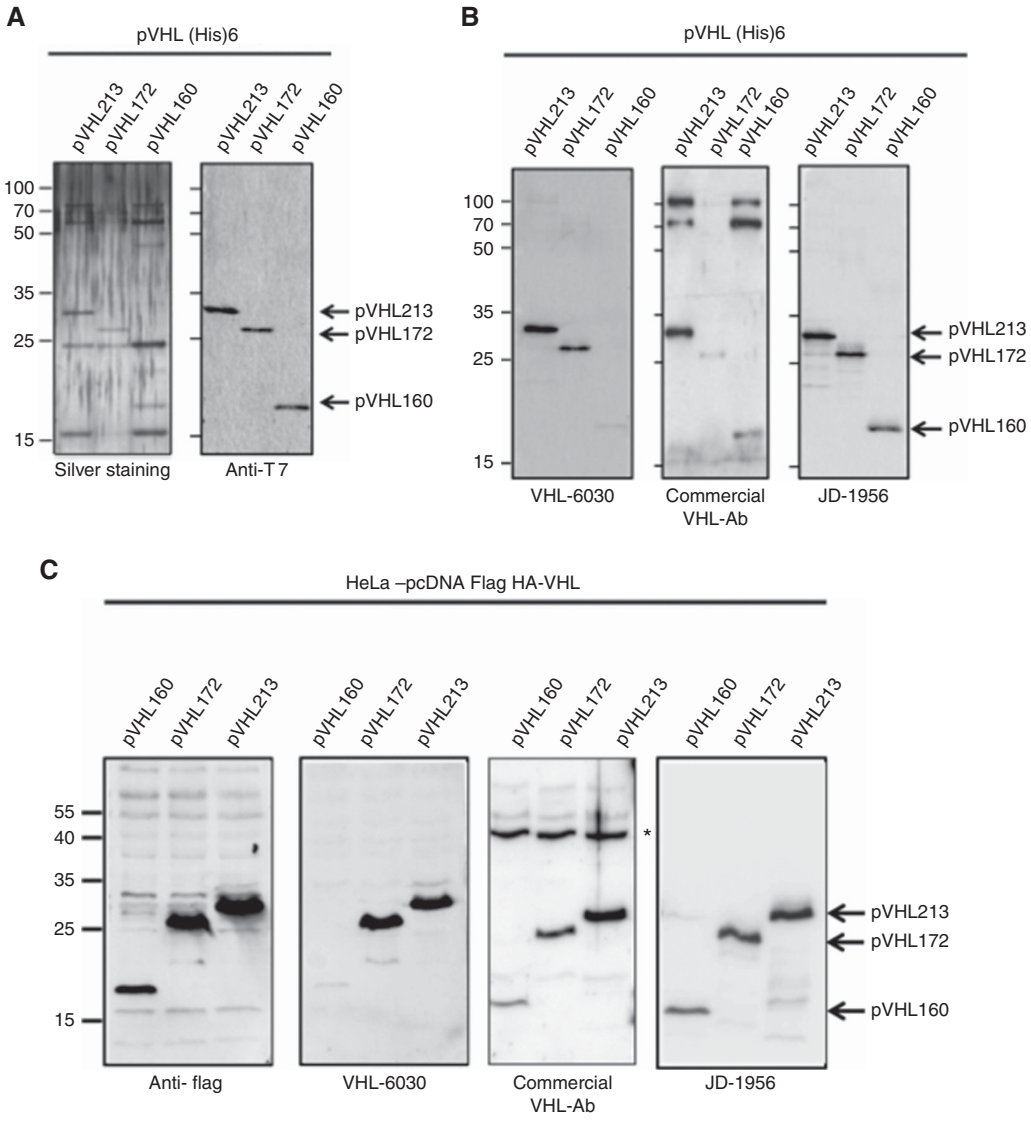


Figure 2. Immunodetection of recombinant pVHL isoforms with different anti-VHL antibodies. (**A**) Loading of the different recombinant pVHL proteins was evaluated by silver staining and by western blotting with an anti-T7 antibody. (**B**) Immune detection of the three recombinant pVHL proteins was performed using the polyclonal antibody VHL-6030 (1 : 1500), a commercial polyclonal anti-VHL antibody from Santa Cruz Biotechnology (1 : 500) and the JD-1956 monoclonal antibody (1 : 100). (**C**) Total protein extracts from HeLa cells transiently transfected with pCDNA-FlagHA plasmids encoding $pVHL_{213}$, $pVHL_{172}$ and $pVHL_{160}$ were analysed by western blotting using an anti-Flag antibody, the commercial polyclonal anti-VHL (1 : 500), VHL-6030 (1 : 1500) and JD-1956 (1 : 100).

We then assessed how efficiently these antibodies could detect Flag-HA-tagged $pVHL_{213}$, $pVHL_{172}$ and $pVHL_{160}$ in cell lysates. HeLa cells were transfected with the corresponding plasmids (Figure 2C) and cell lysates were then immunoblotted with the three antibodies against pVHL. All three antibodies detected $pVHL_{213}$ and $pVHL_{172}$. In contrast, the $pVHL_{160}$ isoform was detected only with the commercial antibody (Santa Cruz Biotechnology) and the monoclonal JD-1956 antibody. However, non-specific bands were not observed when using the JD-1956 antibody, differently from the commercial antibody (*, panel commercial VHL-Ab of Figure 2C). These results indicate that only the mouse monoclonal antibody JD-1956 is appropriate to detect all known pVHL isoforms with high specificity in complex protein extracts.

Characterisation of the antibody specificity. Western blot analysis of HeLa cell extracts (Figure 3A, left panel) showed that the JD-1956 antibody detects three bands that could correspond to the three pVHL isoforms based on their respective electrophoretic mobility (*, $pVHL_{213}$; **, $pVHL_{172}$ and ***, $pVHL_{160}$). Pre-incubation of the antibody with an excess of $(His)_6$-tagged recombinant $pVHL_{213}$, fully abolished immunodetection of the three bands by western blotting (compare the left and right panels of Figure 3A). To further investigate the specificity of the JD-1956 antibody, HeLa cells were transfected with siRNAs to downregulate all variants (siRNA VHL) or only $pVHL_{172}$ (siRNA VHL-172). Western blot analysis of HeLa cell extracts 24 h after transfection showed that, in cells transfected with the siRNA VHL, the intensity of the three bands corresponding to the three pVHL isoforms was strongly decreased compared with non-transfected cells (Figure 3B, lanes 3 and 1, respectively). Conversely, in cells transfected with the siRNA VHL-172, only the intensity of the band expected to correspond to $pVHL_{172}$ was reduced compared with non-transfected cells (Figure 3B, asterisk in lane 2). This effect was dose-dependent as indicated by the progressive reduction in the intensity of the band corresponding to $pVHL_{172}$ in cells transfected with increasing amounts (25–75 nM) of siRNA VHL-172

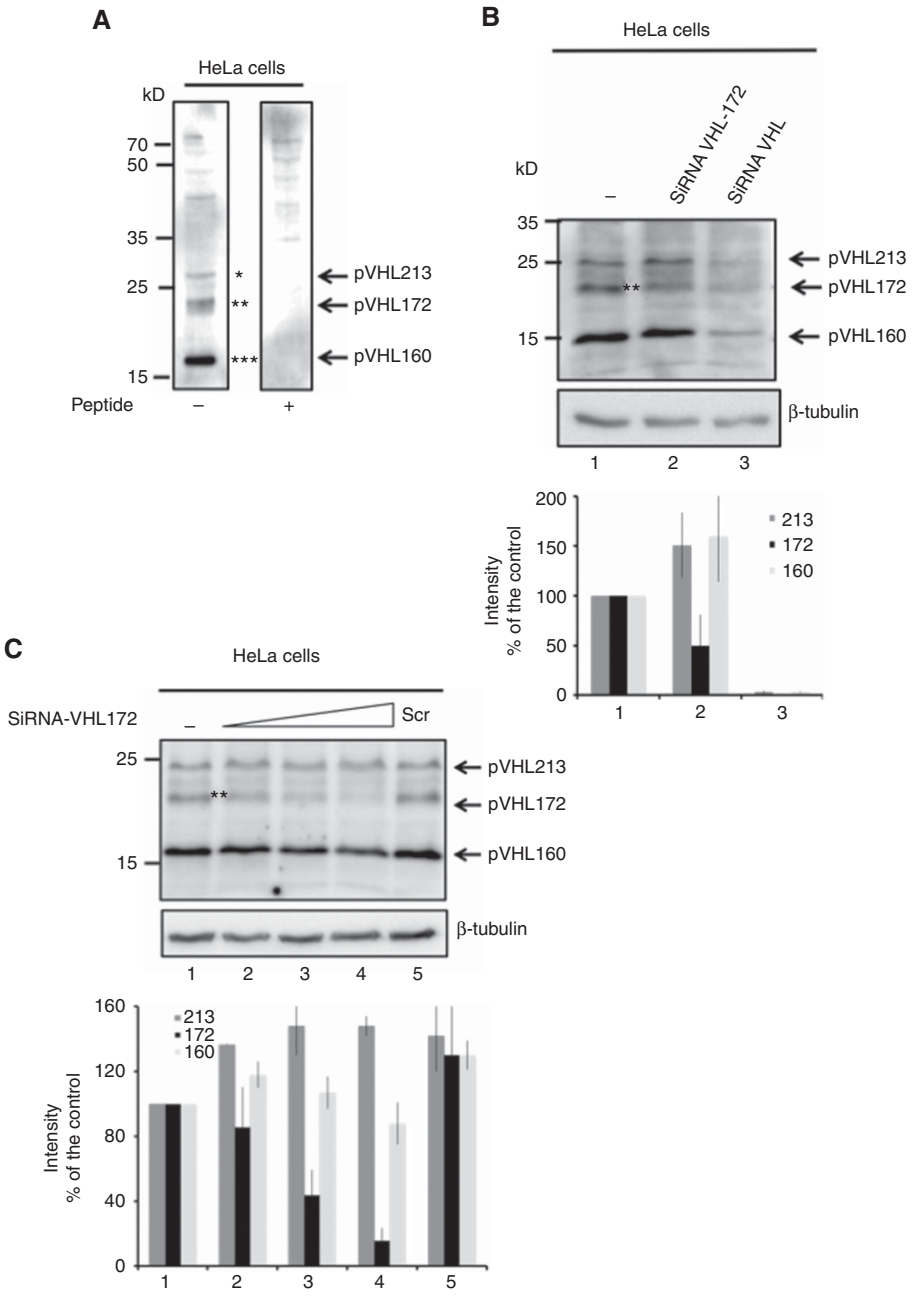


Figure 3. Characterisation of the JD-1956 antibody. (**A**) HeLa cell total protein extracts were analysed by western blotting with the JD-1956 antibody (−, left lane) or with JD-1956 after incubation with an excess of recombinant $pVHL_{213}$ protein at 4 °C for 30 min (+, right lane). (**B**) HeLa cells were transfected with siRNAs (60 pmol) to downregulate all the three pVHL isoforms (siRNA VHL) or $pVHL_{172}$ only (siRNA VHL-172). Cells were harvested 24 h after transfection and the expression level of the different pVHL isoforms was investigated by western blotting using the JD-1956 antibody (1 : 100). Non-transfected HeLa cells (−) were used to assess the expression of the endogenous pVHL isoforms. (**C**) HeLa cells were transfected with increasing amounts of siRNA VHL-172 (50–150 pmol) for 30 h and then the expression of the pVHL isoforms was investigated by immunoblotting using JD-1956 (1 : 100). Non-transfected HeLa cells (−) or transfected with scrambled siRNA (Scr, 100 pmol) were used as controls. In **B** and **C**, signals corresponding to VHL isoforms (as well as tubulin) were quantified using ImageQuant software (GE Healthcare Europe, Velizy-Villacoublay, France), VHL signals were normalised against tubulin and results presented as mean percentages ± s.e.m. ($n = 3$) of the signal (for each pVHL isoform) observed in control HeLa cells.

(Figure 3C). Transfection with scrambled siRNA (50 nM) had no effect on the detection of the three bands (Figure 3C). Again, these pVHL isoform-specific knockdowns indicate that antibody JD-1956 is directed against all three pVHL isoforms (recombinant or endogenously expressed) with high specificity.

Finally, epitope mapping study showed that JD-1956 recognises an amino acid sequence located between amino acids 46 and 72 (peptides #10–12), which is an epitope region shared by all pVHL isoforms (Supplementary Figure S2).

pVHL expression profiling in tumour cell lines and tumour tissues. We then sought to determine whether the JD-1956 antibody could also recognise endogenous pVHL expressed in different cell lines derived from primary ccRCC tumours (786-O, R-180, R-305, Caki-1 and RCC4 + cells), HEK-293T cells and non-tumoral endothelial cells (Huvec). Figure 4A shows the VHL status of all tested cell lines. First, we checked by RT–PCR analysis whether the two *VHL* variants (V1 and V2) were expressed in selected wild-type *VHL* (HeLa and Huvec) and mutated/deleted

A

Cell lines	Hela	786-0	R-180	R-305	Caki-1	RCC4+	HEK-293T	HUVEC
VHL status	wt/wt	mut/del	mut/del	mut/del	wt/wt	wt	wt/wt	wt/wt

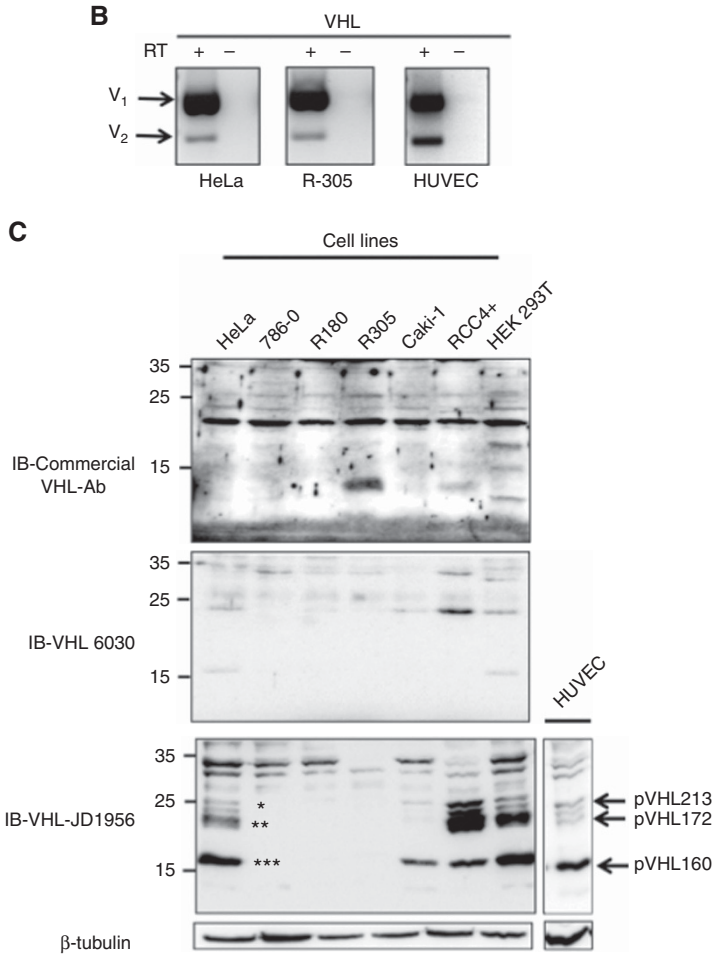


Figure 4. Detection of the VHL isoforms (RNA and proteins) in cell lines. (**A**) VHL status of the different cell lines. (**B**) Total RNA was reverse-transcribed and PCR amplified using VHL primers for V1 (V_1, 308 bp) and V2 (V_2, 185 bp). (**C**) 40 μg of total protein extracts from HeLa cells, kidney cell lines (786-O, R-180, R-305, Caki-1, RCC4 + and HEK-293T) and HUVEC cells were analysed by western blotting with the commercial anti-VHL antibody (1 : 500), VHL-6030 (1 : 1500) and JD-1956 (1 : 10). β-tubulin was used as a loading control.

VHL (R-305) cell lines. Two bands of the expected molecular size (308 bp for V1 and 185 bp for V2) were detected (Figure 4B). Western blot analysis using the commercial antibody revealed a single major band with a molecular weight of about 23 kDa in all tested cell lines (upper panel, Figure 4C), although 786-0 cells do not express full-length pVHL (Gnarra *et al*, 1994). Thus, this band likely corresponds to a non-specific signal. Two additional faster migrating bands were detected in R-305 and RCC4 + cells, but their molecular weight did not correspond to the theoretical molecular weight of the smaller pVHL isoforms (Figure 4C, upper panel). The VHL-6030 antibody detected bands that theoretically corresponded to $pVHL_{213}$ and $pVHL_{160}$, but not to the putative $pVHL_{172}$ isoform, in most of the tested cell extracts (Figure 4C, middle panel). On the other hand, the JD-1956 antibody revealed three strong bands (*, $pVHL_{213}$; **, $pVHL_{172}$ and ***, $pVHL_{160}$) in HeLa, RCC4 + (*VHL*-deficient RCC cells transfected with a plasmid coding for full-length $pVHL_{213}$) and HEK-293T cell extracts. As expected, no band was observed in lysates from 786-0 cells in which a *VHL* mutation has introduced a stop codon in the middle of the mRNA coding sequence (Figure 4B, lower panel). These results show that the JD-1956 antibody can efficiently recognise the three different pVHL isoforms in various contexts (recombinant proteins, overexpressed or endogenous proteins). The expression profile of non-tumoral Huvec cells was similar to that of HeLa cells. The JD-1956 antibody was then used to examine the relative expression of the different pVHL isoforms in ccRCC samples with different *VHL* status (Figure 5A). HeLa cell extract was used as a positive control (Figure 5B right lane). Western blot analysis with the JD-1956 antibody revealed three bands that likely corresponded to *, $pVHL_{213}$; **, $pVHL_{172}$ and ***, $pVHL_{160}$, based on their electrophoretic mobility. The relative intensity of each of the three bands could vary within and between samples, particularly if their *VHL* status was different (Table 1). The intensity of the lowest band (theoretically corresponding to $pVHL_{160}$) ('***' in Figure 5B) was higher compared with the other bands particularly in tumour samples in which *VHL* was deleted in one allele and mutated in the other (del/mut) (Figure 5B, lanes 2, 5 and 8). These results suggest that JD-1956 is a suitable tool for

A

ID	N°	T	N	M	Fuhrman grade	VHL mutation type	Mutation site	VHL deletion	VHL promoter methylation	
D03 1561	1	3	0	0	2	FRAMESHIFT	EXON 1	NO	NO	mut/wt
D03 5472	2	1	0	0	2	MISSENSE	EXON 3	NO	NO	mut/wt
D03 4033	3	1	0	0	2	STOP	EXON 1	YES	NO	mut/del
D04.1130	4	3	1	0	4	MISSENSE	EXON 1	YES	NO	mut/del
D04.1664	5	3	0	1	4	FRAMESHIFT	EXON 1	NO	NO	mut/wt
D04.3372	6	2	0	0	3	MISSENSE	EXON 1	YES	NO	mut/del
D05.407	7	3	0	1	3	FRAMESHIFT	EXON 1	YES	NO	mut/del
D05.781	8	3	0	0	2	MISSENSE	EXON 3	YES	NO	mut/del
D05.1955	9	4	0	1	4	NO MUTATION		NO	NO	wt/wt

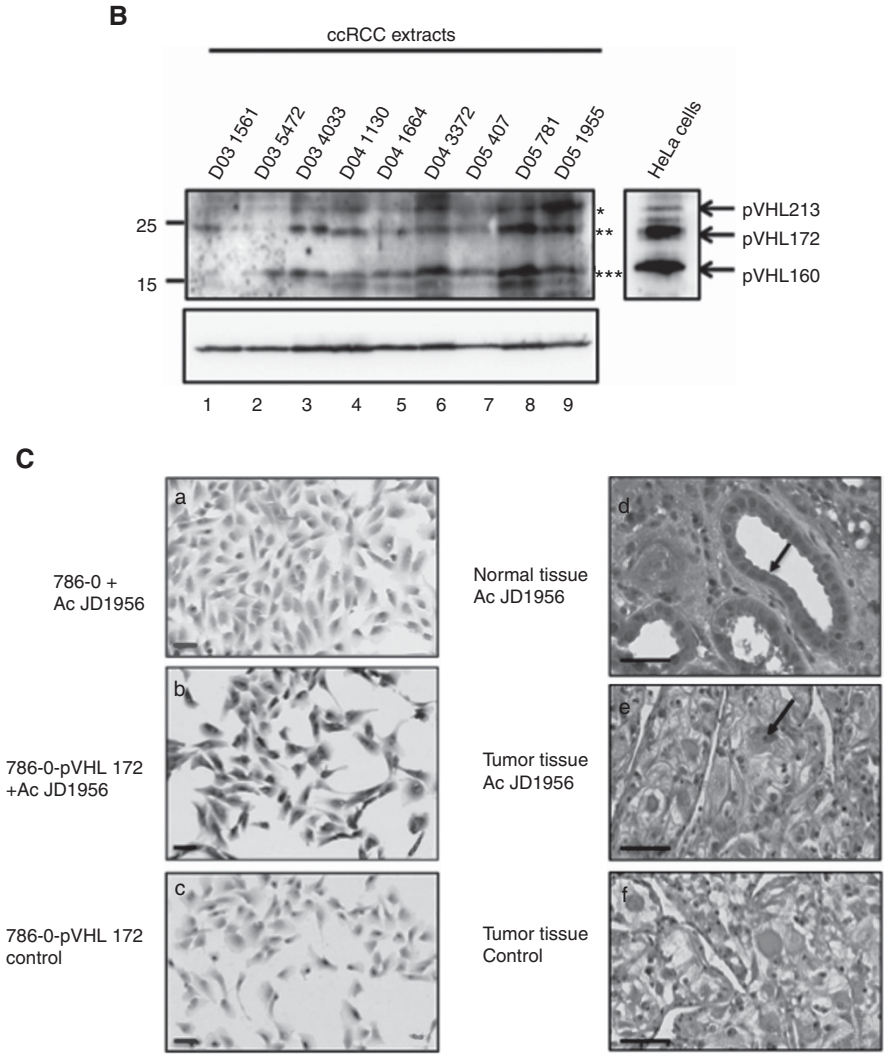


Figure 5. Immunodetection of endogenous pVHL isoforms in tumour tissues. (**A**) Histo-pathological characteristics of the ccRCC samples used for VHL immunodetection. (**B**) Western blot analysis of protein extracts from ccRCC tissue samples using the JD-1956 monoclonal antibody (1 : 100). β-tubulin was used as loading control. Expression of endogenous pVHL isoforms in HeLa cells was added (right panel) as a migration control of the three pVHL isoforms which are indicated by asterisks. The asterisks (*, **, ***) indicate $pVHL_{213}$, $pVHL_{172}$ and $pVHL_{160}$, respectively. (**C**) Microtome sections of formalin-fixed and paraffin-embedded 786-0 cells (a), 786-0 cells overexpressing $pVHL_{172}$ (b and c), normal kidney tissue (d) and ccRCC samples (e and f) were stained with (a, b, d and e) or without (c and f) the monoclonal antibody JD-1956 (1 : 100). Images were acquired with an Olympus microscope and processed with ImageJ 1.4 software. Scale bars, 20 μm (cells) and 50 μm (tumour sections). The arrows indicate the localization of VHL in the cytoplasm (d) and the nucleus (e).

tracking the expression of specific pVHL isoforms and to establish relative expression ratios in a variety of cell lines and tumour tissues.

Immunocytochemical and immunohistochemical detection of pVHL isoforms with the JD-1956 antibody. The JD-1956 antibody was further tested using IHC in cells that overexpress or not the $pVHL_{172}$ isoform and in paraffin-embedded normal kidney and ccRCC tissue samples. The antibody JD-1956 revealed a strong signal in cells that overexpress $pVHL_{172}$ (Figure 5Cb). Likewise, the antibody detected pVHL expression in epithelial cells of renal tubules in healthy kidney samples, mostly in the cytoplasm (Figure 5Cd). In ccRCC tumour samples, expression seemed to localise mainly to the cell membranes, more strongly in samples with del/mut *VHL* (panel e) than in normal tissue expressing wild-type *VHL* (Figure 5Cd). In few cells, VHL was detected both in the

Table 1. Intensity of the different bands detected with the anti-VHL antibody JD-1956 in ccRCC samples (from +, low intensity, to ++++, high intensity)

	Band intensity		
Tumor	**$pVHL_{213}$**	**$pVHL_{172}$**	**$pVHL_{160}$**
Sample 1 (mut/wt)	+	++	−
Sample 2 (mut/wt)	+	+	++
Sample 3 (mut/del)	+	++	++
Sample 4 (mut/del)	++	++	++
Sample 5 (mut/wt)	+	+	++
Sample 6 (mut/del)	++	++	++++
Sample 7 (mut/del)	+	+	++
Sample 8 (mut/del)	++	++++	++++
Sample 9 (wt/wt)	++++	++	++

Abbreviations: ccRCC = clear-cell renal cell carcinoma; del = deleted; mut = mutated; VHL = von Hippel–Lindau; wt = wildtype.

cytoplasm and the nucleus (arrow in Figure 5Ce). Control IHC performed on transfected cells as well as tumour tissue in the same experimental conditions but without incubation with primary antibody revealed no signal (Figure 5Cc and f, respectively).

DISCUSSION

The function of $pVHL_{172}$ isoform is still very elusive owing to the lack of analytic tools to detect its expression experimentally. Although detectable levels of mRNA can be measured in tissues, the relative abundance of this isoform was poorly documented in the literature as the use of current anti-VHL antibodies have resulted in conflicting results. Consequently, the role of $pVHL_{172}$ in tumorigenesis and cancer progression is not clearly defined. Since commercially available and lab-made antibodies against pVHL do not allow to discriminate the isoforms generated by V1 and V2 mRNA variants (Supplementary Data S1), we decided to produce a new monoclonal antibody using human recombinant $pVHL_{172}$ as antigen. However, due to the high similarity of the three isoforms (see Figure 1), raising an antibody that would be specific to the $pVHL_{172}$ isoform is very challenging. Indeed, the results of this study show that the selected monoclonal antibody JD-1956 can detect all three isoforms ($pVHL_{213}$, $pVHL_{172}$ and $pVHL_{160}$) by recognising a peptide sequence right after the internal initiation codon. When tested using recombinant proteins, the JD-1956 antibody detected equally well the three isoforms, whereas the others antibodies (a lab-made polyclonal antibody VHL-6030 and a commercial antibody from Santa Cruz Biotechnology) weakly recognised recombinant $pVHL_{160}$. The antibody VHL-6030 was produced using a combination of two antigenic peptides and recognises preferentially the NH2-terminal part of the full-length protein, thus leading to the weak detection of the pVHL160 (Martin *et al*, 2013). As for the commercial antibody, it revealed some non-specific bands of higher molecular weight that would hamper the unambiguous detection of the pVHL isoforms, for instance, in whole cell extracts or tissue sections. Although not uniquely directed to $pVHL_{172}$ isoform, JD-1956 is the first antibody that specifically and unequivocally recognises all pVHL isoforms. JD-1956 represents a valuable tool to accurately and reliable detect changes in VHL expression within tumours.

The detection of $pVHL_{172}$ isoform, now amenable to a variety of complex biological samples, gave rise to some interesting observations. The significant difference between the apparent and the theoretical molecular weight of $pVHL_{172}$ observed when using recombinant proteins produced in bacteria or protein overexpressed in mammalian cells could be explained by the presence of acidic repeats (eight copies of GXEEX) in the NH2-terminal part (codons 1 to 54) of the $pVHL_{213}$ and $pVHL_{172}$, but not in $pVHL_{160}$. Furthermore, analysis of the amino acid composition of the different isoforms revealed that $pVHL_{160}$ has an isoelectric point of 8.7 and $pVHL_{172}$ of 4.7. Electrophysiological alterations and structural changes within pVHL isoforms might explain why they behave anomalously in SDS–PAGE.

The weaker detection of $pVHL_{160}$ compared with the other isoforms by all antibodies in HeLa cells overexpressing the three pVHL proteins could be explained by $pVHL_{160}$ amino acid composition that confers to the protein a strong hydrophobic behaviour. As a consequence, the detection of this isoform requires the use of strong dissociating agents, such as deoxycholate, that were not present in the buffer used for the preparation of the cell extracts, thus leading to an underestimation of the amount of $pVHL_{160}$ in cells (Schoenfeld *et al*, 1998). This confirmed that the weaker immunodetection of $pVHL_{160}$ in cells was not due to protein instability, but rather due to epitope accessibility (Schoenfeld *et al*, 1998).

We then tested the specificity of the JD-1956 antibody in embryonic and cancer kidney cell lines. Indeed, although the expression of the second mRNA variant has been reported in cells and tissues by different groups (including the present work), the expression of the encoded pVHL isoform ($pVHL_{172}$) has never been substantiated due to the absence of a specific antibody. The JD-1956 antibody allowed highlighting the presence of a band that should correspond to $pVHL_{172}$ both in embryonic kidney cells and in different cancer cell lines. The specificity of this detection was confirmed by *VHL* silencing using siRNAs specific for V2. The intensity of the bands corresponding to the different pVHL isoforms varied in the different cell lines. No signal was detected in 786-0 cells in which the *VHL* gene mutation exon 1 generates a very short and unstable protein (Iliopoulos *et al*, 1995). Similarly, other ccRCC cell lines (R-180 and R-305 cells) did not show any signal, confirming that mutations in the *VHL* gene can affect the expression and/or stability of the protein (Dugay *et al*, 2014). Conversely, in HeLa, RCC4 and HEK cells, different bands corresponding to $pVHL_{213}$, $pVHL_{172}$ and $pVHL_{160}$ were observed. In MCF7 and HUVEC cells, only a smear signal corresponding to $pVHL_{172}$ was detected, despite the confirmation of V2 mRNA presence. This indicates that the translation of V2 and/or the stability of the corresponding protein could be regulated differently in various cell lines. Finally, analysis of tumour tissue extracts led to the heterogeneous detection of the three bands corresponding to the pVHL isoforms. The signal variability could be explained by the heterogeneity of the tumour tissues.

In conclusion, we describe herein a new anti-pVHL antibody that allowed confirming, for the first time, the existence of the $pVHL_{172}$ isoform translated from *VHL* V2 in different cell lines and tissues. In the last two decades, researchers have made significant contributions to the understanding of the functions of $pVHL_{213}$/$pVHL_{160}$; however, the interplay of the different isoforms, including $pVHL_{172}$, in tumour development remains unsolved. Indeed, the *VHL* gene encodes a multifunctional protein with a crucial role in the ubiquitin-mediated degradation of HIF alpha. However, alternative functions, independent of HIF, have been identified and other interacting proteins need to be characterised. While it is clear that loss of pVHL can result in the activation of cellular pathways that are strongly associated with tumour initiation and progression, the concomitant presence of the three isoforms in some cells and tissues raises the question about their respective cellular functions and the reason for their concomitant presence. This new antibody represents an important tool for better understanding the tumour-suppressive function of pVHL, the specific role of $pVHL_{172}$ and its critical targets in cancer.

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AUTHORS CONTRIBUTIONS

All authors contributed to the conception and design, acquisition of data or analysis and interpretation of data.

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