Study of mutations inducing *vhl* Exon 2 splicing: $pVHL_{172}$ over-expression and cell transformation

Alusse A[1]., Chesnel F[1]., Schmitt L[1]. and Arlot-Bonnemains Y[1].

1- CNRS-UMR6290-2 Ave du Pr L. Bernard -35043 RENNES

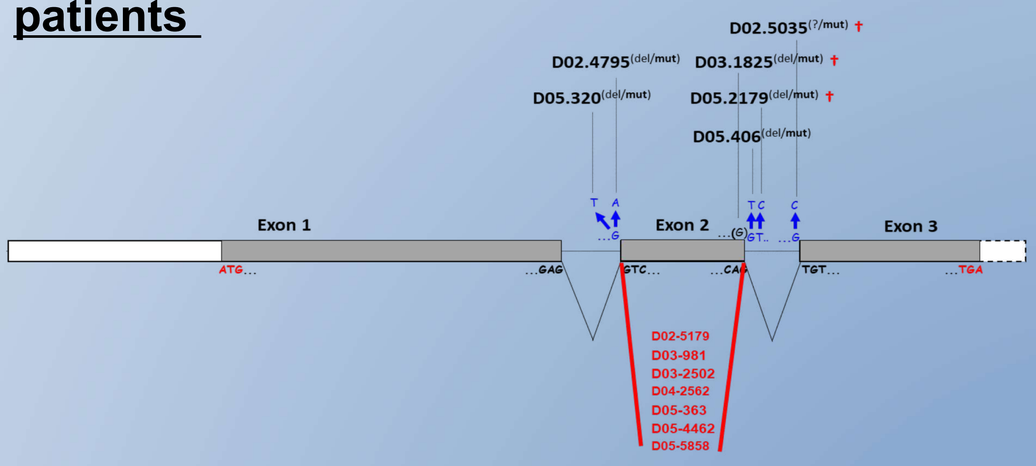
Context

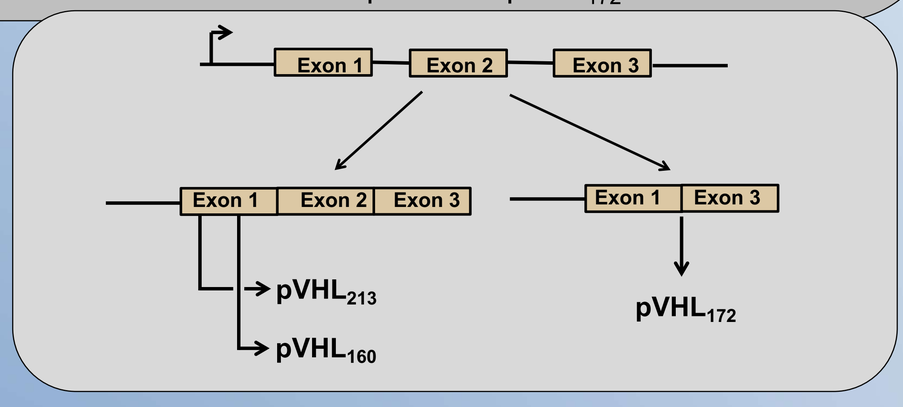
The Von Hippel-Lindau (VHL) tumor suppressor gene *vhl* was identified in 1993. VHL disease is a hereditary, autosomal-dominant, neoplastic disease that is associated with various tumour types, including renal carcinomas (ccRCCs), central nervous system (CNS) and retinal haemangioblastomas, phaeochromocytomas (PCCs) and pancreatic neuroendocrine tumours, in addition to pancreatic and renal cysts. Aberrant VHL protein function is the underlying driver of VHL-related diseases. *Vhl* is inactivated by mutations or hypermethylation in over 85% of ccRCC. Mutations of the *vhl* gene have been reported in a ccRCC database and specific mutations located at the junctions intron1/exon2 and exon2/intron2 were detected.

The *vhl* gene encodes two isoforms of pVHL from a mRNA variant 1 (V1); a 213-amino-acid 30-kDa form (pVHL30) and a 160-amino-acid, 19 kDa form (pVHL19). pVHL19 lacks a 53-amino-acid NH_2-terminal acidic domain and predominates in many tissues. Early functional studies suggested that these two isoforms exert equivalent functions, and that both isoforms have tumour suppressor activity *in vivo*. A second mRNA variant (V2) has been identified which is generated by alternative splicing of exon 2, providing a protein of 172 amino-acids (Chesnel *et al.*, 2015). We previously showed that $pVHL_{172}$ is not a tumour suppressor suggesting an antagonistic function of this pVHL isoform in the HIF-independent aggressiveness of renal tumors compared to $pVHL_{213}$ (Hascoet *et al.*,2017).

In this study, we highlight the impact of mutations identified in patients with kidney cancer on exon 2 splicing. The "minigene" reporter assays demonstrated total or partial exon 2 skipping depending on the mutation and the cell type. The modification of the splicing leads to variations in the V1/V2 ratios in the pathologies. In addition, we have demonstrated by phenotypic analyses the pro-migration features of cells which over-express the $pVHL_{172}$ isoform.

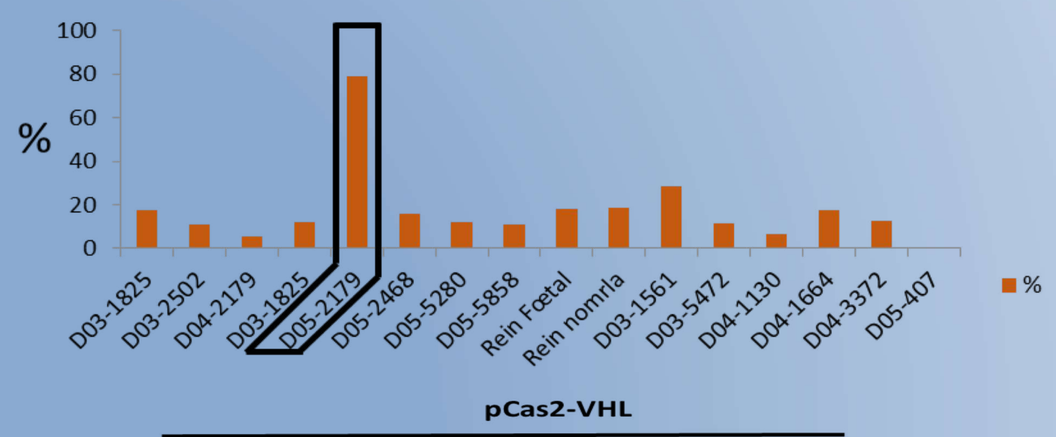
Predicted impact of splicing mutations found in ccRCC patients



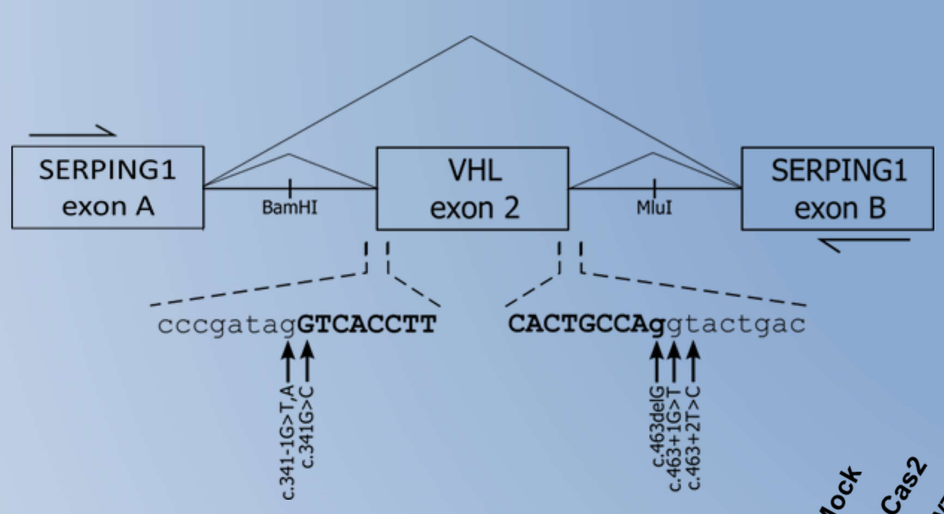


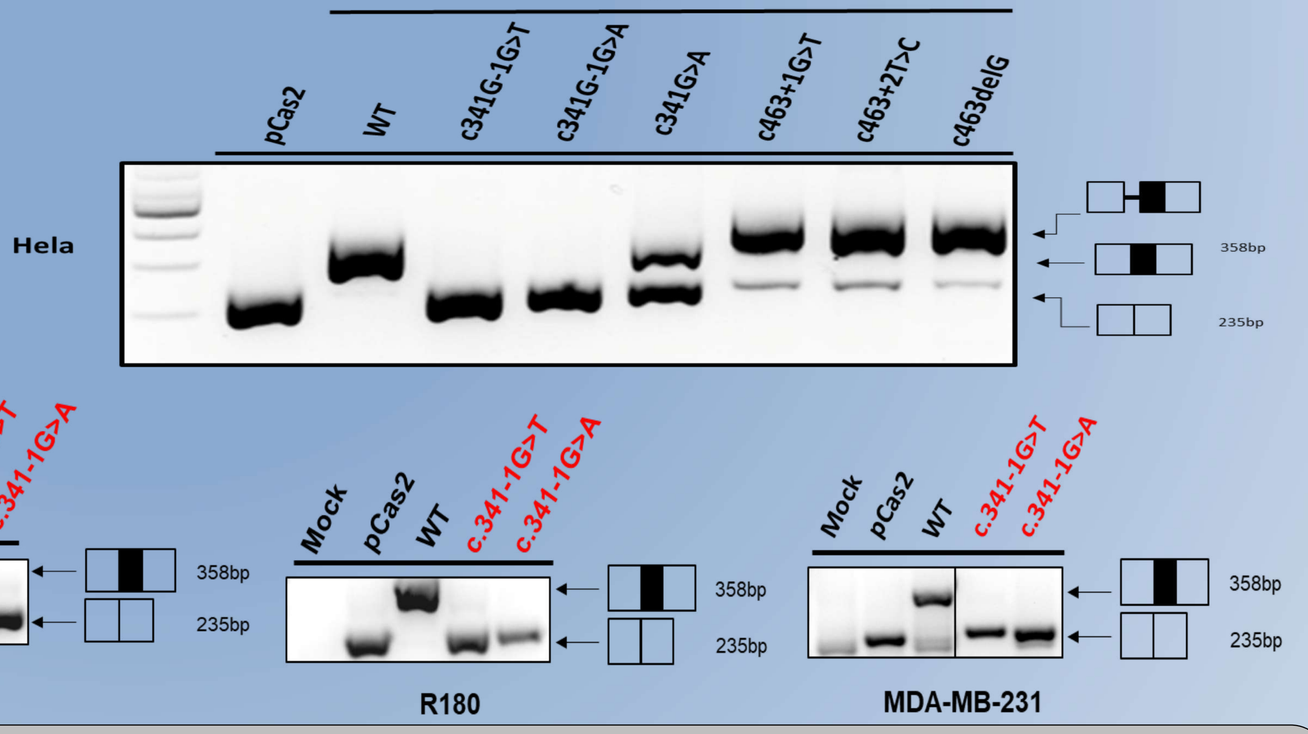
Several mutations identified in patients with ccRCC are carriers of mutations predicted to have an impact on splicing. For one of the patients [D05-2179 ; carrying the *vhl* c.463+2 T>C mutation], a modification of the V1/V2 ratio is observed.

Analysis of VHL V1/V2 ratio in patients with ccRCC samples



Splicing analysis by minigene experiments

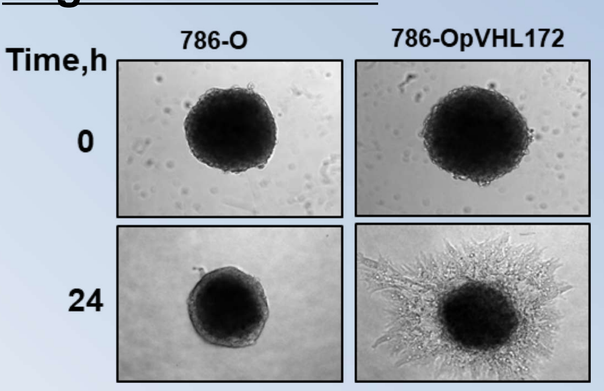


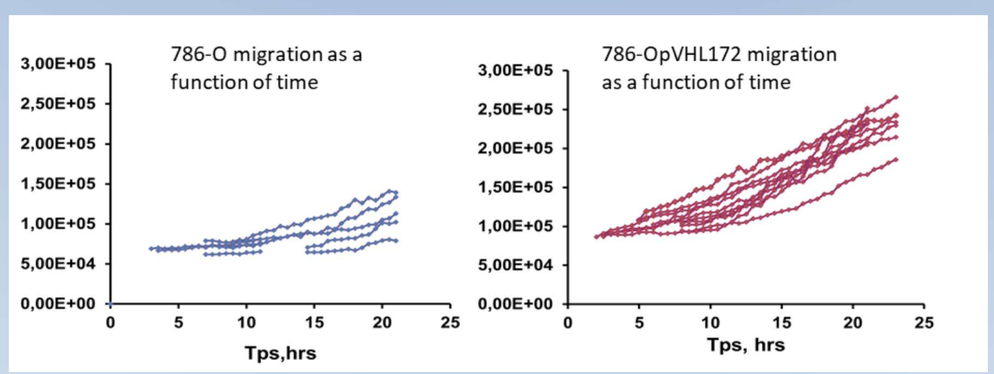


The pCas2 plasmids containing wild-type or muted intron1-exon2-intron2 sequence of *vhl* have been transfected in different cell lines. After 24-h incubation, the ARN were extracted from transfected cells and the splicing pattern of the minigene was assessed by RT–PCR using specific primers.

The RT-PCR analysis revealed the integration of the exon 2 WT in all studied cell lines
The minigene containing mutations (c.341-1G>A and c.341-1G>T) in intron 1 undergo a complete exon skipping, independently in HeLa cells, kidney cell line (R180) and breast cancer cell (MDAMB-231). The c.341G>A mutation triggers a partial exon 2 skipping .
In kidney cells, mutations of intron 2 has a light effect on exon skipping. The mutations (c.463+1G>T) triggers the expression of a splicing variant longer than the WT.

The expression of $pVHL_{172}$ modifies the migration of cells





	786-O	786-OpVHL172
Invasion	53,3	100
Mean of the begining of invasion	20,8	10,7
Speed invasion	4,8 ± 0,75	8,18 + 2,27
Force invasion	5,75±3,7	20,76 + 6,8

Cells expressing $pVHL_{172}$ or not were induced to form spheroids that were thereafter loaded onto matrigel.
The movement of cells escaping from the spheroids was recorded under microscope for 24 hours. A macro was generated to analyse the force invasion. The cells expressing $pVHL_{172}$ have a 4-fold higher invasion capacity than those which do not express the protein

Conclusion

- Mutants in the 3'end of the intron 1 drive the complete exon skipping whatever the cell lines.
- Mutations within the 5' splice site has a very slight effect on exon 2 skipping and moreover uncover the existence of a cryptic 5' splice site

Specific mutations induce alternative splicing of pre-mRNA and is a key step in the expression of the isoform $pVHL_{172}$. The protein induces changes in cell behaviour and most certainly molecular changes that need to be further explored.