European Journal of Human Genetics (2014) 22, 1149–1152

www.nature.com/ejhg

SHORT REPORT

VHL mosaicism can be detected by clinical next-generation sequencing and is not restricted to patients with a mild phenotype

Lucie Coppin[1,2], Claudine Grutzmacher[1], Michel Crépin[1], Evelyne Destailleur[1], Sophie Giraud[3], Catherine Cardot-Bauters[4], Nicole Porchet[1,2] and Pascal Pigny*[,1,2]

The identification of Von Hippel-Lindau (*VHL*) mosaic mutations by conventional Sanger sequencing requires a labour-intensive enrichment step, thus explaining that mosaicism occurrence is underestimated in patients. Nowadays, it is possible to detect mutation in cell sub-populations by next-generation sequencing (NGS). Here, we described a diagnosis strategy using NGS with high coverage in a series of eight patients who were negative for a *VHL* abnormality by Sanger sequencing and deletion search. In two patients, a mosaic mutation in *VHL* was detected by NGS. One patient with a 5.7% mutated allele frequency had a severe phenotype and an early disease onset. In conclusion, clinical NGS in an hospital molecular oncogenetics laboratory is an efficient tool to identify *VHL* mosaic mutation. Its use may improve patient monitoring and genetic counseling.
European Journal of Human Genetics (2014) **22,** 1149–1152; doi:10.1038/ejhg.2013.279; published online 4 December 2013

Keywords: mosaicism; next-generation sequencing; NGS; Von Hippel-Lindau disease; *VHL*

INTRODUCTION

Von Hippel-Lindau (VHL) disease (OMM #193300) is an autosomal dominant inheritable familial cancer syndrome. It predisposes to a variety of malignant and benign tumours such as cerebellar and spinal haemangioblastoma (HB), pheochromocytoma, renal clear cell carcinoma (RCC), pancreatic endocrine tumours, or visceral cysts.[1] Clinical diagnosis of VHL in a patient without a familial history requires the occurrence of two tumours (two HBs or an HB and a visceral tumour), or a single VHL tumour in case of confirmed familial history of VHL.[1] VHL is caused by a mutation, micro- or macro-deletion in the *VHL* tumour suppressor gene mapped to chromosome 3p25-26 (RefSeq NC_000003.11). In a patient with classical VHL disease, a germline abnormality of *VHL* can be identified in 89[2] to 95%[1] of the cases if the molecular analysis comprises both nucleotide sequencing and PCR-based deletion search. Despite clinical evidence of the disease, this combination of molecular tools can fail to identify a germline mutation of *VHL* thus suggesting low levels of mosaic mutations. Mosaicism is the consequence of a somatic mutation occuring early in the embryo development that leads to co-existence of two cell populations in patient's body. The prevalence of mosaicism in VHL, that is largely under-explored owing to the difficulty of its detection, is currently estimated to be around 5%.[3,4] However, the identification of mosaicism is important since it will influence both patient monitoring and genetic counseling in the relatives.

Here, we report a series of eight patients whose phenotype was suggestive of VHL and who were negative for a *VHL* abnormality both by PCR-Sanger sequencing and by quantitative multiplex PCR of short fluorescent fragments (QMPSF). Since next-generation sequencing (NGS) allows the detection of genetic variants in cell sub-populations,[5,6] we decided to evaluate its efficiency in the identification of mosaic *VHL* mutations in a hospital laboratory setting.

MATERIALS AND METHODS

NGS and data analysis

DNA was isolated from peripheral blood mononuclear cells using the EZ1 DNA Blood kit (Qiagen, Hilden, Germany). Library of amplicons was prepared according to the manufacturer's instruction and sequenced with a ROCHE 454 GS Junior (Roche Diagnostics, Meylan, France), which uses the pyrosequencing technology.[7,8] Robotic protocols were developed on HAMILTON StarLet 8 platforms (Hamilton Robotic, Villebon sur Yvette, France) to perform multiplex amplification of the three exons of *VHL* (RefSeq NC_000003.11) using primers as follows: exon 1 Forward (F): 5′–CCCGGGTGGTCTGGA TCG-3′ and Reverse (R): 5′-CTGGATGTGTCCTGCCTCAAG-3′ (amplicon size: 489 bp), exon 2F: 5′-GTGGCTCTTTAACAACCTTTGC-3′ and R: 5′-TGG AATAACGTGCCTGACATCA-3′ (amplicon size: 272 bp), exon 3F: 5′-CAGTAG TACAGGTAGTTGTTG-3′ and R: 5′-ATCAAAAGCTGAGATGAAACAG-3′ (amplicon size: 315 bp). Then, we performed 'AmPure' purification with magnetic beads, quantification with a spectro-fluorimeter SAFAS (SAFAS Monaco, Monaco), normalization of the amplicons and pooling to prepare the normalized library. During the multiplex amplification step, the different patients DNA was 'tagged' with specific Multiplex Identifiers (MID). A high-depth coverage (1000 ×) was defined to detect mutational events poorly represented into the normalized library generated from DNA samples. The sequencing reads were aligned against the human chromosome 3 reference sequence (NC_000003.11) using SeqNext (JSI medical Systems Gmbh, Kippenheim, Germany) and GenSearch (PhenoSystems, Wallonia, Belgium)

[1]Laboratoire de biochimie 'hormonologie, métabolisme-nutrition, oncologie', Centre de Biologie Pathologie, CHRU de Lille, Lille Cedex, France; [2]INSERM, U837, Team 5, Lille Cedex, France; [3]Laboratoire de génétique, Groupement Hospitalier Edouard Herriot, Lyon, France; [4]Service d'endocrinologie et métabolismes, hôpital Claude-Huriez, CHRU de Lille, Lille Cedex, France
*Correspondence: Professor P Pigny, Biochemistry and Molecular Biology, Centre de Biologie Pathologie, CHRU Lille, Lille 59037, France. Tel: +33 3 204 46 154; Fax: +33 3 204 45 693; E-mail: pascal.pigny@chru-lille.fr
Received 31 May 2013; revised 24 October 2013; accepted 30 October 2013; published online 4 December 2013

Table 1 Summary of clinical and molecular characteristics of the patients included in the panel

Patient	Sex	Date of birth	Age of discovery (years)	Familial history	Phenotype	Number of tumours	Clinical diagnosis of VHL	Molecular analysis
P1	M	1990	16	No	-HB of central nervous system -Right pheochromocytoma (17 years) -Pancreatic endocrine tumour (17 years) -A small left adrenal nodule (17 years)	3	Yes	-No *VHL* deletion and rearrangement by QMPSF -Normal *VHL* Sanger sequencing
P2	M	1989	16.5	No	-Unilateral RCC	1	Suggestive	-No *VHL* deletion by QMPSF -Normal *VHL* and *SDHB* Sanger sequencing
P3	F	1954	54	Yes, Maternal uncle with a renal cancer	-Left RCC -Brain tumour	1	Suggestive	-No *VHL* deletion by QMPSF -Normal *VHL* Sanger sequencing
P4	M	1994	15	No information	Several retinal HBs	2	Suggestive	-No *VHL* deletion by QMPSF -Normal *VHL* Sanger sequencing
P5	M	1965	47	Yes, Uncle with renal cancer	Bilateral RCC	2	Suggestive	-No *VHL* deletion by QMPSF -Normal *VHL* and *SDHB* Sanger sequencing
P6	M	1988	23	No	Unilateral RCC	1	Suggestive	-No *VHL* deletion by QMPSF -Normal *VHL* and *SDHB* Sanger sequencing
P7	F	1958	53	No	-HB of central nervous system -Bilateral renal cysts	1	Suggestive	-No *VHL* deletion by QMPSF -Normal *VHL* Sanger sequencing
P8	M	1939	48	Yes, daughter with a *VHL* mutation (c.481C>T, p.(R161*))	-Bilateral RCC	2	Yes	

Abbreviations: HB: hemangioblastoma; RCC: renal clear cell carcinoma.
P1–P8: Patients whose phenotype is indicative of a classical VHL disease (≥2 tumours or one tumour with a familial context) or suggestive of a VHL disease (1 tumour).
VHL transcript reference number: NCBI NM_000551.3.

Table 2 Summary of the analysis of NGS data by SeqNext software

		VHL			
Analysis	Data generated	Exon 1 Mean coverage	Exon 2 Mean coverage	Exon 3 Mean coverage	Mutations (VHL transcript reference number: NCBI NM_000551.3)
Global data					
-Run 1	44 582 Mb	3204x	4736x	6363x	
-Run 2	45 275 Mb	2328x	5144x	6301x	
Specific data					
P1		3845x	7012x	8902x	c.500 G>A, p.(R167Q)
				Forward reads: 4379	Mutated reads: 510
				Reverse reads: 4523	Allele frequency: 5.7%
P8		1184x	3247x	4059x	c.481C>T, p.(R161*)
				Forward reads: 1653	Mutated reads: 68
				Reverse reads: 2406	Allele frequency: 1.7%

Softwares. *VHL* mutations were defined using NCBI NM_000551.3 as a reference for *VHL* transcript.

Confirmation techniques for mosaicism detection

Pyrosequencing was carried out using a PyroMark MD according to the manufacturer's protocol (Qiagen S.A.S., Courtaboeuf, France). The Primers were designed using the PSQ Assay Design v1.0 Software (Pyrosequencing AB, Uppsala, Sweden). Data obtained were analysed with the PyroMark MD v1.0 Software (Pyrosequencing AB).

SNPs genotyping was performed using ABI PRISM 3130 × 1 Genetic Analyzer with 50-cm capillary Array and the ABI PRISM SnaPshot Multiplex Kit according to the manufacturer's instructions (Applied Biosystems, Foster City, CA, USA).

RESULTS AND DISCUSSION

To conduct our study, we built up a panel of eight patients whose clinical and molecular characteristics are detailed in Table 1. All patients harbour clinical or imaging features of VHL, but only two of them (P1 and P8) had a classical VHL disease. Three patients had a positive familial history but a familial *VHL* mutation was identified for only one (P8).

Two NGS runs were carried out on Roche 454 (Table 2). Patient P8 DNA was simultaneously analysed with his daughter's DNA who had a heterozygous p.(R161*) *VHL* mutation. Results show that patient P1 who has developed three tumours (Figure 1a) presents a mosaic *VHL* mutation (c.500G>A, p.(R167Q)) with a frequency of the mutated allele of 5.7% thus explaining that it was undetectable by Sanger nucleotide sequencing (Figure 1b). To confirm these results, we developed alternative methods using specific pyrosequencing and SnaPshot protocols. Both methodologies confirmed that patient P1 is mosaic for the p.(R167Q) mutation (Figure 1c and d). No mosaic mutation was found in patients P2–P7. Finally, patient P8 who had two RCC was mosaic for the mutation p.(R161*) with a mutated allele frequency of 1.7% (Table 2). We did not develop alternative

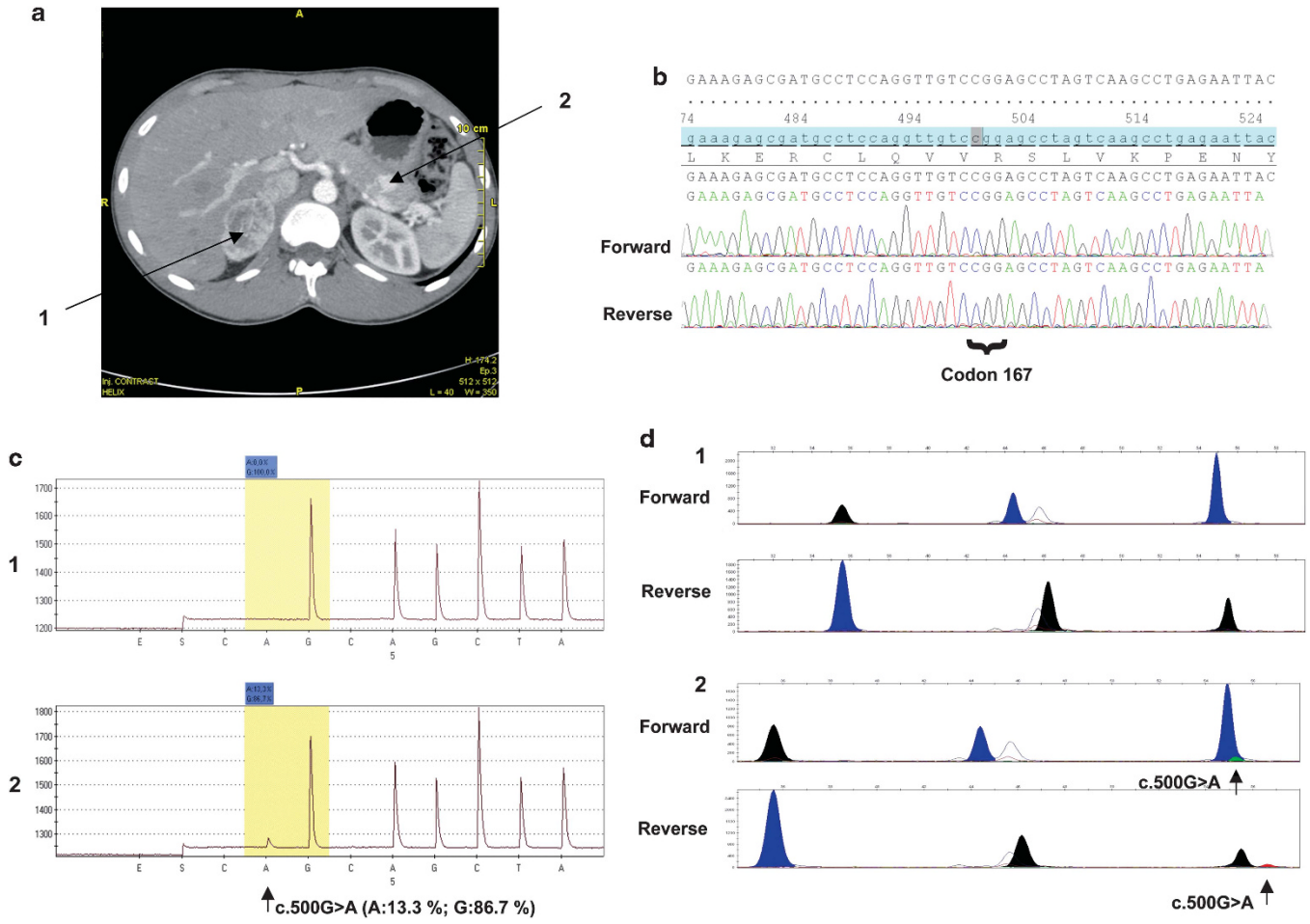

Figure 1 Clinical and molecular features of patient P1. (**a**) Abdominal CT scan of the P1 patient showing (1) a right adrenal pheochromocytoma (33 × 47 mm) and (2) a pancreatic neuro-endocrine tumour. (**b**) Sanger sequence analysis of *VHL*. A zoom is performed on *VHL* exon 3 around the codon 167 that is highlighted by a curly bracket. (**c**) Profile of pyrosequencing of *VHL* around codon 167 for a wild-type patient (1) and for the patient P1 with the c.500G>A *VHL* mosaic mutation (2). Arrow shows the abnormal nucleotide. *VHL* transcript reference number: NCBI NM_000551.3. (**d**) Profile of Snap shot analysis for a wild-type patient (1) and for the patient P1 with the c.500G>A *VHL* mosaic mutation (2). Arrow shows incorporation of the abnormal nucleotide, A in green (Forward sense) and T in red (Reverse sense).

methods to confirm it for two reasons. First, the mutation was identical to those previously identified in his affected daughter. Second, the number of reads for each strand was above 350, that is the lower limit allowing the identification of 1% mosaic mutation with statistical confidence as recently shown by Izawa *et al*.[6]

Previous data showed that Roche 454 sequencing systems allow the detection of rare variants in blood extracted DNA samples, even for those below the Sanger sequencing limit of detection.[9] Our data expand these results by demonstrating that in a hospital laboratory setting, the GS Junior with a high-depth coverage is a valuable alternative to conventional molecular tools for the detection of mosaic mutations in the *VHL* gene.[3,4,10]

By using these tools, Wu *et al*[3] and Santarpia *et al*[10] previously identified mosaic mutations in patients with a mild phenotype and a late onset of the disease (50 and 43 years old, respectively). Our results do not support these observations since patient P1 had a severe phenotype with the first tumour diagnosed at 16 years. Therefore, we suggest that the screening for mosaic *VHL* mutation should not be restricted to criteria of mild phenotype and late onset of the disease. Sgambati *et al*,[4] evaluated the frequency of mosaicism by molecular screening of clinically asymptomatic parents of patients with a putative *de novo VHL* mutation. They found a 5% mosaicism rate (2 families on 42). On the basis of our preliminary data, it seems necessary to re-evaluate the true frequency of mosaic mutations in *VHL* by high sensitive NGS due to its clinical implications in the care of patients.

In conclusion, our study highlights the usefulness of clinical NGS as a diagnostic tool able to detect the presence of mosaic *VHL* mutation and it confirms the possibility to use pyrosequencing or SnaPshot analyses as confirmation techniques. This work also suggests that the research of mosaicism should not be restricted to patients with mild phenotype or late onset of the disease.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGEMENTS

We thank our clinical collaborators who referred their patients to us for molecular evaluation; Wassila Igoudjil, Jerome Gudewicz, Christine Mouton and Lauriane Briois for their expert technical help; and Dr M-F Odou for helpful discussions. Our laboratory is funded by the Institut national du Cancer (InCa) since 2001.

1 Maher ER, Neumann HP, Richard S: von Hippel-Lindau disease: a clinical and scientific review. *Eur J Hum Genet* 2011; **19**: 617–623.
2 Ciotti P, Garuti A, Gulli R, Ballestrero A, Bellone E, Mandich P: Germline mutations in the von Hippel Lindau gene in Italian patients. *Eur J Med Genet* 2009; **52**: 311–314.
3 Wu P, Zhang N, Wang X *et al*: Mosaicism in von Hippel-Lindau disease with severe renal manifestations. *Clin Genet* 2013; e-pub ahead of print 5 February 2013; doi: 10.1111/cge.12092.
4 Sgambati MT, Stolle C, Choyke PL *et al*: Mosaicism in von Hippel-Lindau disease: lessons from kindreds with germline mutations identified in offspring with mosaic parents. *Am J Hum Genet* 2000; **66**: 84–91.
5 Pagnamenta AT, Lise S, Harrison V *et al*: Exome sequencing can detect pathogenic mosaic mutations present at low allele frequencies. *J Hum Genet* 2012; **57**: 70–72.
6 Izawa K, Hijikata A, Tanaka N *et al*: Detection of base substitution-type somatic mosaicism of the NLRP3 gene with >99.9% statistical confidence by massively parallel sequencing. *DNA Res* 2012; **19**: 142–152.
7 Mardis ER: Next-generation DNA sequencing methods. *Annu Rev Genomics Hum Genet* 2008; **9**: 387–402.
8 Ronaghi M, Karamohamed S, Petterson B, Uhlen M, Nyren P: Real time DNA Sequencing using detection of Pyrophosphate release. *Anal Biochem* 1996; **242**: 84–89.
9 Targeted sequencing of leukemia-associated genes using 454 sequencing systems. Sequencing application note. Roche Diagnostics GmBH: Mannheim, Germany, 2012.
10 Santarpia L, Sarlis NJ, Santarpia M, Sherman SI, Trimarchi F, Benvenga S: Mosaicism in von Hippel-Lindau disease: an event important to recognize. *J Cell Mol Med* 2007; **6**: 1408–1415.