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Short report

A novel pathogenic germline chromosome 3 inversion in von Hippel-Lindau disease

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SUMMARY

von Hippel-Lindau (VHL) is an autosomal-dominant hereditary tumour susceptibility disease associated with pathogenic germline variants in the *VHL* tumour suppressor gene. VHL patients are at increased risk of developing multiple benign and malignant tumours. Current CLIA-based genetic tests demonstrate a very high detection rate of germline *VHL* variants in patients with clinical manifestations of VHL. In this report, we describe a large family with canonical VHL manifestations, for which no germline alteration had been detected by conventional germline testing. We identified a novel 291 kb chromosomal inversion involving chromosome 3p in affected family members. This inversion disrupts the *VHL* gene between exon 2 and exon 3 and is thereby responsible for the disease observed in this family.

INTRODUCTION

von Hippel-Lindau (VHL) is an autosomal-dominant hereditary tumour susceptibility disorder associated with pathogenic germline variants in the *VHL* tumour suppressor gene.[1] VHL patients are at increased risk of developing multiple benign and malignant tumours within various organs, including retinal and central nervous system (CNS) haemangioblastomas, clear cell renal cell carcinomas (ccRCCs), pheochromocytoma/paragangliomas, pancreatic neuroendocrine tumours, endolymphatic sac tumours (ELSTs), pancreatic cystadenomas, renal and pancreatic cysts, and epididymal and broad ligament cystadenomas.[2 3]

Current CLIA-based genetic tests demonstrate a very high detection rate of pathogenic germline *VHL* variants in VHL patients,[4 5] including single nucleotide missense and nonsense variants, small insertions and/or deletions, splice site alterations and large deletions encompassing entire exons or the whole gene (UMD-VHL mutations database—http://www.umd.be/VHL/).[6–10] Recently, we identified a balanced constitutional translocation between chromosomes 1 and 3 in a patient with clinical manifestations of VHL who lacked an identifiable *VHL* germline alteration.[11] This translocation was shown to involve a breakpoint within intron 2 of the *VHL* gene, resulting in a germline loss of one copy of *VHL*.[11] This highlighted the potential for rare pathogenic structural variants that are not routinely assessed by standard germline testing.

In the current report, we describe a family in which multiple members showed numerous pathognomonic VHL manifestations, tested germline negative for pathogenic *VHL* variants and for which karyotype analyses of three affected family members were all normal. We identified a novel germline 291 kb inversion within chromosome 3p25.3 that disrupts the *VHL* gene between exon 2 and exon 3, thus accounting for the family's clinical diagnosis of VHL.

MATERIALS AND METHODS

Patient consent

Patients were evaluated at the Urologic Oncology Branch (UOB) of the National Cancer Institute (NCI), National Institutes of Health (NIH) for clinical assessment.

TruSight Oncology 500 analysis

Renal tumours were analysed by the TruSight Oncology 500 (Illumina, California, USA), a next-generation sequencing assay capable of detecting single nucleotide variations, CNVs, gene fusions and indels from 523 cancer driver genes. Formalin-fixed, paraffin-embedded samples were processed and sequenced in the Comprehensive Oncologic Molecular Pathology and Sequencing Service (COMPASS) laboratories within the Laboratory of Pathology, NCI.

PCR and Sanger sequencing

Patient blood and tumour DNA was extracted using Promega Maxwell 16 Blood or Tissue DNA Purification Kits (Promega). Primers were designed in unique sequences adjacent to the Alu repeats containing the estimated inversion boundaries (online supplemental table S1), and a Qiagen Taq PCR Core Kit was used to amplify the deletion boundaries. DNA fragments were electrophoresed on E-Gel SizeSelect or EX Gels (Life Technologies). Bidirectional DNA sequencing was performed using the Big Dye Terminator V.1.1 Cycle Sequencing Kit (Applied Biosystems) according to the manufacturer's specifications and run on an ABI 3130xl or 3730 Genetic Analyzer (Applied Biosystems). Sanger sequencing was conducted at the CCR

Genomics Core at the National Cancer Institute, NIH, Bethesda, Maryland, and sequences were evaluated using Sequencher 5.0.1 (Genecodes). All coordinates are based on the GRCh37/hg19 genome build.

Chromosomal copy number analysis

Chromosomal copy number analysis was performed on available tumour DNA from patients III:1, III:25, and III:35 using the Applied Biosystems OncoScan CNV Assay (Applied Biosystems, California, USA) according to the manufacturer's specifications and compared with provided Affymetrix positive control DNA. Data was analysed using the Chromosome Analysis Suite software provided by Thermo Fisher Scientific (Thermo Fisher Scientific, Massachusetts, USA). The analysis was performed by the NCI Genomics Technology Laboratory at Frederick, Maryland.

RT-PCR-based VHL expression analysis

Normal kidney samples from unrelated VHL patients and ccRCC tumour samples from the presented family were initially cryo-pulverised and the resultant powder was mixed with 1 mL of Trizol Reagent (Invitrogen, California, USA) for total RNA extraction following the manufacturer's protocol. RNA concentration was measured using a NanoDrop 2000 UV-Vis Spectrophotometer (Thermo Fisher Scientific) and stored at −80°C. cDNA was generated from ~1 µg of total RNA in a 20 µL volume using the SuperScript VILO cDNA Synthesis Kit (Invitrogen), as described in the manufacturer's protocol, and the completed reaction was diluted 10-fold with 180 µL of RNase-Free Water. *VHL* expression analysis was performed using the TaqMan gene expression assay Hs001844551_m1 on a ViiA7 Real-Time PCR system (Thermo Fisher Scientific).

RESULTS

A large family with a multi-generational history of clinical manifestations of VHL presented at the UOB of the National Cancer Institute for clinical management. In the first generation, patient I:2 was reported to have had unspecified cancer (figure 1A). Seven of the twelve siblings in the second generation and at least fourteen of the thirty-seven members of the third generation demonstrated VHL clinical manifestations (figure 1A,online supplemental table S2). Many of the family members had not had screening or routine surveillance. We have enrolled fifteen family members, nine of whom have manifestations including CNS haemangioblastomas, ccRCCs and renal cysts,

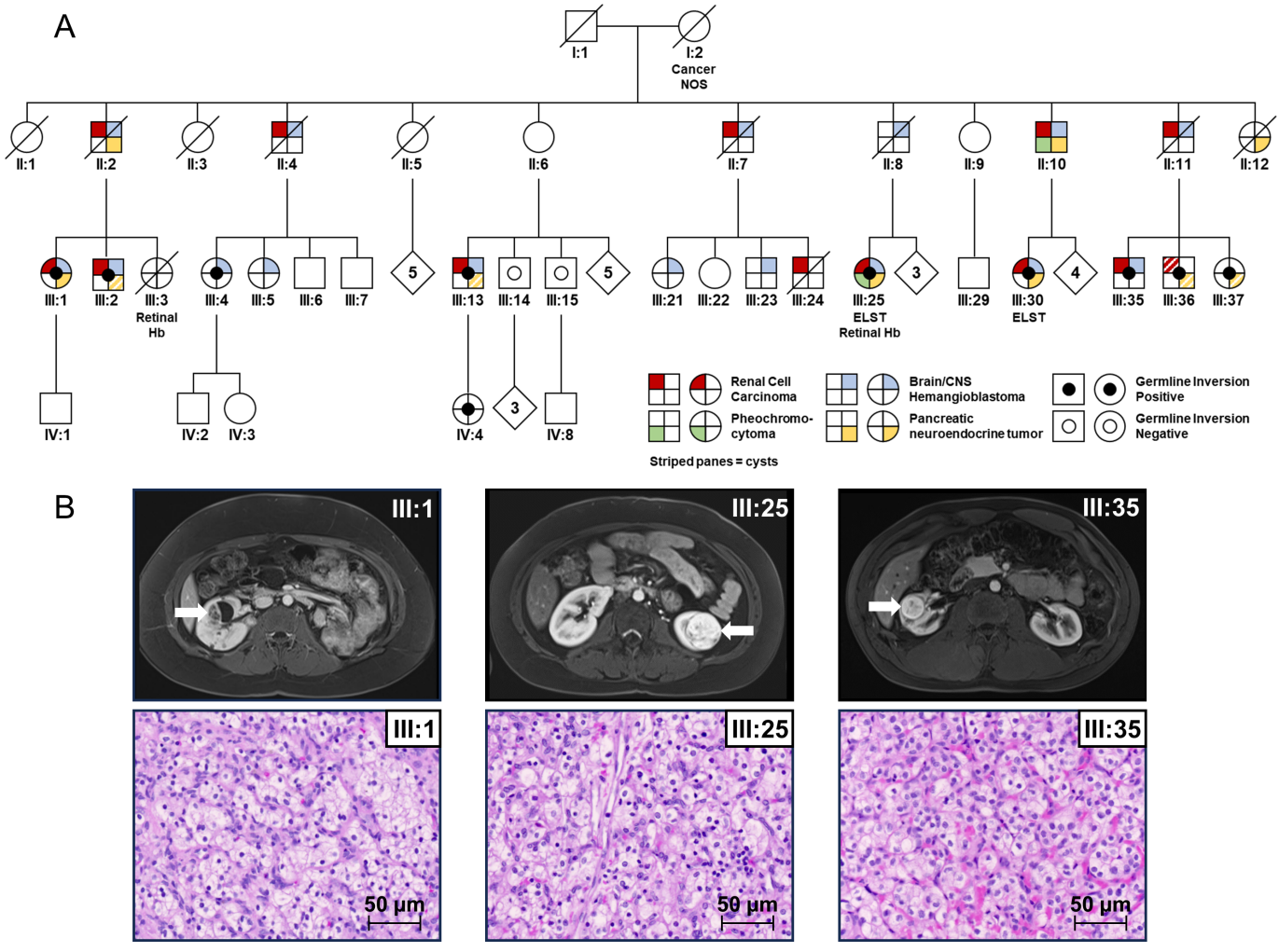

Figure 1 Family pedigree with patient imaging and histology. (A) The family pedigree showed four generations with the clinical features of von Hippel-Lindau highlighted in evaluated patients. The presence of the germline chromosome 3p inversion was denoted by a filled black circle for carriers and an empty circle for those who tested negative for the inversion. (B) Imaging of example kidney tumours is shown for patients III:1, III:25, and III:35 with example histological staining from those patients' tumours showing clear cell renal cell carcinomas. CNS, central nervous system; ELST, endolymphatic sac tumour.

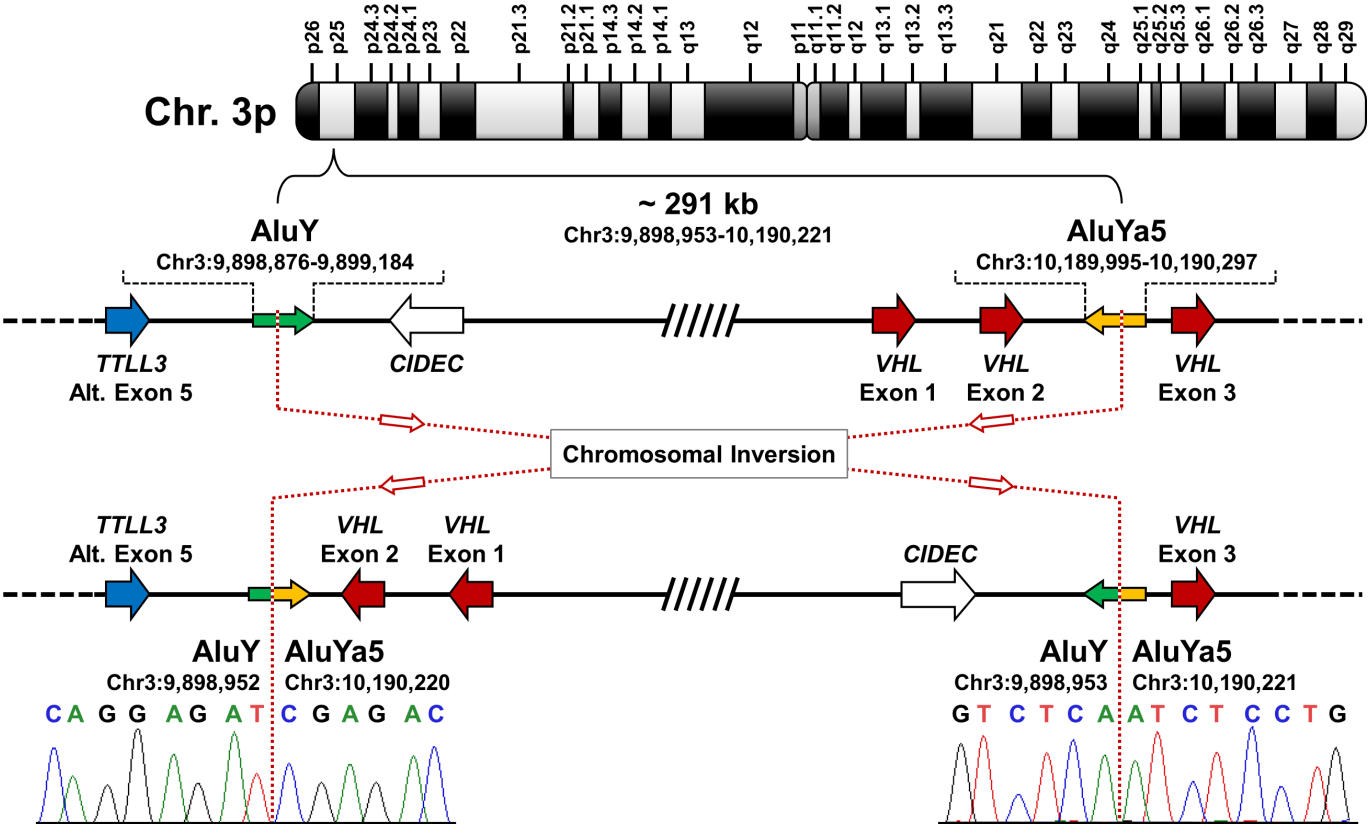

Figure 2 Mapping of germline chromosome 3 inversion. A germline inversion on chromosome 3p was identified resulting in the flipping of approximately 291 kb of DNA that disrupted the *VHL* gene. Both breakpoints were mapped by DNA sequencing and occurred within Alu repeats, *AluY* at Hg19 chr3:9898876–9899184 and *AluYa5* at Hg19 chr3:10189995–10190297.

pancreatic neuroendocrine tumours and/or pancreatic cysts, and less frequently, retinal haemangioblastomas, pheochromocytomas and ELSTs (figure 1A and online supplemental table S2). Patients II:2 and II:11 had historically undergone standardised CLIA germline testing for *VHL* without any detectable alterations, and more recently patients III:1, III:4, and III:25 had both CLIA germline testing and karyotype analysis, with all results demonstrating no *VHL* alterations. Recently, a cryptic *VHL* E1' exon has been identified that demonstrates at least six known germline variants, two of them have been shown to be pathogenic in patients presenting with VHL clinical features.[12] This cryptic exon is not covered by conventional testing and therefore was sequenced on a research basis in eight family members, six affected and two unaffected, and all eight were shown to have the wild-type sequence (online supplemental figure S1).

Patient III:1 underwent a right partial nephrectomy resulting in the resection of three ccRCC WHO/ISUP grade 2 tumours ranging in size from 0.8 to 2.0 in the largest dimension (figure 1B). The 2.0 cm tumour was evaluated using the TruSight Oncology 500 Gene Panel (V.3) and DNA analysis detected no pathogenic alterations in either the *VHL* gene or any other cancer genes (online supplemental table S3). However, RNA analysis identified a unique fusion transcript in four reads, containing the sequences from the end of *VHL* exon 2 and the alternate transcript ENST00000455274 exon 5 of *TTLL3* in a tail–tail configuration (figure 2 and online supplemental table S3). The *TTLL3* (Tubulin Tyrosine Ligase Like 3) gene is located on chromosome 3p25.3 telomeric to *VHL*, suggesting the possibility of a chromosomal inversion with breakpoints within the *TTLL3* and *VHL* genes.

This potential inversion was next investigated at the DNA level in the germline. PCR analysis of germline DNA from patient III:1 using primer sets designed to bind near the potential sites for the inversion breakpoints resulted in a novel PCR product. Sanger sequencing confirmed the chromosome 3p inversion, with both the left and the right breakpoints occurring within *Alu* repeats, an *AluY* at Hg19 chr3:9898876–9899184 and an *AluYa5* at Hg19 chr3:10189995–10190297 (figure 2). This is consistent with a balanced inversion of 291 269 bp between *VHL* intron 2 and the 3'untranslated region (UTR) of *TTLL3* transcript ENST00000455274 (Hg19 chr3:9898953–10190221). The presence of the inversion breakpoints was further confirmed in patients III:4 and III:25.

Since the PCR primers designed to identify the inversion breakpoints would not amplify wild-type DNA, a multiplex PCR strategy was employed to amplify both wild-type and inverted PCR products so that additional family members could be tested along with negative controls. Both wild-type and inverted PCR products were detected in nine patients who had one or more clinical manifestations of VHL (III:1, III:2, III:4, III:13, III:25, III:30, III:35, III:36, III:37). Importantly, patient IV:4, a teenaged female who does not yet exhibit any VHL manifestations, was revealed to inherit the inversion. In contrast, only the wild-type product was detected in two family members with no VHL clinical manifestations (III:14, III:15), as well as in unrelated control DNA samples (online supplemental figure S2).

Patients III:25 and III:35 subsequently had renal surgeries at NCI. The OncoScan CNV Assay was performed on the ccRCCs from these patients as well as III:1 to assess CNVs. All tumours exhibited chromosome 3p loss, and most also exhibited

chromosome 5q gain, which is consistent with VHL-deficient ccRCCs (online supplemental figure S3). A few additional gains and losses were noted (online supplemental table S3). Expression analysis of *VHL* was performed on 13 ccRCCs from patients III:1 (n=3), III:25 (n=3) and III:35 (n=7) and compared with normal kidney samples derived from three independent, unrelated VHL patients, due to no normal kidney tissue being available from patients within this family. All tumours from patients III:25 and III:35 showed reduced expression of VHL, while two tumours from patient III:1 had mildly reduced expression (online supplemental figure S4). This is consistent with the observed loss of chromosome 3p in these tumours and residual *VHL* expression from normal cells within the tumours, such as infiltrating immune cells and tumour vasculature. In addition, patients III:13 and III:25 subsequently underwent surgery at NIH for brainstem and cerebellar haemangioblastomas, respectively.

DISCUSSION

This report highlights the importance of continually refining the methodologies used to evaluate germline alterations in patients with hereditary diseases. The vast majority of VHL patients will have germline variants that are detected with conventional testing, but this family and the recently published case of VHL disease caused by a balanced constitutional translocation[11] highlight the need for germline structural variants to be considered in the absence of other genetic causes. Although patients may be clinically diagnosed with diseases such as VHL, identifying the germline pathogenic cause is an essential component of managing these families. Most importantly, it can be used to identify additional, non-symptomatic members of the family and provide them with the appropriate screening and surveillance necessary to detect symptoms early and provide the best possible care. In the family described here, one teenaged patient (patient IV:4) is now confirmed to be germline positive for the chromosomal inversion we have identified, even before clinical manifestations are apparent. She can now receive surveillance that is appropriate for VHL, as can any additional family members who are confirmed germline positive as testing progresses. Likewise, efforts are being made to provide testing and screening for patient II:6, who despite being asymptomatic, is an obligate carrier since her son and granddaughter (III:13 and IV:4) are both germline positive.

In a previous study identifying breakpoints of germline *VHL* deletions, we reported that 95% (55 of 58) of the fully mapped germline deletions involve *Alu* repeats at both breakpoints and the *AluYa5* at chr3:10189995–10190297 involved in this inversion was the most common site for a breakpoint in these deletions (44.8%).[13] Furthermore, two germline inversions were present in conjunction with deletions that involved *Alu* repeats at all identified breakpoints, suggesting the possibility of an inversion occurring in the absence of a deletion.[13] The identified 291 kb inversion is similar in size to the largest reported *VHL* germline deletion (355 kb)[13] and was confined to a single cytogenetic band (3p25.3), thus would not be detected by conventional karyotype analysis.

Although families with a clinical diagnosis of VHL and no detectable germline alteration are very rare, this study suggests that for such families, additional analysis techniques, such as RNAseq or whole genome sequencing, should be used to screen for these types of structural germline alterations. Ultimately, a complete genetic diagnosis is essential for the effective management of these VHL families, both now and in the future.

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Competing interests None declared.

Patient consent for publication Not applicable.

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REFERENCES

1 Latif F, Tory K, Gnarra J, *et al*. Identification of the von Hippel-Lindau disease tumor suppressor gene. *Science* 1993;260:1317–20.
2 Choyke PL, Glenn GM, Walther MM, *et al*. von Hippel-Lindau disease: genetic, clinical, and imaging features. *Radiology* 1995;194:629–42.
3 Nielsen SM, Rhodes L, Blanco I, *et al*. Von Hippel-Lindau Disease: Genetics and Role of Genetic Counseling in a Multiple Neoplasia Syndrome. *J Clin Oncol* 2016;34:2172–81.
4 Stolle C, Glenn G, Zbar B, *et al*. Improved detection of germline mutations in the von Hippel-Lindau disease tumor suppressor gene. *Hum Mutat* 1998;12:417–23.
5 Hes FJ, van der Luijt RB, Janssen ALW, *et al*. Frequency of Von Hippel-Lindau germline mutations in classic and non-classic Von Hippel-Lindau disease identified

by DNA sequencing, Southern blot analysis and multiplex ligation-dependent probe amplification. *Clin Genet* 2007;72:122–9.
6 Chen F, Kishida T, Yao M, *et al*. Germline mutations in the von Hippel-Lindau disease tumor suppressor gene: correlations with phenotype. *Hum Mutat* 1995;5:66–75.
7 Zbar B, Kishida T, Chen F, *et al*. Germline mutations in the Von Hippel-Lindau disease (VHL) gene in families from North America, Europe, and Japan. *Hum Mutat* 1996;8:348–57.
8 Béroud C, Joly D, Gallou C, *et al*. Software and database for the analysis of mutations in the VHL gene. *Nucleic Acids Res* 1998;26:256–8.
9 Maranchie JK, Afonso A, Albert PS, *et al*. Solid renal tumor severity in von Hippel Lindau disease is related to germline deletion length and location. *Hum Mutat* 2004;23:40–6.
10 Nordstrom-O'Brien M, van der Luijt RB, van Rooijen E, *et al*. Genetic analysis of von Hippel-Lindau disease. *Hum Mutat* 2010;31:521–37.
11 Ricketts CJ, Vocke CD, Lang M, *et al*. A germline 1;3 translocation disrupting the *VHL* gene: a novel genetic cause for von Hippel-Lindau. *J Med Genet* 2022;59:18–22.
12 Buffet A, Calsina B, Flores S, *et al*. Germline mutations in the new E1′ cryptic exon of the *VHL* gene in patients with tumours of von Hippel-Lindau disease spectrum or with paraganglioma. *J Med Genet* 2020;57:752–9.
13 Vocke CD, Ricketts CJ, Schmidt LS, *et al*. Comprehensive characterization of Alu-mediated breakpoints in germline VHL gene deletions and rearrangements in patients from 71 VHL families. *Hum Mutat* 2021;42:520–9.