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Original Article

Unexpected information of DNA deletions in genome: analysis of one VHL case with Array-CGH

Yang Yang[1*], Changwei Ji[1*], Xiangyu Zhu[4], Huimei Chen[2,3], Hongqian Guo[1]

[1]Department of Urology, Nanjing Drum Tower Hospital, Medicine School of Nanjing University, Institute of Urology, Nanjing University, Nanjing 210008, Jiangsu, China; [2]Department of Medical Genetics, Nanjing University School of Medicine, Nanjing, Jiangsu, China; [3]Jiangsu Key Laboratory of Molecular Medicine, Nanjing University, Nanjing, Jiangsu, China; [4]Prenatal Diagnosis Center, Departments of Obstetrics and Gynecology, Nanjing Drum Tower Hospital, The Affiliated Hospital of Nanjing University School of Medicine, Nanjing, Jiangsu, China. [*]Equal contributors and co-first authors.

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Abstract: Background: Von Hippel-Lindau (VHL) disease is an autosomal dominant familial cancer syndrome that occurs as a consequence of an inactivation of the VHL gene. Point mutations in VHL are a major cause for VHL disease, and fragment deletions contribute to ~30% of VHL patients. Array Comparative Genomic Hybridization (Array-CGH) is a powerful tool for the identification of genomic deletions, and often reveals unexpected information as a non-targeted method. Method: We extracted the DNA from the patients' blood andscreened for point mutations in VHL by PCR and large deletions by Array Comparative Genomic Hybridization (Array-CGH). Real-time quantitative PCR was used to confirm the deletion of VHL gene and expression of VHL in tumor tissue. Results: We present a family case of VHL syndrome, showing hemangioblastomas in the central nervous system and retina, multiple pancreatic cysts and clear-cell renal cell carcinoma. After excluding point mutations in VHL gene exons and conjunctive regions, we identified a ~9 kb deletion of chromosome 3p25.3 with Array-CGH analysis. Real-time PCR confirmed a heterozygous deletion in exon 3 of the VHL gene. Additional 90 DNA deletions were detected ranging from 5 kb to 160 kb in size. Moreover, 70.3% of the breakpoints were located in Alu elements. Conclusions: This study confirmed that DNA deletions contribute to familial VHL disease. It also highlights that coding deletions appear to be more frequent than expected, and that Alu-mediated recombination is the major mechanism.

Keywords: Von Hippel-Lindau, array comparative genomic hybridization, coding deletion, alu element

Introduction

Von Hippel-Lindau (VHL) disease is a hereditary cancer syndrome that is characterized by multiple benign and malignant neoplasias in many organs, especially hemangioblastomas in the central nervous system (CNS) and retina at a young age [1]. It also presents with clear-cell renal cell carcinoma (ccRCC) and cysts, pheochromocytomas, multiple pancreatic cysts or tumors, epididymal/ovarian cystadenomas and endolymphatic sac tumors [2]. The inactivation of the VHL gene (OMIM 608537), a well-known tumor-suppressor gene, directly leads to familial VHL diseases, as well sporadic cases. The VHL gene is located on chromosome 3p26-25, spans a 10 kb region consisting of three exons [3]. VHL mRNA encodes a 213 amino acid protein (pVHL) with a molecular weight of ~24 to 30 kDa (VHL_{30}) [4], as well a second pVHL isoform of approximately 19 kDa (VHL_{19}) [5]. Both isoforms appear to retain tumor suppressor activity [6]. Inactivation of VHL leads to an accumulation of hypoxia-inducible factor (HIF), the target of pVHL, which contributes to the overproduction of vascular endothelial growth factor (VEGF) [7] and erythropoietin (Epo) [8], and, ultimately, vascular proliferation and tumorigenesis.

In 1894, Treacher et al. [9] first described a mutation in the VHL gene in two retina hemangioblastoma patients. Thereafter, patients with visceral cysts and tumors were also reported to be resultant of VHL mutations, such as clear-cell renal carcinomas, pheochromocytomas,

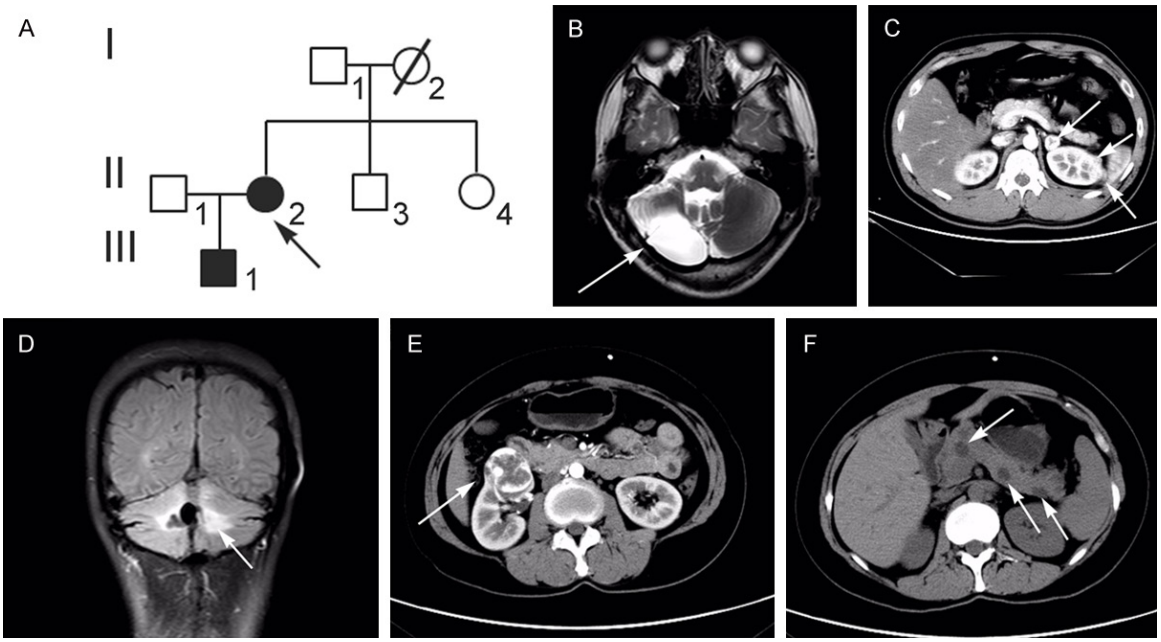

Figure 1. Clinical presentation of the two VHL patients in this family. A. Genograms of the VHL family. B. Head MRI of III1 showed hemangioblastoma at right cerebellar hemispheres (arrows) C. Abdomen CT scan of III1 showed multiple and bilateral renal tumors on right renal and right adrenal tumors (arrows). D. Head MRI of II2 showed hemangioblastoma at cerebellar hemispheres (arrows). E. Abdomen CT scan of II2 showed left renal tumor (arrows). F. Abdomen CT scan of II2 showed multiple pancreatic cysts (arrows).

pancreatic tumors and epididymal/ovarian cystadennoma and endolymphatic sac tumor [10]. Generally, germline mutations in the VHL gene account for approximately 100% classical non-mosaic VHL disease and somatic from 17.9% to 71% of ccRCC [11-13]. Point mutations are the major cause of inactivation of VHL gene, including 30% to 38% for missense mutations and 23% and 27% for nonsense or frameshift mutations, respectively. Germline rearrangements have frequently been reported to account for approximately 20 to 37% of VHL patients, especially large or partial fragment deletions [10, 14]. Karyotyping analysis and multiplex ligation-dependent probe amplification (MLPA) are often used to detect these deletions. Array Comparative Genomic Hybridization (Array-CGH) was recently developed to identify genomic imbalances. As a conventional method, it is promising in clinical diagnostics. Compared to karyotyping, Array-CGH has a higher resolution, detecting 50-100 kb deletions, while karyotyping is unreliable for subtle copy number changes of 5 Mb in size or smaller [15]. MLPA can only detect deletions or duplications on specific genomic regions [16]. Array-CGH analysis, however, can also reveal unexpected information of DNA recombinations a non-targeted method of scanning the whole genome [17].

In the present study, we screened potential mutations in a familial case with VHL disease and identified a fragment deletion in VHL genes. Moreover, unexpected DNA deletions were detected by Array-CGH analysis. The characteristics of these genome deletions and underlying mechanisms were therefore investigated.

Patients, samples and method

Subjects

Members of a Chinese multigeneration family (**Figure 1A**) underwent surgical therapy for renal tumors in Nanjing Drum tower hospital. Clinical features were carefully reviewed and imaging and hematological examinations were performed before the surgery. Blood specimens were collected from all family members, and tissues from renal tumors of cancer patients in this family were also collected. Samples from sporadic renal cancers served as tumor controls, and adjacent normal renal tissues as negative controls.

Table 1. Primers used in mutation screening of the VHL gene

	Forward primer 5'→3'	Backward primer 5'→3'	Tm (°C)
Exon 1	GCGCGCGAAGACTACGGAGGTC	TCCCCGTCTGCAAAATGGAC	58.5
Exon 2	GTGGCTCTTTAACAACCTTTG	CCTGTACTTACCACAACAACCTTATC	60
Exon 3	TCTTTAACCTAAAGTGAGATCCATCA	GAAACTAAGGAAGGAACCAGTCC	60

Table 2. The clinical features and onset age in the patients

	II-2	III-1
Gender	F	M
First sign of onset	Hemangioblastoma	Hemangioblastoma
Hemangioblastoma	in CNS/RA	in CNS
Age of diagnosis	33/45 yr	9 yr
Treatment	Tumorectomy/Tumorectomy	Tumorectomy
ccRCC	R:	L:/R:
Age of diagnosis	47 yr	27 yr
Treatment	Tumorectomy	Tumorectomy & Scheduled follow-up later
Pheochromocytoma		
Age of diagnosis	--	26
Treatment	--	Tumorectomy
PCE		
Age of diagnosis	--	24
Treatment	--	Scheduled follow-up later
Pancreatic cysts	Multiple	
Age of diagnosis	47	--
Treatment	Scheduled follow-up later	--

Abbreviations: CNS: central nervous system, RA: retina, ccRCC: clear-cell renal cell carcinoma, PCE: papillary cystadenoma of the epididymis.

Screening for point mutations in VHL

Genomic DNA was extracted from peripheral blood samples (TIANamp Blood DNA Kit, Beijing, China). DNA samples were subjected to mutation screening with all of the exons and exon-intron boundaries of VHL (NC_000003.12) amplified by polymerase chain reaction (PCR). The primers for PCR are listed **Table 1**. The products were examined on a 2% agarose gel, purified by a universal DNA purification kit (TIANGEN, Beijing, China) and then subjected to direct DNA sequencing using an ABI Prism 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA).

Array-CGH analysis

Array-CGH microarray analysis was performed using an Affymetrix Cytoscan HD GeneChip at an effective resolution of 5 kb. Data analysis was performed using the Chromosome Analysis Suite (ChAS) 2.0 software following the standard protocol. Abnormalities detected were compared with the RefSeq genome build hg19, via the database browser at http://genome.ucsc.edu/cgi-bin/hgGateway.

Quantitative PCR analysis

Real-time quantitative PCR was used to confirm the deletion of VHL gene, with SYBR Green I detection (Roche, Mannheim, Germany) on an ABI StepOne Sequence Detection System (PE Applied Biosystems, Foster City, CA). The primers used were as follow: forward 5'GGTCGCTCTACGAAGATCTGGA3', reverse 5'GAAATCTTCAATCTCCCATCCG3' and 18SrRNA was used as an internal reference. Real-time quantitative PCR was also used to detect the expression of *VHL*. Briefly, after total RNA was isolated from tissues and complementary DNA (cDNA) was synthesized using a reverse transcription kit (Transgen Biotech, Beijing, China), quantitative PCR was performed with Green I detection using the primers: forward 5'GGAGCCTAGTC-

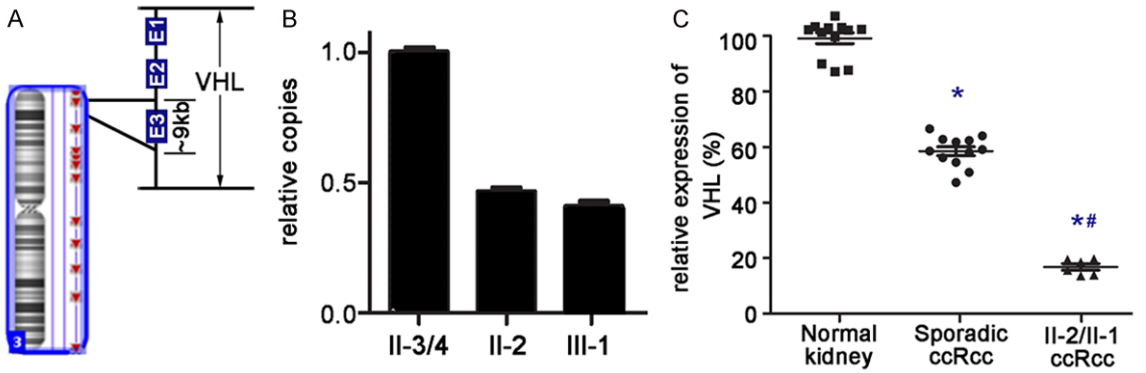

Figure 2. Deletions in the VHL gene were detected by various methods. A. Array-CGH enabled the detection of a 9 kb deletion at 3p25.3, where the VHL gene is located. B. Q-PCR showed that the VHL copies in patients II3/4 were double than that of II2 and III1. C. Q-PCR showed a lower expression of pVHL in VHL associated ccRCCs than that found in sporadic ccRCCs (P=0.001), and expression of pVHL in these sporadic ccRCCs was also lower than that in corresponding adjacent normal tissue (P=0.001).

AAGCCTGAGA3', reverse 5'CATCCGTTGATGTG-CAATGCG3'. All of the PCRs were performed in triplicate and data analysis was performed using the ΔΔCT method.

Statistical analysis

All statistical analyses were carried out using the statistical program SPSS, version 17.0. The non-parametric test was used to evaluate the deletions in and expression of the VHL gene between patients and normal individuals. The chi-square test was performed to analyze the relationship between deletion polymorphisms, Alu-related deletions and coding deletions. Pearson correlation analysis was utilized to evaluate the association between the length of the deletions, the length of the whole chromosome, and the number of Alu elements on each chromosome. All tests were two-sided and $P \leq 0.05$ was considered statistically significant.

Result

Germline VHL deletion

Two of the four subjects in this family were diagnosed with VHL disease (**Figure 1A**, II-2 & III-1). The II2 was a 47 years old female with a history of surgery for hemangioblastomas of CNS and retina (**Table 2**). Imageological diagnosis as a routine test was performed and revealed a right renal tumor and multiple pancreatic cysts (**Figure 1B**, **1C**). Patient III1 had similar symptoms, with CNS hemangioblastomas and a renal tumor, as well as pheochromocytomas and epididymal cystadenoma (**Figure 1D-F**). Both of the two patients underwent laparoscopic renal tumor resection, and pathological analysis revealed clear-cell renal cell carcinoma.

After sequencing of the VHL gene, we did not identify any point mutations (missense mutation or small insertion and deletion) in II2 and III1. Array-CGH was further performed to detect potential fragment deletions. Focusing on chromosome 3p and the VHL gene (**Figure 2A**), a 9 kb deletion was detected and located in exon 3 of the VHL gene. Further Q-PCR showed that VHL copies of II3/4 were double than that of II2 and III1, as considered as VHL deletion occurred in the two patients (**Figure 2B**). VHL deletion also leaded to lower expression of pVHL in VHL associated ccRCCs than that in sporadic ccRCCs. (P=0.001), while expression of pVHL in these sporadic ccRCCs was also lower than that in corresponding adjacent normal tissue (P=0.001) (**Figure 2C**).

Coding deletion in genome

The Array-CGH also showed a common distribution of deletions and duplication across whole genome at a 5 k resolution. The distribution of these deletions was imbalance (**Figure 3A**). Chromosome 1, 3, 4 and X were high ladled with markers for deletions, followed chromosome 8, 11, 9 and 11. The chromosome 21 had none markers and some chromosomes short arms didn't, either. In addition to of the

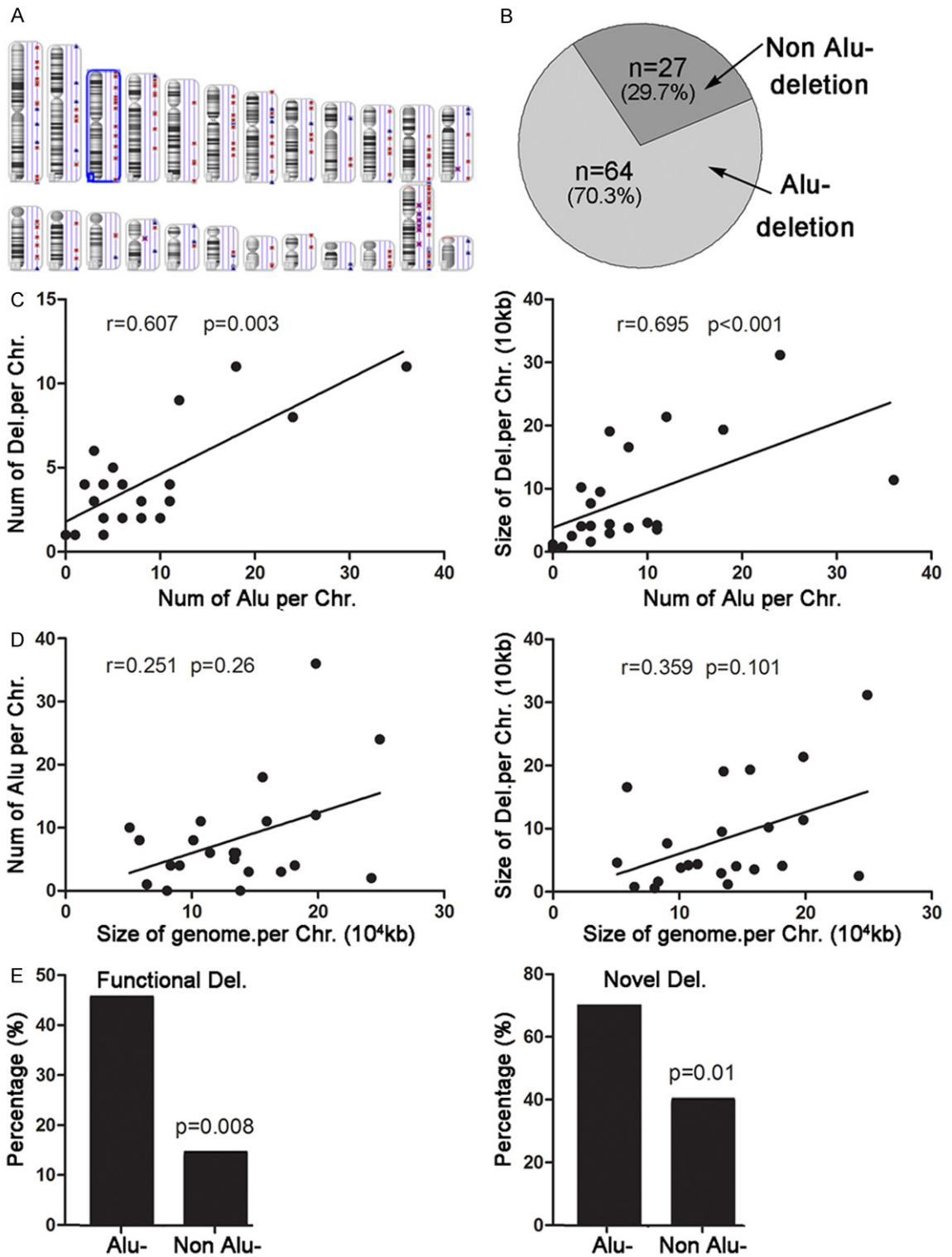

Figure 3. A. Deletions detected by Array-CGH acrossthe whole genome of II2. B. The majority of deletions were mediated by Alu elements. C. There was a liner correlation between the number of deletions and the number of Alu elements at each chromosome (r=0.607, P=0.003), as well as the sizes of deletions and number of Alu elements at each chromosome (r=0.695, P<0.001). D. The size of the whole chromosome was not related with the number of Alus or the size of deletions. E. A total of 45.3% of deletionsmediated by Alu elements were coding deletions, and 14.8% of deletions that were not mediated by Alu elements were coding deletions (P=0.008). Of the Alu-associated deletions, 70.3% led to novel deletions, while only 40.7% non-Alu associateddeletions led to novel deletions (P=0.01).

Table 3A. Detected coding deletions in II2

Deletion		Involved genes
Polymorphism	Allele frequency <0.5%	SMR3A, SMR3B, DMBT1, ZNF826P
	Allele frequency: 0.5%-5%	OR2T3, OR2T5, OR2G6, OR2T29, OR2T34, TUSC3
	Allele frequency >5%	SCAP, TRY6, PRSS2, OR4P4, OR4S2, OR4C6, TRIM49, ACOT1, SIGLEC14, LILRA3, SIRPB1
Novel deletion	Diseased related	MAP3K2, VHL, DACH1, PEX1
	Unknown	SLMAP, FLJ34503, NBPF6, EFHA2, FLT3, SLC15A4, KCNK10, PAK6, PLA2G4F, PDXDC1, PIR, KRTAP4-12, SLC25A17, MAGEA9, MAGE-A9B, RFPL1, ASB9

Table 3B. Virulence genes involved with deletions

Gene	OMIM	Inactivation causing disease	
VHL	608537	VHL syndrome	Autosomal dominant inheritance
MAP3K2	609487	Glioblastoma	Complex disease
PEX1	602136	Zellweger syndrome	Autosomal recessive inheritance
TUSC3	601385	Loss of heterozygosity is common in epithelial tumor, nonsyndromic mental retardation	Complex disease autosomal recessive inheritance
DMBT1	601969	Medulloblastoma, glioblastoma multiforme	Complex disease
DACH1	603803	breast cancer, endometrial cancer, ovarian cancer	complex disease

deletion on VHL, The CGH analysis together showed 91 deletions and 24.18% (22/91) were less than 8 K in size. Among these deletions, the deleted size ranged from 5 to 160 kb, and 35 of 91 were deletion polymorphisms listed in the Database of Genomic Variants (http://dgv-beta.tcag.ca/dgv/app/index.html), including 18 (51.4%) coding deletions.

Moreover, there were 52 deletions located at 60 known genes across whole genome and 33 coding deletions at exon regions, indicating deteriorating the function of these involved 42 genes. Interestingly, dysfunction of 21 genes was reported as polymorphism in the database (datas from Database of Genomic Variants http://dgvbeta.tcag.ca/dgv/app/index.html and allele frequency from 1000 Genomes Consortium Phase 1) and deletions involved 11 genes were even common (allele frequency >5%) among population (**Table 3A**). Other deletions were at first detected Particularly, the dysfunction of 6 genes in novel or rare deletions was unequivocally related with different diseases (**Table 3B**). VHL is an autosomal dominant gene that contributed to the patients investigated. Two genes, PEX1 and TUSC3, were related with autosomal recessive inheritance (AR). Complete lack of PEX1 protein is associated with severe Zellweger syndrome. In an Iranian family, a homozygous deletion involving the first exon of TUSC3 gene was detected, while all unaffected parents of patients were heterozygous and all of the unrelated controls did not have this deletion. However, we did not know how the dysfunction of these genes would contribute to the phenotypes in this family.

Alu elements distribution with DNA deletions

Regarding the breakpoints of the deletion, Alu elements appeared frequently through 1000 bp of the upstreams and downstreams. The Alu elements were detected at bilateral breakpoints in 32 of 91 deletions and unilateral in another 32 of 91 deletions (**Figure 3B**). In addition, bilateral breakpoints of 4 deletions were located at homologous sequences and 1 deletion at a tandem repeat sequence.

As shown in **Figure 3C**, there was a liner correlation between the number of deletions and the number of Alu elements at each chromosome ($r=0.607$, $P=0.003$), as well as the sizes of deletions and the number of Alu elements at each chromosome ($r=0.695$, $P<0.001$). But, the size of the whole chromosome was not related to the number of Alu elements or the size of the deletions (**Figure 3D**). Moreover, 45.3% (29/64) of deletions mediated by Alu were coding deletions, and the percentage was significantly higher than that of deletions not mediated by

Alu elements (4/27, 14.8%) (P=0.008). Of the Alu-associated deletions, 70.3% (45/64) led to novel deletions, while only 40.7% (11/27) of non-Alu associated deletions led to novel deletions (P=0.01) (**Figure 3E**).

Discussion

Array-CGH is a powerful method used to identify genomic imbalances, deletions or duplications. It is helpful for identifying additional, unexpected deletions and insertions associated with diseases, which has been largely ignored previously. In the present study, we identified 91 fragment deletions in a patient with VHL, suggesting that gene deletion is a relatively more common issue than has been previously considered, and it was consistent with other published studies [18, 19]. This study further showed that DNA deletions most often result from Alu element-mediated rearrangements, which contribute to the novel mutations.

Regarding the patients with VHL syndrome, Array-CGH demonstrated a heterozygosis deletion on chromosome 3p25 where VHL gene locals. Familial VHL is a typical autosomal dominant heritable disease. Heterozygotes point mutation or germline deletion VHLleads to the inactivated allele and insufficient VHL protein [6]. The deletion investigated in this study has been confirmed to be associated with insufficient *VHL* expression. The molecular genetic contribution to the clinical phenotype of VHL is: decreased pVHL led to an accumulation of HIF, overproduction of VEGF and Epo, and ultimately tumorigenesis.

Coding deletions are typically deleterious, such as the deletion in VHL. However, additional coding deletions were detected without significance on disease, and such deletions are even considered to be a common issue. We found that 36.3% (33/91) of deletions detected in this patient were coding deletions. McCarroll et al. [18] found 10 expressed genes contained deletions and their research also demonstrated that coding deletions are common in healthy individuals. Hinds et al. [19] found several deletions that resulted in loss of exons in genes in unrelated individuals.

The PEX1 [20, 21] and TUSC3 [22, 23] genes were previously demonstrated to be autosomal recessive genes. A deletion in one copy may lead to low expression, but it is not enough to result in obvious clinical phenotypes. The remaining allele may be sufficient under normal conditions, but these deletions increase the risk for disease and the genetic load among a population. The functions of other genes are not clear, and the association of their heterozygous inactivation with clinical phenotypes is not known. Even homozygous null genotypes were detected in some functional genes without a related phenotype, and the deletion frequency varied across different races [18]. These findings highlight the functional deletions that generally exist in various populations. The significance of coding deletions in genetic evolution and disease risk requires further clarification.

Another interesting finding of in the present study is that Alu elements are the most elements involved in the genome deletions. The deletion in the VHL gene appeared to be the result of Alu-Alu recombination, as Alu elements were detected frequently at both breakpoints. Of the 91 deletions, 64 (70.33%) were located within Alu elements. A liner correlation was found between the number of deletions and the number of Alu elements at each chromosome, as well as the sizes of the deletions and the number of Alu elements at each chromosome. An increasing number of studies are demonstrating that Alu elements are widely distributed throughout the whole genome, and they are known to be hot spots for recombination events. Alu element-mediated deletions have been widely detected in various genes. Franke's research demonstrated that 90% of deletions that involved the *VHL* gene were located in Alu elements [24]. Coutinho's study also observed that Alu-Alu recombination mediated a large homozygous intragenic GNPTAB gene deletion in a mucolipidosis patient [25]. Our findings further provide evidence that Alu-Alu recombination is the main mechanism of mediating large deletions.

Conclusion

Array technology is a powerful test for the identification of deletions of the VHL gene, which contributes to haploinsufficiency of VHL and ultimately leads to cancer. Array-CGH also reveals the profile of additional genomic imbalances that co-exist in the patient, although the

underlying pathogenesis is unclear. Moreover, investigating these fragment variations is helpful for a better understanding of the universality of coding deletions, as well the mechanism of DNA recombination by Alu-Alu elements. The findings of such studies enrich knowledge regarding the molecular mechanisms underlying large deletions of chromosomes, and enable us to recognize and identify such genetic phenomena.

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Disclosure of conflict of interest

None.

Address correspondence to: Huimei Chen, Department of Medical Genetics, Nanjing University School of Medicine, Nanjing, Jiangsu, China. E-mail: chenhuimei@nju.edu.cn; Hongqian Guo, Department of Urology, Nanjing Drum Tower Hospital, Medicine School of Nanjing University, Institute of Urology, Nanjing University, Nanjing 210008, Jiangsu, PR China. E-mail: dr.guohongqian@gmail.com

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