Mutations in *KIF27*, *GNAS* and *IFT140* genes in a patient with VACTERL association: A case report

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ABSTRACT

VACTERL association is a rare genetic disorder involving at least three of the following congenital malformations: vertebral defects (V), anal atresia (A), cardiac defects (C), trachea-oesophageal fistula with or without oesophageal atresia (TE), renal anomalies (R) and limb abnormalities (L). Until now, the aetiology of VACTERL association is unknown, particularly at the molecular level. Here, we performed whole exome sequencing (WES) of an infant with VACTERL association. The patient was delivered prematurely at 30 weeks and had 4/6 of the VACTERL malformations. Trio-WES analysis was performed using Torrent Suite and ANNOVAR. Polymorphisms with an allele frequency of >0.01 were excluded, and the remaining variants were filtered based on *de novo* mutations, autosomal recessive, X-linked and di-genic inheritance traits. In this patient, no homozygous, compound heterozygous or X-linked mutations was associated with VACTERL. However, we identified two heterozygous mutations; *KIF27* (ENST00000297814: c.3004A> C:p.N1002H) and *GNAS* (ENST00000371098: c.205C>A:p.H69N) genes that were inherited from her father and mother respectively. A *de novo*, *IFT140* gene mutation (ENST00000426508: c.683C>G:p.S228C) was also identified in this patient. The VACTERL phenotype in this patient may due to heterozygous mutations affecting *KIF27* and *GNAS* genes, inherited via autosomal recessive trait. In addition, the *IFT140* gene mutation may also be involved. These genes are known to be directly or non-directly involved in the sonic hedgehog signalling that is known to be implicated in VACTERL. This is the first report of these genetic mutations in association with VACTERL.

Introduction

VACTERL association (OMIM#192350) is a rare, non-random co-occurrence of at least three of the following congenital malformations: vertebral defects (V), anorectal anomalies (A), cardiac defects (C), trachea-oesophageal fistula with or without oesophageal atresia (TE), renal anomalies (R) and limbs defects (L) [1]. The prevalence of VACTERL association among infants ranges from 1/10,000 to 1/40,000 births with a male predominance [1]. Until now, the aetiology of the VACTERL is not fully understood thus making the diagnosis rather challenging. Currently, VACTERL is diagnosed based on clinical manifestations of the above malformations after exclusion of other syndromes [2]. Heterogeneous phenotypes of VACTERL could lead to overlapping defects with other diseases such as CHARGE, Feingold, McKusick-Kaufman, Pallister-Jall, Alagille, and Fanconi Anemia [1]. Although the clinical diagnosis of VACTERL is mostly definite, molecular diagnosis may assist the clinicians for disease prognostication and management. Currently, genetic counselling for families with VACTERL is based on the existing knowledge; however it is still unsatisfactory. Exploratory genetic and environmental studies will allow for personalised treatment to improve the survival rates of children with multiple congenital anomalies.

Previous studies have shown that about 80% of VACTERL association cases are due to sporadic mutations during embryogenesis, whereas 20% of them are due to genetic inheritance via autosomal recessive or X-linked recessive traits [3]. Several genetic mutations have been identified in VACTERL

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association or VACTERL-like association including *HOXD13* [4], *ZIC3* (X-linked) [5], *PTEN* [6], *FANCB* [7], *FOXF1* [8] *PCSK5* [9] and *TRAP1* [10] genes. In the experimental mouse model of VACTERL, the sonic hedgehog (SHH) signalling pathway has been identified to be implicated in VACTERL pathogenesis, particularly with the loss of function in *SHH* and *GLI* genes [11]. Similarly, the intraflagellar transport (IFT) pathway has also been linked to the VACTERL pathogenesis [12], as the IFT pathway is needed for mammalian SHH signal transduction [13]. In addition, mitochondrial dysfunction including complex IV deficiency and A3243G mitochondrial DNA mutation has also been associated with VACTERL association [14], thus suggesting the heterogeneity in VACTERL causative candidate genes. Therefore, determination of these genetic factors and their consequences are important to add further information on the aetiology and possibly identify the molecular mechanisms involved in the pathogenesis of the VACTERL association.

Previously, genetic screening studies have been limited to the identification of candidate genes only. With the advent of next-generation sequencing, screening of the whole genome can be done in a more cost-effective manner. One such technique is whole exome sequencing (WES), which is a powerful tool in the investigation of disease-causing-mutations at the genome-wide level and has uncovered novel mutations associated with various rare diseases [15]. Here, we report the genetic data of an infant girl who was diagnosed with VACTERL association via the trio genetic analysis using the whole exome sequencing (WES) platform. We aimed to add to the knowledge of the pathogenesis of VACTERL association..

Materials and methods

Subject and clinical findings

An infant girl was delivered prematurely (Gestation age = 30 weeks) by emergency Caesarean section due to breech presentation and multiple congenital anomalies, with a birth weight of 1.48 kg. The patient's family history was without known inborn abnormalities. She was the first child of a non-consanguineous marriage between two phenotypically normal individuals. Her mother and father were 33 and 39 years old respectively when she was born. Her mother was diagnosed with Type 2 Diabetes Mellitus when she was 25 years old. At birth, the patient was diagnosed with VACTERL association. Anomalies observed were; 1) sacral agenesis with no presence of spina bifilda, 2) high type of anorectal anomaly, 3) situs ambiguous with single ventricle, unrestricted pulmonary blood flow and patent ductus arteriosus (PDA) and 4) right thumb syndactyly, rocker bottom feet with overlapping toes and right congenital talipes equinovarus (CTEV). There were no tracheoesophageal fistula, oesophageal atresia or renal anomalies. At 2 days of life, the patient underwent a left transverse loop colostomy for her anorectal anomaly. When the patient was treated at the neonatal intensive care unit (NICU), she developed heart failure, and was treated with frusemide, spironolactone and captopril. She also had sepsis and was treated with antibiotics but later developed disseminated intravascular coagulation. The patient died at 27 days of life due to the severe sepsis with underlying multiple congenital anomalies.

Cytogenetic analysis

Blood was sampled from the patient to culture the lymphocytes according to standard protocol. Giemsa-banded chromosomes from 20 metaphases were performed. Further investigation using fluorescence *in situ* hybridisation (FISH) was performed using probes for 22q11.2 (N25 and TUPLE1, DiGeorge/velocardiofacial syndrome critical region) and chromosomes 13 and 18 (Vysis Abbott Molecular Inc., USA).

DNA isolation, library construction, and exome sequencing

Following genetic counselling, the parents provided written informed consent for exome sequencing. About 3ml of peripheral blood was taken from the patient and both of her parents to extract the genomic DNA using salt extraction method. The DNA quality and purity were assessed using agarose gel electrophoresis and NanoDrop unit (Thermo Fisher Scientific, USA) respectively. DNA concentration was measured using a Qubit dsDNA BR Assay Kit (Thermo Fisher Scientific, USA). DNA libraries were prepared using an Ion AmpliSeq™ Exome RDY Kit (Thermo Fisher Scientific, USA) according to the manufacturer's instructions. Briefly, 100 ng of genomic DNA was mixed with the 5X Ion AmpliSeq™ HiFi Mix and loaded into the Ion AmpliSeq™ Exome RDY plate for library amplification and barcoding according to manufacturer's recommendation. Amplified libraries were confirmed for their quality and quantity using the High Sensitivity DNA kit (Agilent Technologies, USA) on Bioanalyzer (Agilent Technologies, USA). The libraries were then sequenced using the Ion Proton™ System (Thermo Fisher Scientific, USA) according to the manufacturer's recommendations.

Bioinformatics data analysis

Read mapping and variant calling was performed using Ion TorrentSuite™ v5.0.4 software (Thermo Fisher Scientific, USA) using the default parameters. The reads were aligned to the human reference genome hg19, followed by variant calling using TorrentSuite™ Variant Caller v5.0.13. Next, variants with single nucleotide polymorphism (SNP) quality ≤ 30 were filtered out using SnpSift [16] followed by annotation with ANNOVAR [17]. Only non-synonymous variants in coding regions (exonic, splicing) with read depth > 5X were retained for further analysis. To identify the disease-causing mutations, polymorphisms with the allele frequencies > 0.01 reported in the 1000 Genomes Project, NHLBI Exome Sequencing Project (ESP) Exome Variant Server, Exome Aggregation Consortium (ExAC), Complete Genomics (cg69) and maximum population frequency were filtered out. Subsequently, the putative disease-causing mutations were identified by filtering based on *de novo*, autosomal recessive, X-linked recessive and di-genic inheritance traits. Variants that fulfilled the above criteria were manually inspected using Integrative Genomics Viewer (IGV) to filter out false positive variants [18, 19] and further validated with exome data of 50 healthy controls to confirm the presence of the mutations. The effects of the confirmed variants were predicted using SIFT [20], PolyPhen-2[21], Mutation Taster [22], FATHMM [23], CADD [24], PROVEAN [25], and DANN [26]. Candidate mutations which were predicted to be deleterious by one of the above tools were further studied through searching literature databases. Identification of the implicated pathway was performed using the pathway enrichment analysis based on the Kyoto Encyclopaedia of Genes and Genomes (KEGG) database [27] and also other pathway databases including PANTHER [28] and PathCards [29].

Results

Cytogenetic analysis

For the patient, giemsa-banded chromosomes from 20 metaphases showed a normal female karyotype (46, XX). Analysis of 200 nuclei and metaphases showed no deletion of the N25 and TUPLE1 regions and no evidence of mosaicism for trisomy13 and 18 (Figure 1).

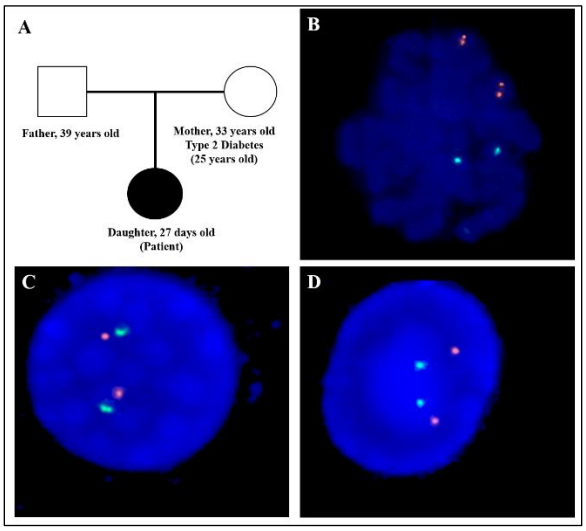

Figure 1: (A) Family pedigree of the patient. Open symbols, unaffected parents; closed symbol, affected patient. Normal fluorescence in-situ hybridisation (FISH) results of locus specific (LSI) probes for (B) chromosome 13 (orange) and 18 (green), and chromosome 21, (C) TUPLE1 (orange) and (D) N25 (orange) with arylsulfatase A (green, control) in affected patient.

Variants detected via whole exome sequencing (WES)

About 39 million reads were mapped to the hg19 genome base for all the samples, with an average of 83.98% of bases covered at > 20X coverage (Table 1). The lowest mean depth of coverage was 97.1X and the highest was 127.9X (Table 1). The average uniformity across the samples was 82.09% (Table 1).

Table 1. Descriptive findings of the summary for whole exome sequencing of the patient and her unaffected parents.

Summary of the whole exome sequencing parameters for the patient and her unaffected parents.

Parameter	Patient	Father	Mother
Mapped reads	39114273	45956362	34359247
Percentage of on-target (%)	95.90	92.48	92.98
Mean depth coverage	116.7X	127.9X	97.1X
20X coverage (%)	92.12	83.79	76.03
Uniformity (%)	90.45	79.16	76.65
No. of total variants identified	41500	41802	37602
No. of total variants with quality score ≥ 30	31035	33652	29303

Heritable mutations in VACTERL association

To identify the heritable mutations associated with VACTERL association, the list of mutations was filtered based on autosomal recessive and X-linked recessive traits. Table 2 shows the mutations identified based on di-genic inheritance, autosomal recessive and de-novo mutations in the patient and her parents. Based on the inheritance traits, we identified three heterozygous mutations, in which one was inherited from the father affecting Tenascin XB (*TNXB*) gene. However, this *TNXB* gene is not reported previously to be associated with VACTERL association. Interestingly, the patient inherited two heterozygous mutations affecting genes that are involved in the SHH pathway (Table 2 and Figure 2), i.e., *KIF27* (ENST00000297814: c.3004A>C:p.N1002H) and *GNAS* (ENST00000371098: c.205C>A:p.H69N) from her father and mother respectively, which were inherited via autosomal recessive trait. These mutations were predicted to be deleterious by SIFT, Polyphen-2, MutationTaster, FATHMM, CADD, PROVEAN or DANN. Other genetic mutations inherited from her father and mother are listed in Tables 3 and 4, respectively.

Table 2. List of the homozygous and compound heterozygous mutations detected in this study.

Descriptive summary of the selected homozygous, compound heterozygous and heterozygous mutations identified in the study. Homo, homozygous, CompHet, compound heterozygous, Het, heterozygous mutation.

Mutation	Mutation Type	Patient	Father	Mother
Inheritance				
TNXB: ENST00000375247: c.2882A>G:p.Q961R	Het	AG	AG	AA
KIF27: ENST00000297814: c.3004A>C:p.N1002H	Het	AC	AC	AA
GNAS: ENST00000371098: c.205C>A:p.H69N	Het	CA	CC	CA
***De novo* mutations**				
FLG: ENST00000368799: c.6603_6604CA	Het	CT/AG	TG_ HOM	CC/A G
DIEXF: ENST00000491415: c.508 A>G:p.T170A	Het	AG	AA	AA
SEPN1: ENST00000354177: c.505 A>G:p.T169A	Het	AG	AA	AA
CTBS: ENST00000465118: c.29_30TT	Het	AA/AC	GA/C C	GA/C A
SORBS1: ENST00000371247: c.3557C>G:p.P1186R	Het	GC	GG	GG
IFT140: ENST00000426508: c.683C >G:p.S228C	Homo	CC	GG	GC
PRR25: ENST00000301698: c.1175C>T:p.A392V	Het	CT	CC	CC
SUPT5H: ENST00000599117: c.698C>T:p.T233I	Het	CT	CC	CC
ACSS2: ENST00000360596: c.814A>G:p.R272G	Het	AG	AA	AA
MAP3K1: ENST00000399503: c.917G>A:p.R306H	Homo	AA	GG	GA
ZNF425: ENSG00000204947: c.146-1G>C	Het	GC	GG	GG
Autosomal Recessive (*De novo*)				
SH2B1: ENST00000322610: c.853C>T:p.P285S	Homo	TT	CT	CT
KRT33A: ENST00000007735: c.440C>A:p.T147N	Het	GT	GG	TT

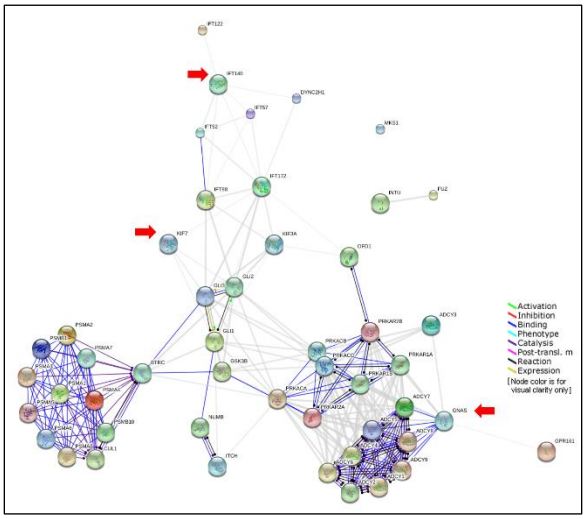

Figure 2: Off-state of the sonic hedgehog signalling (SHH) pathway generated by PathCards database showing the interaction between the KIF7 (paralog to KIF27), GNAS and IFT140 (red arrow pointed). SHH pathway has been shown to be implicated in VACTERL and VACTERL-like association.

De novo mutations in VACTERL

A total of 14 *de novo* mutations were identified in the patient (Table 2). Among them, one missense mutation was identified in the intraflagellar transport 140 (*IFT140*) gene (ENST00000426508: c.683C>G:p.S228C), a homozygous mutation that was also predicted to be deleterious (Figure 2). Other *de novo* genetic mutations were also identified in the patient (Table 2). None of these genes are associated with VACTERL or VACTERL-like phenotypes

Table 3. List of the other compound heterozygous and heterozygous mutations inherited from the father

Descriptive summary of the identified other compound heterozygous and heterozygous mutations inherited from the father. CompHet, compound heterozygous, Het, heterozygous mutation.

Mutation	Mutation Type	Patient	Father	Mother
ADAMTS18: ENST00000449265:c.406 C > G:p.Q136E	Het	GC	GC	GG
ADGRB1: ENST00000521208:c.3122 C > T:p.T1041M	Het	CT	CT	CC
AHNAK2: ENST00000333244:c.16252 G > C:p.A5418P	Comp Het	CG	CG	CC
AHNAK2: ENST00000333244:c.14839 G > A:p.E4947K	Comp Het	CT	CT	CC
ANKRD12: ENST00000262126:c.1720 A > G:p.M574V	Het	AG	AG	AA
ARHGEF28: ENST00000545377:c.4071 G >C:p.E1357D	Het	GC	GC	GG
ASAP3: ENST00000336689:c.908 C > T:p.T303M	Het	GA	GA	GG
BACH1: ENST00000399921:c.2033 c > G:p.A678G	Het	CG	CG	CC
BAHD1: ENST00000561234:c.33 G > A:p.M11I	Het	GA	GA	GG
BCKDHA: ENST00000540732:C.1262 G > A:p.R421H	Het	GA	GA	GG
BIRC6: ENST00000421745:c.14494 A > G:p.T4832A	Het	AG	AG	AA
BTN2A1: ENST00000312541:c.1406 G > A:p.R469K	Het	GA	GA	GG
BTN2A2: ENST00000416795:c.1201 C > G:p.H401D	Het	CG	CG	CC
BTN3A3: ENST00000339789:c.1154 C > T:p.P385L	Het	CT	CT	CC
C6orf106: ENST00000374023:c.874 C > G:p.P292A	Het	GC	GC	GG
CAPN5: ENST00000529629:c.349 T > G:p.Y117D	Het	TG	TG	TT
CASP7: ENST00000369321:c.200 C > A:p.S67Y	Het	CA	CA	CC
CD248: ENST00000311330:c.1235 C > G:p.P412R	Het	GC	GC	GG
CDK11A/B: ENST00000479362:c.302 A > G:p.K101R	Het	TC	TC	TT
CLEC1A:ENST00000414501:c.171 G > C:p.L57F	Het	CG	CG	CC
CNTNAP2: ENST00000361727:c.3106 G > A:p.A1036T	Het	GA	GA	GG
COA4: ENST00000537289:c.20 A > G:p.Q7R	Het	TC	TC	TT
COL2A1: ENST00000380518:c.196 G > A:p.D66N	Het	CT	CT	CC
COQ4: ENST00000609948:c.134 C > G:p.S45C	Het	CG	CG	CC
CTNNAL1: ENST00000374595:c.983 G > A:p.R328H	Het	CT	CT	CC
CXCL16: ENST00000574412:c.218 T > A:p.F73Y	Het	AT	AT	AA
DAG1: ENST00000545947:c.220 G > A:p.V74I	Het	GA	GA	GG
DBNDD2: ENST00000372723:c.455 A > G:p.D152G	Het	AG	AG	AA
DCAF13: ENST00000519682:c.626 T > C:p.F209S	Het	TC	TC	TT
DDX60L: ENST00000511577:c.2140 G > A:p.G714R	Het	CT	CT	CC
DHRS1: ENST00000558340:c.22 C > T:p.Q8X	Het	GA	GA	GG
DIEXF: ENST00000491415:c.1601 T > A:p.F534Y	Het	TA	TA	TT
DNAH5: ENST00000265104:c.7452 C > A:p.F2484L	Het	GT	GT	GG
DPCR1: ENST00000462446:c.869 G > A:p.G290E	Het	GA	GA	GG
DUOXA1: ENST00000558422:c.77 G > A:p.S26N	Het	CT	CT	CC
ELFN1: ENST00000424383:c.23 C > T:p.A8V	Het	CT	CT	CC
EMC1: ENST00000477853:c.1862 G > A:p.R621H	Het	CT	CT	CC
ESRRA: ENST00000468670:c.22 A > T:p.I8F	Het	AT	AT	AA
FAM124A: ENST00000280057:c.1471 G > C:p.A491P	Het	GC	GC	GG
FAT2: ENST00000261800:c.9805 C> T:p.R3269C	Het	GA	GA	GG
FHOD1: ENST00000258201:c.3130 C > T:p.R1044W	Het	GA	GA	GG
FKBP10: ENST00000489591:c.274 C > G:p.L92V	Het	CG	CG	CC
FOXP1: ENST00000493089:c.160 G > A:p.A54T	Het	CT	CT	CC
FXR1: ENST00000305586:c.1484 T > A:p.I495K	Het	TA	TA	TT
GDPD5: ENST00000529721:c.1814 G > A:p.R605H	Het	CT	CT	CC
GPATCH8: ENST00000434000:c.3934 G > A:p.A1312T	Het	CT	CT	CC
HLAC: ENST00000383329:c.595 G > A:p.G199R	Het	CT	CT	CC
HLADMA: ENST00000456800:c.708 C > A:p.H236Q	Het	GT	GT	GG
HYDIN: ENST00000393567:c.13925 G > A:p.R4642H	Het	CT	CT	CC
IGSF10: ENST00000282466:c.3104 G > C:p.R1035T	Het	CG	CG	CC
IGSF9B: ENST00000533871:c.3530 C > T:p.P1177L	Het	GA	GA	GG
IRX6: ENST00000290552:c.1147 G > C:p.G383R	Het	GC	GC	GG
ISM1: ENST00000262487:c.476 G > A:p.R159Q	Het	GA	GA	GG
KAZN: ENST00000376030:c.1566 G > T:p.E522D	Het	GT	GT	GG
KDM3B: ENST00000314358:c.3604 A > C:p.N1202H	Het	AC	AC	AA
KIAA1614: ENST00000367588:c.3455 A > T:p.N1152I	Het	AT	AT	AA
KLB: ENST00000257408:c.2491 G > T:p.V831L	Het	GT	GT	GG
KMT2D: ENST00000301067:c.10009 C > A:p.H3337N	Het	GT	GT	GG
LAG3: ENST00000203629:c.1333 C > T:p.L445F	Het	CT	CT	CC
LAMA3: ENST00000399516:c.2402 G > T:p.G801V	Het	GT	GT	GG
LDAH: ENST00000440866:c.272 A > T:p.D91V	Het	TA	TA	TT
LGI3: ENST00000517694:c.604 T > C:p.Y202H	Het	AG	AG	AA
LOC81691: ENST00000568894:c.133 G > A:p.A45T	Het	GA	GA	GG
LRFN1: ENST00000248668:c.1051 G > C:p.G351R	Het	CG	CG	CC
LRRC43: ENST00000425921:c.1119 C > G:p.D373E	Het	CG	CG	CC
MAST4: ENST00000404260:c.5840 A > C:p.N1947T	Het	AC	AC	AA
MCM3AP: ENST00000397708:c.507 C > A:p.S169R	Het	GT	GT	GG
MRPS5: ENST00000272418:c.1157 C > T:p.S386F	Het	GA	GA	GG
MS4A63: ENST00000532756:c.106 G > C:p.A36P	Het	GC	GC	GG
MTFMT: ENST00000560717:c.44 G > C:p.G15A	Het	CG	CG	CC
NBEAL1: ENST00000449802:c.1211 C > T:p.T404I	Het	CT	CT	CC
NLRP12: ENST00000535162:c.629 C > T:p.P210L	Het	GA	GA	GG
NRN1L: ENST00000339176:c.32 G > C:p.C11S	Het	GC	GC	GG
NUP85: ENST00000245544:c.1823 C > T:p.T608M	Het	CT	CT	CC
OR5B2: ENST00000302581:c.319 A > G:p.T107A	Het	TC	TC	TT
PAX1: ENST00000444366:c.185delC:p.S62fs	Het	DEL	DEL	NO DEL
PCDHA: ENST00000532602:c.A2713 A > G:p.N905D	Het	AG	AG	AA
PCDHA6: ENST00000529310:c.2027 C > T:p.P676L	Het	CT	CT	CC
PEX16: ENST00000378750c.187 G > A:p.G63R	Het	CT	CT	CC
PKHD1L1: ENST00000378402:c.2867 C > A:p.A956D	Het	CA	CA	CC
PLCE1: ENST00000260766:c.6691 T > C:p.F2231L	Het	TC	TC	TT
PLEKHA5: ENST00000429027:c.2077 A > G:p.M693V	Het	AG	AG	AA
PLEKHG3: ENST00000394691:c.1337 G > A:p.R446Q	Het	GA	GA	GG
PON3: ENST00000451904:c.289 G > A:p.A97T	Het	CT	CT	CC
PRRC2A: ENST00000376033:c.4316 G > A:p.R1439Q	Het	GA	GA	GG
PRUNE2: ENST00000428286:c.2438 T > C:p.L813P	Het	AG	AG	AA
PYGM: ENST00000164139:c.2290 A > G:p.N764D	Het	TC	TC	TT
RAB3GAP2: ENST00000358951:c.1660 A > G:p.M554V	Het	TC	TC	TT
RAD52: ENST00000541619:c.34 C > T:p.R12C	Het	GA	GA	GG
RASL10A: ENST00000401450:c.208 G > A:p.G70R	Het	CT	CT	CC
RBFOX2: ENST00000438146:c.473 G > T:p.G158V	Het	CA	CA	CC
RBM47: ENST00000515053:c.366 C > G:p.H122Q	Het	GC	GC	GG
REP15: ENST00000310791:c.624 T > G:p.C208W	Het	TG	TG	TT
RRAGA: ENST00000380527:c.702 C > G:p.I234M	Het	CG	CG	CC
SAG: ENST00000409110:c.1159 C > A:p.L387M	Het	CA	CA	CC
SCNN1D: ENST00000379116:c.661 C > T:p.R221W	Het	CT	CT	CC
SEMA5A: ENST00000382496:c.1568 C > T:p.T523M	Het	GA	GA	GG
SFTPA1: ENST00000419470:c.698 G > T:p.G233V	Het	GT	GT	GG
SH3BP2: ENST00000511747:c.914 C > T:p.P305L	Het	CT	CT	CC
SKIV2L2: ENST00000230640:c.2102 G > A:p.R701H	Het	GA	GA	GG
SLC20A2: ENST00000520262:c.787 G > A:p.V263I	Het	CT	CT	CC
SOGA1: ENST00000357779:c.3214 C > T:p.R1072W	Het	GA	GA	GG
SPANXC/D:ENST00000370515:exon2:c.G253A:p.E85K	Het	CT	CT	CC
SPIDR: ENST00000518074:c.1574 G > A:p.R525H	Het	GA	GA	GG
SPZ1: ENST00000296739:c.1000 A > G:p.R334G	Het	AG	AG	AA
SSFA2: ENST00000431877:c.673 C > G:p.Q225E	Het	CG	CG	CC
ST6GALNAC4: ENST00000335791:c.491 G > A:p.R164H	Het	CT	CT	CC
STRIP2: ENST00000435494:c.367 C > T:p.R123W	Het	CT	CT	CC
SYNGR1: ENST00000381535:c.62 A > G:p.Q21R	Het	AG	AG	AA
TAF1L: ENST00000242310:c.944 A > G:p.Y315C	Het	TC	TC	TT
TKTL2: ENST00000280605:c.70 C > T:p.R24W	Het	GA	GA	GG
TMPRSS9: ENST00000332578:c.3020 G > A:p.G1007D	Het	GA	GA	GG
TPRKB: ENST00000409716:c.362 A > G:p.N121S	Het	TC	TT	TC
TRIM47: ENST00000587339:c.115 G > A:p.A39T	Het	CT	CT	CC
TSSC4: ENST00000437110:c.151 C > A:p.P51T	Het	CA	CA	CC
TTC7A: ENST00000409245:c.335 G > A:p.R112Q	Het	GA	GA	GG
USP17L2: ENST00000333796:c.1238 C > T:p.P413L	Het	GA	GA	GG
USP42: ENST00000306177:c.1660 C > A:p.P554T	Het	CA	CA	CC
WASF1: ENST00000392589:c.1048 C > T:p.P350S	Het	AA	AA	GG
WDPCP: ENST00000409562:c.1310 G > T:p.S437I	Het	CA	CA	CC
ZNF573: ENST00000339503:c.475 A > G:p.T159A	Het	TC	TC	TT
ZNF740: ENST00000416904:c.550 T > G:p.S184A	Het	TG	TG	TT
ZNF766: ENST00000439461:c.1351 A > C:p.S451R	Het	AC	AC	AA
ZNF865: ENST00000568956:c.2297 G > T:p.G766V	Het	GT	GT	GG

Table 4. List of the other compound heterozygous and heterozygous mutations inherited from the mother

Descriptive summary of the identified other compound heterozygous and heterozygous mutations inherited from the mother. Homo, homozygous, CompHet, compound heterozygous, Het, heterozygous mutation.

Mutation	Mutation Type	Patient	Father	Mother
ABCA7: ENST00000263094:c.1193 G > A:p.G398D	Het	GA	GG	GA
ADAMTS7: ENST00000388820:c.2731 G > A:p.V911M	Het	CT	CC	CT
ADGRL2: ENST00000370725:c.4183_4197del:p.1395_1399del	Hom	DEL	NO DEL	DEL
AKAP6: ENST00000557354:exon4:c.A1406G:p.N469S	Het	AG	AA	AG
AMZ1: ENST00000312371:c.1226 G > A:p.R409Q	Het	GA	GG	GA
ARRB2: ENST00000575877:c.788 G > A:p.R263H	Het	GA	GG	GA
BCL7A: ENST00000538010:c.428 C > T:p.P143L	Het	CT	CC	CT
C8orf34A: ENST00000512294:c.76 G > A:p.G26R	Het	CT	CC	CT
CACNA1G: ENST00000416767:c.4630 G > A:p.V1544I	Het	GA	GG	GA
CANT1: ENST00000591773:c.205 C > T:p.P69S	Homo	AA	GG	AA
CARD6: ENST00000254691:c.943 T > C:p.C315R	Het	TC	TT	TC
CCDC102E: ENST00000584775:c.220 C > T:p.R74C	Het	CT	CC	CT
CD163L1: ENST00000416109:c.1828 T > C:p.F610L	Het	AG	AA	AG
CDY1: ENST00000328908:c.887 A > G:p.N296S	Het	AG	AA	AG
CERS4: ENST00000558331:c.791 T > G:p.L264R	Het	TG	TT	TG
CES2: ENST00000417689:c.343 G > A:p.A115T	Het	GA	GG	GA
CFAP61: ENST00000245957:c.1666 G > A:p.G556R	Comp Het	GA	GG	GA
CFAP61: ENST00000245957:c.3328 G > A:p.A1110T	Comp Het	GA	GG	GA
CHPF2: ENST00000495645:c.341 G > T:p.R114L	Het	GT	GG	GT
COLQ: ENST00000603808:c.476 G > C:p.G159A	Het	CG	CC	CG
CPAMD8: ENST00000443236:c.4402 G > A:p.V1468M	Het	CT	CC	CT
CTNNA3: ENST00000433211:c.1231 A > G:p.I411V	Het	TC	TT	TC
CUBN: ENST00000377833:c.10215 C> A:p.N3405K	Het	GT	GG	GT
DAGLA: ENST00000257215:c.2210 C > T:p.S737L	Het	CT	CC	CT
DDOST: ENST00000602624:c.815 A > G:p.Y272C	Het	TC	TT	TC
DNAH10: ENST00000409039:c.4897 G > A:p.A1633T	Het	GA	GG	GA
EGLN1: ENST00000366641:c.832 G > A:p.D278N	Het	CT	CC	CT
ENTPD7: ENST00000370489:c.1541 C > T:p.T514M	Het	CT	CC	CT
ERP29: ENST00000261735:c.205 A > C:p.K69Q	Het	AC	AA	AC
FCAMR: ENST00000400962:c.445 A > G:p.R149G	Het	TC	TT	TC
FCMR: ENST00000367091:c.1128 G > C:p.M376I	Het	CG	CC	CG
FFAR2: ENST00000599180:c.299 T > C:p.L100P	Het	TC	TT	TC
GAL: ENST00000265643:c.178 C > T:p.L60F	Het	CT	CC	CT
GAPVD1: ENST00000495955:c.2102 C > T:p.P701L	Het	CT	CC	CT
GLIS1: ENST00000312233:c.172 C > T:p.P58S	Het	GA	GG	GA
GPR37L1: ENST00000367282:c.194 C > T:p.A65V	Het	CT	CC	CT
HIST3H3: ENST00000366696:c.47 C > T:p.A16V	Het	GA	GG	GA
HIVEP1: ENST00000379388:c.5858 T > C:p.L1953S	Het	TC	TT	TC
IRS2: ENST00000375856:c.2657 G > T:p.R886L	Het	CA	CC	CA
ITGA11: ENST00000423218:c.2654 A > G:p.E885G	Het	TC	TT	TC
KIAA2026: ENST00000399933:c.1517 G > A:p.R506Q	Het	CT	CC	CT
KIR2DL3: ENST00000434419:c.197 T > A:p.F66Y	Het	TA	TT	TA
KLHL14: ENST00000359358:c.961 A > G:p.K321E	Het	TC	TT	TC
KLHL42: ENST00000539176:c.958 G > A:p.E320K	Het	GA	GG	GA
KRT6A: ENST00000330722:c.701 G > A:p.R234H	Het	CT	CC	CT
KRTAP5-1: ENST00000382171:c.656 G > A:p.S219N	Het	CT	CC	CT
LIMCH1: ENST00000513024:c.20 A > G:p.D7G	Het	AG	AA	AG
LINC01619: ENST00000549802:c.187 G > A:p.V63M	Het	CT	CC	CT
LRRIQ4: ENST00000340806:c.223 G > T:p.V75F	Het	GT	GG	GT
MAP2: ENST00000360351:c.4912 G > A:p.V1638M	Het	GA	GG	GA
MARK3: ENST00000416682:c.1729 C > T:p.R577C	Het	CT	CC	CT
MUC16: ENST00000397910:c.35080 C> T:p.P11694S	Comp Het	GA	GG	GA
MUC16: ENST00000397910:c.35060 T > C:p.M11687T	Comp Het	AG	AA	AG
MUC16: ENST00000397910:c.30964 G > A:p.E10322K	Comp Het	CT	CC	CT
MUC16: ENST00000397910:c.30274 G > A:p.D10092N	Comp Het	CT	CC	CT
MUC16: ENST00000397910:c.24941 G > A:p.S8314N	Comp Het	CT	CC	CT
MUC22: ENST00000561890:c.4840 G > A:p.V1614I	Het	GA	GG	GA
MUM1: ENST00000344663:c.869 C > G:p.S290W	Het	CG	CC	CG
MYOM3: ENST00000374434:c.1232 G > A:p.R411Q	Het	CT	CC	CT
NDUFS8: ENST00000453471:c.4 C > T:p.R2C	Het	CT	CC	CT
NEUROD1: ENST00000295108:c.751 G > T:p.A251S	Het	CA	CC	CA
NEXN: ENST00000334785:c.512 T > C:p.I171T	Het	TC	TT	TC
NFATC1: ENST00000592223:c.815 C > T:p.P272L	Het	CT	CC	CT
NKAPL: ENST00000343684:c.176 A > C:p.D59A	Het	AC	AA	AC
NLRP7: ENST00000588756:c.251 G > A:p.C84Y	Het	CT	CC	CT
OBSCN: ENST00000570156:c.20155 G > A:p.D6719N	Het	GA	GG	GA
OR4K2: ENST00000298642:c.451 A > G:p.M151V	Het	AG	AA	AG
PCDHA4: ENST00000530339:c.214 G > C:p.G72R	Comp Het	GC	GG	GC
PCDHA4: ENST00000530339:c.218 G > A:p.G73D	Comp Het	GA	GG	GA
PCDHGA10: ENST00000398610:c.1897 G > A:p.A633T	Het	GA	GG	GA
PCDHGA11: ENST00000518882:c.1382 A > G:p.Y461C	Het	AG	AA	AG
PCM1: ENST00000519253:c.3520 A > G:p.T1174A	Het	AG	AA	AG
PDCD4: ENST00000393104:c.502 G > A:p.G168R	Het	GA	GG	GA
PEX26: ENST00000329627:c.427 G > A:p.A143T	Het	GA	GG	GA
PHLPP1: ENST00000262719:c.1184 G > T:p.R395L	Het	GT	GG	GT
PIGV: ENST00000449950:c.499 C > G:p.L167V	Het	CG	CC	CG
PKD1: ENST00000423118:c.8611 G > A:p.A2871T	Het	CT	CC	CT
PKHD1: ENST00000371117:c.5959 G > A:p.A1987T	Het	CT	CC	CT
PLEKHA4: ENST00000263265:c.1712 G > A:p.R571H	Het	CT	CC	CT
PLEKHG1: ENST00000367328:c.2329 T > G:p.C777G	Het	TG	TT	TG
PREX1: ENST00000396220:c.1169 A > T:p.E390V	Het	TA	TT	TA
PRSS3: ENST00000457896:c.194 T > A:p.I65N	Comp Het	TA	TT	TA
PRSS3: ENST00000457896:c.198 C > G:p.S66R	Comp Het	CG	CC	CG
PRSS3: ENST00000457896:c.202 C > T:p.Q68X	Comp Het	CT	CC	CT
PRX: ENST00000324001:c.641 C > G:p.P214R	Het	GC	GG	GC
PTPN13: ENST00000436978:c.5018 C > A:p.A1673E	Het	CA	CC	CA
RHAG: ENST00000371175:c.64 T > G:p.L22V	Het	AC	AA	AC
RHBDF2: ENST00000592123:c.325 C > T:p.R109C	Het	GA	GG	GA
RPL13: ENST00000452368:c.359 G > A:p.R120Q	Het	GA	GG	GA
RPL13A: ENST00000391857:c.302 G > A:p.R101H	Het	GA	GG	GA
RPL3L: ENST00000268661:c.745 C > T:p.R249C	Het	GA	GG	GA
RPTN: ENST00000316073:c.460 A > G:p.R154G	Het	TC	TT	TC
RSPO1: ENST00000401068:c.512 G > C:p.G171A	Het	CG	CC	CG
SAP18: ENST00000450573:c.16 G > A:p.A6T	Het	GA	GG	GA
SGK494: ENST00000584196:c.188 A > G:p.Y63C	Het	CC	TT	CC
SIM2: ENST00000430056:c.232 A > G:p.K78E	Het	AG	AA	AG
SLC24A4: ENST00000531433:c.502 G > A:p.V168I	Het	GA	GG	GA
SLC25A15: ENST00000338625:c.147 C > G:p.D49E	Het	CG	CC	CG
SP100: ENST00000264052:c.2242 G > A:p.E748K	Het	GA	GG	GA
SPHK2: ENST00000600537:c.793 G > T:p.A265S	Het	GT	GG	GT
SSC5D: ENST00000587166:c.2116 C > T:p.R706X	Het	CT	CC	CT
SVEP1: ENST00000401783:c.5595 T > G:p.F1865L	Het	AC	AA	AC
TAS2R42: ENST00000334266:c.694 G > A:p.A232T	Het	CT	CC	CT
TEKT5: ENST00000283025:c.640 C > T:p.L214F	Het	GA	GG	GA
TEX15: ENST00000256246:c.6874 A > G:p.T2292A	Het	TC	TT	TC
THEMIS: ENST00000368250:c.1019 A > T:p.K340M	Het	TA	TT	TA
THSD4: ENST00000355327:c.1064 G > A:p.R355H	Het	GA	GG	GA
TMEM176A: ENST00000484928:c.455 G > A:p.R152H	Het	GA	GG	GA
TMEM219: ENST00000561899:c.688 C > T:p.R230C	Het	CT	CC	CT
TMEM74: ENST00000297459:c.164 T > C:p.M55T	Het	AG	AA	AG
TNKS1BP1: ENST00000528882:c.11 C > G:p.S4C	Het	GC	GG	GC
TONSL: ENST00000409379:c.3347 G > A:p.R1116H	Het	CT	CC	CT
TOR2A: ENST00000458505:c.98 T > C:p.L33P	Het	AG	AA	AG
TPR: ENST00000367478:c.5770 C > G:p.Q1924E	Het	GC	GG	GC
TRANK1: ENST00000429976:c.2324 C > T:p.T775M	Het	GA	GG	GA
UBASH3B: ENST00000284273:c.1049 G > A:p.R350Q	Het	GA	GG	GA
USP35: ENST00000529308:c.2351 C > T:p.S784L	Het	CT	CC	CT
ZC3HAV1: ENST00000464606:c.3046 A > G:p.T1016A	Het	TC	TT	TC
ZPBP: ENST00000419417:c.346 G > A:p.A116T	Het	CT	CC	CT

Discussion

In the present study, we have identified several possible mutations that may contribute to the VACTERL association in an infant girl. These included two inherited heterozygous mutations in *KIF27* and *GNAS* genes, as well as the one *de novo* missense mutation of the *IFT140* gene, in which these affected genes are involved in the SHH pathway. *KIF27* and *GNAS* gene mutations are inherited via the autosomal recessive pattern. From the trio WES analysis, we identified a mutation of the *KIF27* gene (ENST00000297814: c.3004A>C:p.N1002H) which is inherited from the father, and a mutation in the *GNAS* gene (ENST00000371098: c.205C>A:p.H69N) which is inherited from the mother. These mutations could be causative of the VACTERL association seen in the patient. To the best of our knowledge, we are the first to describe these two genetic mutations in association with the VACTERL phenotype. Currently, the genetic aetiology of VACTERL association is not well described as its phenotypes are too heterogeneous [1]. Thus, until now, there is no specific genetic marker to diagnose VACTERL association and also to help identify the carriers. Therefore, our findings of these possible genetic mutations in our Malaysian infant girl diagnosed with VACTERL association may provide a better understanding of the genetic architecture of VACTERL phenotypes and hence may offer the information needed for familial screening of VACTERL carriers and genetic counselling.

Two digenic inherited heterozygous mutations in our VACTERL patient are involved in SHH signalling. *KIF27* is a member of the kinesin 4 superfamily, and its paralog protein is *KIF7* [30]. Although no specific function was identified for KIF27 protein in VACTERL pathogenesis, both of KIF27 and KIF7 proteins are needed to fulfil the same role of a single Drosophila melanogaster of kinesin-like protein Costal-2 (*Cos2*); which is an important negative regulator in SHH signalling [30]. SHH signalling has been implicated as the key signal in developmental biology particularly in regulating the ventral neural tube, the anterior–posterior limb axis and the ventral somites formation [31]. Mice knockout of the *SHH* gene and the transcription factor, *GLI* genes, resulted in mutant mice that exhibited the similar VACTERL phenotypes [32]. Thus, suggesting the possible genetic and molecular pathogenesis of VACTERL association via *KIF27* mutation and SHH signalling. As for *GNAS* gene that encodes the α-subunit of the heterotrimeric stimulatory G protein (Gsα), this GNAS protein is responsible for molecular switching of the various peptide hormones binding the G-protein-coupled receptors (GPCR) [33]. Interestingly, a knockout mice study of gain- and loss-of-function of the *GNAS* gene revealed that Gsα protein inhibits SHH signalling via the cAMP-dependent pathway to regulate GLI3 processing and GLI2 activation [34]. Therefore, these findings may imply that any mutation in the *GNAS* gene can contribute to VACTERL and VACTERL-like phenotype via interacting with SHH signalling. However, to what extent these heterozygous inherited mutations in *KIF27* and *GNAS* genes in our patient caused the VACTERL or VACTERL-like phenotypes is unknown and would require more functional studies to evaluate the impact of the mutations in SHH and VACTERL development.

In the present study, we also identified a homozygous, *de novo* mutation that may also be associated with VACTERL or VACTERL-like association via SHH signalling. A missense mutation in the *IFT140* gene (ENST00000426508: c.683C>G:p.S228C) was identified in our patient. *IFT140* gene encodes a subunit of intraflagellar transport A (IFTA) complex that is responsible for the movement of molecules from the axon to the cell body or known as the retrograde transport in primary cilia [35]. Mutations in the genes that encode the components of IFTA complex can affect the skeleton development and maintenance [36], thus suggesting that IFTA complex play a role in VACTERL pathogenesis. Mutant mice that lacked IFTA complex expression showed a loss of SHH activity with severe disruptions of the cilia structure and membrane protein trafficking [37]. Another study of mutant mice with low expression of *IFT172* gene (another component of IFT) also showed that these mice developed VACTERL and VACTERL-like phenotypes (12), due to dysregulation of GLI activation and repression [13, 38]. However, how this *de novo* mutation in *IFT140* gene can cause VACTERL or VACTERL-like phenotypes in our patient is unknown and whether the mechanisms of IFT140 dysregulation contributing to the VACTERL phenotypes are similar to the IFT172 mode of action also needs further confirmation.

Our present study has a few limitations that needed to be addressed. One is that we cannot conclude that the three genetic mutations in association with VACTERL phenotypes were causative mutations. Functional studies will be required to identify which of those mutations (*KIF27*, *GNAS* and *IFT140*) contribute to the pathogenesis of VACTERL. We were also not able to validate the findings with other VACTERL subjects and using other sequencing platforms. This is particularly important as there are many possible technical challenges in WES analysis to discover accurate and true genetic mutations (false positive), and also to apply such mutations in the biologically

meaningful information or disease pathogenesis [39]. However, many other studies did show that the WES technique has high sensitivity in detecting true genetic variants and also has high replicability across different platforms [40], which suggests that the WES findings in this study warrant further investigation in VACTERL pathogenesis. Furthermore, the patient's mother has a history of diabetes mellitus, which is a risk factor for the development of VACTERL in the offspring [41, 42]. The exact mechanism of how diabetes mellitus contributes to the congenital malformations is unknown, though hyperglycemia, oxidative stress and mitochondrial dysfunction are thought to play a role in disturbing the certain key developmental pathways in the foetus [14, 42]. Even so, no strong evidence is reported to show a causal relationship between maternal diabetes and VACTERL development in these infants, as many of the VACTERL infants are not born to women with diabetes [42, 43]. Despite these limitations, the fact that those three genetic mutations in *KIF27*, *GNAS* and *IFT140* genes are associated with SHH signalling have provided additional knowledge and also the additional candidate genes involved in the pathogenesis of VACTERL and VACTERL-like association.

Conclusion

In the present study, we performed the trio-genetic analysis to discover the mutations of VACTERL association in our Malaysian infant patient together with her parents using the WES technique. We identified three mutations in *KIF27*, *GNAS* and *IFT140* genes that may be responsible for the VACTERL association, possibly via a disruption in SHH pathway. We also identified a *de novo* missense mutation in the *IFT140* gene which may also contribute to the molecular pathogenesis of VACTERL in our patient. This is the first time that these three genetic mutations are reported in association with VACTERL and VACTERL-like phenotypes. The identification of these genetic mutations may offer new knowledge for future studies to understand the molecular mechanisms for VACTERL pathogenesis and potentially to improve the diagnosis and genetic screening for VACTERL.

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Statement of Ethics

Informed consent was obtained from all subjects involved in this study for the WES and publication of the results. The test was performed under the molecular diagnostic services offered by the UKM Medical Molecular Biology Institute (UMBI) for those with rare diseases treated at the UKM Medical Centre.

Disclosure Statement

The authors declare that they have no competing interests.

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