1 INTRODUCTION

Robinow Syndrome (RS), firstly described by Robinow et al.,1 is a rare genetic disorder characterized by dysmorphic craniofacial features resembling a “fetal face,” mesomelic limb shortening, kyphoscoliosis, hemivertebrae, hypoplastic external genitalia, and renal anomalies.2 Both autosomal dominant and autosomal recessive patterns of inheritance have been reported. Among which, ROR2-related Robinow Syndrome (ARRS; MIM #268310) is the autosomal recessive form caused by pathogenic variations in the
ROR2 (MIM *602337) gene located at 9q22. Symptoms of ARRS are more severe than those of autosomal dominant RSs (ADRS; MIM #180700, #616331, and #616894) caused by WNT5A (MIM *164975), DVL1 (MIM *601365), or DVL3 (MIM *601368). So far, less than 200 cases of ARRS have been reported, mainly in consanguineous families, for example, those of Turkish, Omani, Egyptian origin.

The human ROR2 gene comprises nine exons and eight introns, including 226kb bases. Thus far, 26 mutations associated with ARRS have been reported in the ROR2 gene (http://101.200.211.232/skeletonogenetics/), most of which are stopgain or deletion variations in the 5-9 exons.

Herein, we described the clinical, anatomical, and molecular findings of a fetus with typical ARRS manifestation from a non-consanguineous couple with normal phenotype. Two heterozygous variants were identified including a novel one in the ROR2 gene. Moreover, the results of ROR2 protein expression detection were consistent with this finding, which confirmed the pathogenicity of this variation.

2 | MATERIALS AND METHODS

2.1 | Subjects

This study was approved by the Ethics Committee of Haidian Maternal and Child Healthcare Hospital (No. 2018-10), and informed consent was signed by the recruited couple.

A 39-year-old pregnant woman gravida 5 para 0 referred to the center of prenatal diagnosis in Haidian Maternal and Child Healthcare Hospital. She and her husband had been through 4 times of adverse pregnancies including twice spontaneous abortions at first trimester and twice pregnancies with multiple malformations terminated at 23 and 25 gestational weeks, respectively (Pedigree shown in Figure 1). Conventionally, amniocentesis was conducted at 19 weeks of gestation. Afterwards, ultrasonography revealed multiple malformations in the fetus at 22 weeks of gestation. Fetal stillbirth occurred at 24+2 weeks, thus, induced labor operation was conducted.

2.2 | Genetic analysis

Conventional karyotyping by G-banding and fluorescence in situ hybridization (FISH) were performed on amniotic fluid cells (AFCs) according to standard operation procedures to detect overall chromosomal anomalies. Chromosomal microarray analysis (CMA) with CytoScan 750K SNP Array (Affymetrix Inc) was conducted according to the manufacturer’s manual workflow on DNA extracted from AFCs, so that to investigate genomic copy number variants with clinical significance. Data were collected and analyzed by GeneChip Scanner 3000 with AGCC software.

After induced abortion, more DNA was extracted from umbilical cord sample and then used for WES detection. DNA fragments hybridization (by IDT’s XGen Exome Research Panel, Integrated DNA Technologies), library quality testing, and WES sequencing (using Novaseq6000 platform, Illumina) were performed as described in our previous study. Sanger sequencing was used to verify the variation of suspected pathogenicity. The conservation of specific amino acid in various species was obtained from NCBI blast (https://blast.ncbi.nlm.nih.gov/Blast.cgi). The pathogenicity index of specific variant was analyzed using Sorting Intolerant From Tolerant (SIFT) (http://sift.bii.a-star.edu.sg/) and Polymorphism Phenotyping V2 (http://genetics.bwh.harvard.edu/pph2/).

2.3 | Western blotting and IHC

The body of the fetus was sent for pathological examination. Then, the fetal skin tissue sample from the proband was used for WB testing, along with a ROR2 wild type sample at similar gestational age as normal control. Meanwhile, the paraffin sections of fetal auricular finger osteochondrocytes were subjected to IHC detection, along with a costal cartilage tissue sample from an aborted fetus (ROR2 wild type) at similar gestational age as normal control. Both of these experiments were performed with monoclonal anti-ROR2 antibody (Abcam; with beta Actin antibody #119716 as inner control). All experimental procedures were carried out according to the manufacturer’s protocols (https://www.abcam.com/ror2-antibody-nt-2535-2835-ab190145.html). Image processing was performed with Image J software. Statistical analysis was performed with Prism 6 software (GraphPad). Two-tailed Student’s t test was used to determine the difference between two groups.

3 | RESULTS

3.1 | Clinical data

The result of ultrasonographic diagnosis at 22 weeks indicated that the proband fetus had multiple structural abnormalities, including micrognathia, rachischisis, limbs shortening, and genital abnormality. Pathological examination result suggested that the anomalies of the fetus were: macrocephaly, frontal bossing, splay eyebrows, ocular hypertelorism, depressed nasal bridge, anteverted nares, inverted V-shaped mouth, micrognathia, low-set ears; mesomelia; external genital dysplasia; scoliosis, thoracic 9, 12 hemivertebra, thoracic 2, 3, 4, 5, 7, and 11 vertebral dysplasia, thoracic 10 vertebral loss, left rib 8, 9, and 10 partial fusion deformity, right rib 9 and 10 fusion deformity; and bilateral polycystic kidneys (Figure 1).

3.2 | Results of genetic analysis

Results of karyotyping and CMA were both normal (Figure 2A).

Two compound heterozygous variants in ROR2 comprising c.613C > T(p.Arg205*) and c.904C > T(p.Arg302Cys) were identified by WES and confirmed by Sanger sequencing (Figure 2B and C). In addition, Sanger sequencing also revealed that the father was a carrier of heterozygous c.613C > T, while the mother was a carrier of heterozygous c.904C > T. As a newly detected variant with potential pathogenesis, c.904C > T has a very low frequency in variation.
databases, namely 0 in 1000 Genome (https://www.internationalgenome.org/) and 1.772e-05 in ExAC (http://exac.broadinstitute.org/variant/9-94495436-C-T). The ROR2: Arg302 amino acid among mammal species is highly conserved (Figure 2D). Besides, prediction results from SIFT and PolyPhen V2 both suggested that c.904C > T was a deleterious missense variation.

3.3 | Results of WB and IHC

Immunohistochemistry result indicated that the expression level of ror2 protein was significantly increased in the osteochondral tissues of the proband (Figure 3A). However, as shown in Figure 3B and C, Western blotting result demonstrated that the ror2 expression level difference between the proband and wild type in skin tissue was not significant (P = .656).

4 | DISCUSSION

As a type of severe and rare disorder involving skeletal dysplasia, Robinow syndrome has strong clinical and genetic heterogeneity and is difficult to distinguish from other semblable conditions. In this study, the manifestations of the proband were consistent with previous reports, so it can be clinically diagnosed as Robinow syndrome. However, the fetus ended up stillborn, which is not very common in ARRS cases, and the reason is still unclear. It may be due to the visceral extrusion caused by the abnormal thoracic development.

All genes that have been identified to cause this disorder, whether in an autosomal dominant or recessive pattern, are in the Wnt signaling pathway and interact strongly with each other. ROR2, the causative gene of ARRS, is a member of the receptor tyrosine kinase-like gene family and encodes an orphan membrane-bound tyrosine kinase which acts as a co-receptor of WNT5A in the process of osteoclastogenesis. The ROR2 transmembrane protein comprises several distinct motifs including immunoglobulin-like (Ig), cysteine-rich (CRD), and kringle domains in the extracellular region; a transmembrane section; and an intracellular region with tyrosine kinase (TK), serine/threonine-rich, and proline-rich structures. In an initial study, ROR2 gene was reported to cause brachydactyly type B (BDB1, MIM #113000); Then, Ali et al first described that homozygous missense mutations in ROR2 proteins, both intracellular and extracellular, and homozygous nonsense mutations that caused truncation at downstream of TK domain can lead to ARRS.
The compound heterozygous variation detected in our study contains two variants. Among which, c.613C > T has been reported by van Bokhoven et al. and is a definite pathogenic variant; on the other hand, c.904C > T located in the frizzled domain (cysteine-rich domain) was a novel missense variation, which has not been reported. According to the ACMG variant interpretation criteria, we determined it as a likely pathogenic variation with evidence level of PM2 + PM3 + PP2 + PP3 + PP4.
Ali et al.\textsuperscript{19} and Mehawej et al.\textsuperscript{20} demonstrated that mutations in the frizzled domain led to loss of function on account of retention of the mutated ror2 protein inside the endoplasmic reticulum (ER) due to misfolding. The protein is then degraded using ER-associated protein degradation and does not reach the plasma membrane. Thus, it was speculated that the effect of c.904C \textgreater T on ror2 protein should conform to this mechanism. It was also reported that ror2 expressed at high levels during early embryonic development, then the expression levels dropped strongly around day 16 and there were only very low levels in adult tissues (https://www.abcam.com/oror2-antibody-nt-2535-2835-ab190145.html). In this case, the expression levels of ror2 protein in the fetus' osteoid cartilage were abnormally elevated at 24 weeks of gestation, most likely due to missense mutation activating the expression of one allele in the presence of an early terminated translation of the other allele that disrupted gene function. Yet, the mechanism of the abnormal high expression of the missense allele and its effect on the downstream molecules in its pathway still need further study. Moreover, there was no difference between the expression of ror2 in skin samples of the proband and the control, indicating that the abnormal high expression may have tissue specificity. In addition, many studies pointed out that ROR2 gene was highly expressed in a variety of malignant tumors\textsuperscript{21-23} and was listed as a potential target for treatment.\textsuperscript{24} Therefore, our study may provide an idea for the research on the treatment and mechanism of these malignant tumors.

Since the couple in this case were both carriers of pathogenic ROR2 variants, their future pregnancy still has a 25% risk of being affected. So, their subsequent pregnancies should be followed by genetic counselling, and family plan options such as prenatal diagnosis, preimplantation genetic testing, or gamete donation should be considered.

At the present stage, the time and economic cost of WES have been profoundly reduced, the interpretation ability of its results has been gradually improved, and the corresponding disease databases have been continuously improved, all of which provide a prerequisite for its application in prenatal diagnosis in the near future.

In conclusion, we identified a novel heterozygous compound variation in ROR2 gene leading to ARRS, which expanded the mutation spectrum of the disease. The high efficiency and sensitivity of WES are very helpful in identifying genetic causes of fetal congenital malformations with complex and ambiguous phenotypes.

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