Dear Editor,

46,XY disorders/differences of sex development (DSD) includes a broad spectrum of disorders caused by genetic mutations, changes in the production of testicular hormones, or altered peripheral responses to testicular hormones, resulting in varying degrees of undervirilization.1,2 Infants with 46,XY DSD might exhibit a variety of ambiguous genital phenotypes, and the management of these individuals can prove challenging.

Achieving the correct diagnosis as early as possible is known to be an essential factor for optimizing gender assignment, which can be of great concern to parents.3 The classic diagnostic approach emphasizes obtaining serial laboratory and imaging assessments before conducting genetic analyses,4 and is expensive and laborious. The new approach proposes genetic testing as the first-line investigation after karyotyping and selective subsequent investigation to detail the phenotype.5 We implemented both approaches in parallel in the diagnosis of a Chinese infant with 46,XY DSD. Here, we describe the diagnostic process and share our experience with the aim of bringing more attention to infants with ambiguous genitalia.

The diagnostic process (Figure 1a) was extensive. The baby was raised as a girl because of the ambiguous genitalia present at birth. The first comprehensive evaluation of the infant was at 2 months of age, at which point there were no symptoms or signs of adrenal insufficiency. Ambiguous genitalia were characterized by a micropenis and dysplasia of the scrotum. Increased gonadotropin levels, especially of follicle-stimulating hormone, indicated hypergonadotropic hypogonadism. The karyotype was 46,XY and sex determining region Y (SRY) was present. An ultrasound examination showed suspected bilateral perineal testes and the absence of uterus. These clinical data supported a clinical diagnosis of 46,XY DSD. Copy number variation sequencing results were negative (Figure 1b).

To achieve a molecular diagnosis, we captured and sequenced 360 endocrine disease-related genes using high-throughput sequencing combined with gene capture technology. This identified a novel de novo c.46T>C (p.C16R) missense mutation in the nuclear receptor subfamily 5 group A member 1 gene (NR5A1) that was present in the baby after 2 months but not in the parents; findings were confirmed by Sanger sequencing (Figure 1c).

The baby had second and third evaluations at 4 months and 11 months of age, respectively (Supplementary Table 1), when there were still no symptoms or signs of adrenal insufficiency. The external masculinization score6 was 6. Gonadotropin levels were higher than those at the first evaluation, and levels of anti-Müllerian hormone (AMH) and inhibin-B were lower than normal. The influence of mini-puberty (typically occurring 15–90 days after birth)4 on increasing gonadotropin levels could not be completely excluded. The 3-day human chorionic gonadotropin stimulation test showed that the testes of the baby maintained an endocrine function. The 7-day human menopausal gonadotropin stimulation test7 was negative, indicating poor ovary function (Supplementary Table 1).

The multidisciplinary DSD team communicated with the baby’s parents three times regarding gender re-assignment. The baby was too young to be evaluated by a gender-related psychological test, but the parents decided to raise it as a boy. The baby underwent urethroplasty at the age of 11 months. Because testes with a normal appearance were visible to the naked eye, the pediatric urologists thought it unnecessary to perform a biopsy of the gonads. The child is still under follow-up. This study was carried out with approval from the Ethics Committee of Shengjing Hospital of China Medical University (Shenyang, China; Ethical No. 2021PS115K).

Written informed consent was obtained from the parents.

NR5A1 is an essential transcription factor that regulates steroidogenesis, reproductive development, and reproductive function.8 NR5A1 mutations are a common cause of 46,XY DSD, occurring with a frequency of around 15%–20%,9 so genetic screening of NR5A1 is recommended for patients with ambiguous genitalia. The phenotypes of DSD caused by NR5A1 variations encompass an entire spectrum from complete female to male external genitalia. DSD can notably be distinguished from androgen insensitivity syndrome and 5α-reductase deficiency during diagnosis.

The NR5A1 mutation identified in this report was heterozygous, as seen in most NR5A1 mutations,9 and was a missense mutation which reportedly accounts for 58% of all NR5A1 mutations.10 To the best of our knowledge, the NR5A1 c.46T>C (p.C16R) mutation has not been reported in the past literatures. Bioinformatics analysis suggested that the mutation causes the substitution of a neutral amino acid with a positively charged one, thus changing the three-dimensional structure of NR5A1 (Figure 1d). We constructed an in vitro functional NR5A1-overexpression system by transfecting the NR5A1 open reading frame (GeneCopoeia, Guangzhou, China) within a Myc-tagged eukaryotic expression vector (Forevergen Biosciences, Guangzhou, China) into the 293T cell line (ScienCell, Carlsbad, CA, USA) using
Figure 1: (a) A summary approach to diagnosis of the baby with ambiguous genitalia. (b) CNV-seq analysis showed that it was 46,XY, and no chromosome aneuploidy and genome copy number variation above 100 kb were found. (c) Sanger sequencing analysis of the \textit{NR5A1} mutation in the baby and parents. The mutation site was identified by the red arrow. (d) Conformational changes. Hydrogen bonds are formed by Cys13 and Lys59 in the WT protein, while Cys16 of Mut protein forms multiple hydrogen bonds with Cys13, Val15, Lys59, and Arg69. (e) \textit{NR5A1} mRNA expression in the four groups were revealed via real-time quantitative reverse transcription polymerase chain reaction analysis. (f) \textit{NR5A1} expression in the four groups were shown via western blot. (g) Nuclear localization of mutant \textit{NR5A1}. The cells exposed to different treatments were subjected to immunocytochemical analysis using an anti-Myc antibody (scale bars=50 µm). (h) DNA binding of mutant \textit{NR5A1}. Nuclear extracts prepared from cells in the four groups were subjected to electrophoretic mobility shift assay using biotin-labeled probes. (i) Transcriptional activity of mutant \textit{NR5A1}. According to different transfected plasmids, the cells in the dual-luciferase reporter assay were divided into three groups as shown in the histogram. Abbreviation definitions are shown in Supplementary Information.

JetPRIME® transfection reagent (Polyplus-transfection SA, Illkirch, France). Quantitative real-time (RT)-PCR and western blot analyses suggested that the p.C16R variant did not impair \textit{NR5A1} mRNA or protein expression compared with wild type (Figure 1e and 1f).
Immunofluorescence analysis localized the NR5A1 p.C16R mutant to the cell nucleus, the same as the wild-type protein (Figure 1g). Electrophoretic mobility shift assays showed that p.C16R decreased the DNA-binding affinity (Figure 1h), while dual-luciferase reporter assays found that the mutant reduced the transactivation capacity of AMH (Figure 1i) resulting in the degradation of Müllerian structures. Thus, this novel NR5A1 variant appears to change the protein structure, impeding the effect of NR5A1 on the regulation of gonadal development.

In conclusion, this novel missense mutation of c.46T>C (p.C16R) in a Chinese infant with ambiguous genitalia increases our knowledge of the NR5A1 mutational spectrum. Implementing the classic and new approaches in parallel to the diagnosis of an infant with ambiguous genitalia offered many advantages. These included using genetic analysis to improve the rate of correct diagnosis and reduce diagnostic delay, obtaining an early molecular diagnosis to allow for an objective decision on gender assignment, and reducing the pressure of repeated tests on parents through selective investigations.

AUTHOR CONTRIBUTIONS
DZ and YJT conceived and designed the study; DZ and LZM performed all the experiments related to the functional studies; MYL performed Sanger sequencing and site-directed mutagenesis; DZ and YX collected clinical information about the patient and parents; LZM performed bioinformatics analysis; DZ and YX drafted the manuscript; and YJT reviewed and edited the manuscript. All authors read and approved the final manuscript.

COMPETING INTERESTS
All authors declare no competing interests.

Supplementary Information is linked to the online version of the paper on the Asian Journal of Andrology website.
## Clinical characteristics of a 46,XY baby with a NR5A1 c.46T>C (p.C16R) mutation

| Clinical indicator | Values (2 months old) | Values (4 months old) | Values (11 months old) | Standard value |
|-------------------|-----------------------|-----------------------|------------------------|----------------|
| ACTH (8:00)      | 49.93 pg ml⁻¹         | 45.29 pg ml⁻¹         | 33.69 pg ml⁻¹          | 7.2–63.3 pg ml⁻¹ |
| Cortisol (8:00)  | 1.33 ug dl⁻¹          | 6.63 ug dl⁻¹          | 12.32 ug dl⁻¹          | 6.02–18.4 ug dl⁻¹ |
| Potassium        | 5.29 mmol dl⁻¹        | 4.7 mmol dl⁻¹         | 5.00 mmol dl⁻¹         | 3.5–5.5 mmol dl⁻¹ |
| Sodium           | 135 mmol dl⁻¹         | 142 mmol dl⁻¹         | 139 mmol dl⁻¹          | 135–155 mmol dl⁻¹ |
| LH                | 1.35 mIU ml⁻¹         | 1.64 mIU ml⁻¹         | 1.02 mIU ml⁻¹          |                 |
| FSH               | 15.3 mIU ml⁻¹         | 16.93 mIU ml⁻¹        | 12.57 mIU ml⁻¹         |                 |
| T                 | <0.1 ng ml⁻¹          | <0.1 ng ml⁻¹          | <0.1 ng ml⁻¹           |                 |
| E2                | <20 pg ml⁻¹           | <20 pg ml⁻¹           | <20 pg ml⁻¹            |                 |
| Progesterone      | 0.97 ng ml⁻¹          | 1.20 ng ml⁻¹          | 0.73 ng ml⁻¹           |                 |
| 17-OHP            | 2.52 ng ml⁻¹          | -                     | -                      | 3.8–13.7 ng ml⁻¹ |
| Androstenedione   | <0.3 ng ml⁻¹          | -                     | -                      |                 |
| AMH               | -                     | 9.19 ng ml⁻¹          | 11.84 ng ml⁻¹          | 55.37–439.45 ng ml⁻¹ |
| Inhibin-B         | -                     | 34.4 pg ml⁻¹          | 41.9 pg ml⁻¹           | 274.23–602.34 pg ml⁻¹ |
| Renin             | -                     | 13.64 ng ml⁻¹ per h   | 3.66 ng ml⁻¹ per h     |                 |
| Aldosterone       | -                     | 235.7 pg ml⁻¹         | -                      | 387 pg ml⁻¹     |

**HCG stimulation test**

Before stimulation

| T: 170 pg ml⁻¹ |
| DHT: 59.59 pg ml⁻¹ |
| T/DHT: 2.85 |

After stimulation

| T: 340 pg ml⁻¹ |
| DHT: 73.67 pg ml⁻¹ |
| T/DHT: 5.43 |

**HMG stimulation test**

Before stimulation

| E2: <20 pg ml⁻¹ |
| LH: 0.23 mIU ml⁻¹ |
| FSH: 0.38 mIU ml⁻¹ |
| T: 0.20 ng ml⁻¹ |

After stimulation

| E2: <20 pg ml⁻¹ |
| LH: 0.33 mIU ml⁻¹ |
| FSH: 6.37 mIU ml⁻¹ |
| T: <0.1 ng ml⁻¹ |

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**Abbreviation definitions of Figure 1:** Control: non-transfected 293T cells; Vector: cells transfected with an empty vector; WT: Myc-tagged wild type; Mut: NR5A1 mutation. G2P2: Gravidity twice and Parity twice; DRE: Digital rectal examination; ACTH: Adrenocorticotropic hormone; E2: Estradiol; 17-OHP: 17-hydroxyprogesterone; AMH: Anti-Müllerian hormone; DHT: Dihydrotestosterone; HCG: Human chorionic gonadotropin; HMG: Human menopausal gonadotropin.

Abbreviation definitions of Supplementary Table 1: Using luminescence; Using chemiluminescence; Using electrochemiluminescence; Using ELISA; Using DAPI; Using thymidine kinase promoter-Renilla luciferase reporter plasmid.