INTRODUCTION

Haemophilia A (HA) is the most common X-linked recessive bleeding disorder and is characterized as a deficiency or dysfunction of coagulation factor VIII. Patients with HA show symptoms of prolonged bleeding after injury or trauma. In the coagulation pathway, factor VIII and factor IX form an active complex (tenase complex) which activates subsequent factors, including factor X. The prevalence of HA among live male births worldwide is approximately 1/5000. Clinically, the phenotype of HA is classified based on residual factor VIII activity (FVIII:C), as severe (<1%), moderate (1%-5%), or mild (>5%). The FVIII gene (F8), located at the distal end of the long arm of the X chromosome, spans 186 kb of genomic DNA and is comprised of 26 exons. The F8 gene is translated into a 2315 amino acid
polypeptide chain with a 19 amino acid leader peptide. The mature polypeptide consists of six structural domains, namely A1-A2-B-A3-C1-C2, and is activated following the dissociation of the B domain.\(^4,5\) In peripheral blood, the FVIII protein is bound to the von Willebrand factor (vWF), which protects it from degradation.

HA is characterized by extremely high mutational heterogeneity. To date, 3171 mutations of the \(F8\) gene identified as disease causing have been recorded in the HGMD® Professional 2020.1 database (https://portal.biobase-international.com/hgmd/). Overall, missense and nonsense variants are responsible for more than 50% of HA cases, followed by small deletions, duplications or insertions. However, inversions in intron 22 (Inv22) and intron 1 (Inv1) are the most frequent gene defects found in severe HA patients, occurring in 45–50% and 0.5–5% of cases, respectively.\(^6,7\) It has been reported that both Inv22 and Inv1 may be correlated with the formation of inhibitors that interfere with treatment.\(^8\)

The aim of this research is to produce a mutation spectrum for the \(F8\) gene based on 485 unrelated Chinese HA carrier families using a systematic approach, including LD-PCR, multiplex PCR, NGS and MLPA. Additionally, the accuracy of prenatal diagnosis of HA from the analysis of 274 foetal samples is reported.

2 | MATERIALS AND METHODS

2.1 | Participants

The study was approved by the ethics committee of the First Affiliated Hospital of Zhengzhou University. A total of 485 unrelated HA cases were included, comprising 381 severe, 72 moderate, and 32 mild clinical patients. The clinical phenotype was mainly classified based on factor VIII activity via biochemical characterization or analysis of bleeding characteristics. Once an informed consent document was signed, 2 mL of peripheral blood was collected in EDTA tubes from each participant.

2.2 | DNA extraction

Genomic DNA was obtained from 500 \(\mu\)L of whole blood using a Lab-Aid Nucleic Acid (DNA) Isolation Kit (Zeesan, Xiamen, China) according to the manufacturer’s instructions. DNA purity and concentration was determined using UV spectrophotometry (Nanovue Plus, GE healthcare, Buckinghamshire, UK).

2.3 | Inversion detecting

Samples from severe cases were first tested for Inv22. The analysis was performed using an improved method based on the traditional Long-Distance PCR method,\(^9\) which involves performing two PCR reactions concurrently in two distinct reaction tubes. The PCR products obtained were analysed using 0.7% agarose gel electrophoresis. Subsequently, samples identified as negative for Inv22 were subjected to multiplex PCR to evaluate Inv1, according to the procedure reported previously.\(^10\) The PCR products obtained herein were analysed using 1% agarose gel electrophoresis.

2.4 | Next-generation sequencing (NGS)

DNA from inversion-negative patient samples with severe as well as moderate-to-mild HA were subjected to NGS of the essential regions of the \(F8\) gene, including all exons, both the 5’- and 3’-UTRs, and exon-intron junction regions. The entire procedure was conducted using the Life Technologies PGM platform (Carlsbad, CA, USA) according to the standard operating procedure.

2.5 | Multiplex ligation-dependent probe amplification (MLPA)

For patient samples lacking mutations, large deletions and duplications within the 26 exons of the \(F8\) gene were detected using MLPA. The process was carried out using the SALSA MLPA Probemix P178-A1 \(F8\) Kit (MRC-Holland, Amsterdam, Netherlands) according to the manufacturer’s instructions.

2.6 | Prenatal diagnosis

Two hundred and thirty-five pregnant probable HA carriers were confirmed to carry the familial mutation of the \(F8\) gene and applied for prenatal diagnosis. A total of 274 samples, including chorionic villi, direct amniocytes, or umbilical cord blood cells, were collected. Potential maternal contamination of foetal samples was identified using Goldeneye™ DNA ID System 20A Kit (PEOPLESPOT, Beijing, China). Subsequently, all foetal samples underwent the same genetic analysis as the probands.

2.7 | Data analysis

The data resulting from the PGM runs was analysed using Ion Torrent sequencing data analysis software from Torrent Suite (Life Technologies, Carlsbad, CA, USA) to generate sequence reads. Finally, all resulting gene variations were collected and analysed.

2.8 | Mutation verification and software prediction

Sanger sequencing was performed to verify the nucleotide sequence of all generated variants. To determine the pathogenicity of novel
identified missense variants, a variety of functional prediction software packages were used, including Polyphen_2\textsuperscript{11} (http://genetics.bwh.harvard.edu/pph2/), PROVEAN\textsuperscript{12} (http://provean.jcvi.org/index.php) and MutationTaster\textsuperscript{213} (http://www.mutationtaster.org/).

## 3 RESULTS

Following the molecular diagnostic workflow described above, 237 different alterations of the \textit{F8} gene were identified throughout all 26 exons and 15 introns, in 478 out of 485 (98.56%) unrelated HA patients. Upon comparison of the results with available databases, including dbSNP, Factor VIII Variant (http://www.factorviii-db.org/) and HGMD\textsuperscript{®} Professional 2020.1, 146 known mutations and 91 novel mutations were identified. The distribution of mutational characteristics of the \textit{F8} gene mutations identified is shown in the Table S1.

In 374 of the 381 severe cases, inversion tests identified 167 patients with Inv22 and 10 patients with Inv1, representing 44.65% and 2.67% of the severe cases evaluated, respectively. Frameshift mutations encompassed the second most common mutation type identified and accounted for 62 of the severe cases studied (16.58%). These mutations included 40 small deletions, 19 small insertions and t indels representing 10.70%, 5.08%, 0.80% of the severe cases evaluated, respectively. In addition, we observed 53 missense mutations (14.17%), 41 nonsense mutations (10.96%), 17 splice site mutations (4.55%), 16 gross deletions (4.28%), five gross insertions (1.34%) and three complex mutations (0.80%). Notably, a single female patient with a severe phenotype was found to carry an Inv1 mutation. Among the moderate cases, the dominant mutation was missense, which contributed 50 cases, representing 69.44% of all moderate phenotypes studied. Less common mutation types identified within this HA phenotype class included Inv22 (4.17%), small deletion (12.5%), splice site mutation (8.33%) and gross duplication (1.39%). Among patients with a mild phenotype, the mutation spectrum consisted of 29 cases of missense mutation (90.63%), one nonsense mutation (3.13%), one small insertion (3.13%) and one gross deletion (3.13%). The two female patients with mild HA symptoms were found to carry a missense mutation and a nonsense mutation. The data described above are summarized in Table 1.

Based on their clinical phenotype, 235 pregnant HA carriers provided 274 prenatal samples. The results of the prenatal diagnosis are presented in Table 2. Via sex identification, there were 200 males and 74 females. Of the 200 male foetuses, 103 were found to carry the familial HA mutation. Of the 74 female foetuses, 38 were carriers while the other 36 were mutation free.

## 4 DISCUSSION

In this study, we used a variety of analytical methods to evaluate the HA mutation spectrum in Chinese families comprising all phenotypic classes. Specifically, LD-PCR and multiplex PCR was first used to detect Inv22 and Inv1, respectively, followed by NGS for point mutations, MLPA for deletion and duplication of exons, and Sanger sequencing for sequence verification. In total, 478 pathogenic variants in 485 HA patients were successfully identified with an efficiency of 98.56%. Despite the use of multiple techniques, no mutations were

### TABLE 1 Frequencies of mutation type by severity

| Mutation type | Severe N (% of total) | Moderate N (% of total) | Mild N (% of total) |
|---------------|-----------------------|------------------------|---------------------|
| Inv22         | 167 (44.65)           | 3 (4.17)               | 0                   |
| Inv1          | 10 (2.67)             | 0                      | 0                   |
| Missense      | 53 (14.17)            | 50 (69.44)             | 29 (90.63)          |
| Nonsense      | 41 (10.96)            | 3 (4.17)               | 1 (3.13)            |
| Small deletion| 40 (10.70)            | 9 (12.5)               | 0                   |
| Small insertion| 19 (5.08)            | 0                      | 1 (3.13)            |
| Small indels  | 3 (0.80)              | 0                      | 0                   |
| Splicing      | 17 (4.55)             | 6 (8.33)               | 0                   |
| Gross deletion| 16 (4.28)             | 0                      | 1 (3.13)            |
| Gross duplication| 5 (1.34)        | 1 (1.39)               | 0                   |
| Complex       | 3 (0.80)              | 0                      | 0                   |
| Total         | 374 (100)             | 72 (100)               | 32 (100)            |

Note: Inv1: inversion in intron 1; Inv22: inversion in intron 22; Small deletion, small insertion, small indel: 20 bp or less.

### TABLE 2 Consequence of prenatal diagnosis

| Mutation type | Normal male foetus | Male patient | Normal female foetus | Female carrier | Total |
|---------------|--------------------|--------------|----------------------|---------------|-------|
| Inv1          | 2                  | 1            | 1                    | 1             | 5     |
| Inv22         | 29                 | 43           | 6                    | 18            | 96    |
| Missense      | 32                 | 25           | 11                   | 10            | 78    |
| Nonsense      | 10                 | 9            | 3                    | 3             | 25    |
| Frameshift    | 15                 | 16           | 12                   | 5             | 48    |
| Splicing      | 4                  | 8            | 3                    | 1             | 16    |
| Gross deletion| 5                  | 1            | 0                    | 0             | 6     |
| Total         | 97 (35.40)         | 103 (37.59)  | 36 (13.14)           | 38 (13.87)    | 274   |

Note: Inv1: inversion in intron 1; Inv22: inversion in intron 22; Frameshift includes small deletion, small insertion and small indel.
identified in the remaining seven individuals. A possible explanation for
this finding includes the presence of mutations located deep
within introns or in regions outside of the F8 gene that are important
for its expression.\textsuperscript{14}

Inv22 and Inv1 mutations in patients with severe HA have been
reported in the ranges of 40–50\% and 0.5–5\%, respectively. In this
study, the prevalence of Inv22 and Inv1 mutations were found to
be similar to those described in other populations.\textsuperscript{10,15} Intron 22 is
the largest intron in the F8 gene, and it has been established that its
inversion impedes the formation of full-length FVIII messenger RNA
(mRNA), thus precluding the synthesis of the FVIII protein, and re-
sulting in severe HA.\textsuperscript{16} Nonetheless, three cases of Inv22 mutations
were detected in patients with moderate phenotypes. This could
be due to clinical variability and gene variation among different in-
dividuals. A milder bleeding phenotype has been reported in men
with severe haemophilia A. To date, there have been a few reports
of a milder bleeding phenotype in patients with severe haemophilia
A.\textsuperscript{17,18} Researchers have suggested the following potential reasons
for this observation: (i) DNA replication/RNA transcription errors
resulting in restoration of the reading frame, (ii) ‘ribosomal frame-
shifting’, and (iii) \textit{de novo} mutations with somatic mosaicism that
occurred post-zygotically.\textsuperscript{19,20} However, alternative causes, such as
mutations in the anti-coagulation genes, may also be contributing
factors. Similarly, a large deletion within the F8 gene resulting in a
mild phenotype could result from similar factors. Therefore, it is nec-
essary to collect accurate clinical materials (e.g. FVIII:C, inhibitors,
family history and arthropathy) and focus on phenotypic and geno-
typic data in genetic analysis. In addition, further studies addressing
the incongruity between phenotype and genotype are necessary.

Among the severe cases, a female patient whose parents did not
carry the familial HA mutation was confirmed to have the Inv1 muta-
tion, suggesting that the mutation must have occurred \textit{de novo} in the
paternal germline. Due to the absence of a second mutation in this
female patient, the severe symptoms of her might be associated with
a nonrandom pattern of inactivation of the maternally derived X chromo-
some.\textsuperscript{21} Of the two female patients with mild symptoms, one was
identified as having a missense mutation, and the other a nonsense
mutation. The father of the female with a missense mutation is also
an HA patient, and his clinical presentation is moderate. The female,
as the unique patient in her family, with a nonsense mutation was ver-
ified to have inherited the mutation from her mother. This single-nu-
cleotide substitution is in the B domain, a region lacking procoagulant
activity and is partially spliced from the mature protein.\textsuperscript{22} Due to the
combination of individual differences with respect to the mechanism
of a nonrandom pattern of inactivation of the maternally derived X chromo-
some, the female presented mild clinical symptoms.

In this study, a total of 160 types of single-nucleotide substitu-
tions were identified in 253 unrelated families, including 101 mis-
sense mutations in 132 HA families, 38 nonsense mutations in 45
HA families and 21 splice site mutations in 23 HA families. Of the
160 single-nucleotide substitutions identified, 51 were either not
included in the available databases or not previously reported in the
literature. All novel single-nucleotide substitutions were predicted
to be damaged using computational analysis. Since some missense
mutations may present polymorphisms,\textsuperscript{23} further evaluation may be
necessary. Moreover, a nonsense mutation, c.1063C>T, was identi-
fied in four unrelated families, as well as two missense mutations,
c.2167G>A and c.6506G>A, that were detected in six and four unre-
lated families, respectively. These three mutations may be regarded as
recurrent mutations for single-nucleotide substitutions.

Frameshift mutations were prominently observed in this study,
and included small deletions, insertions and indels. All frameshift
mutations detected, except two (c.599_601delAAAC and c.7031delG),
resulted in premature stop codons. Since exon 14 is the longest exon
in the F8 gene and possesses a large number of poly-A runs, it is con-
sidered a hot spot for such mutations.\textsuperscript{24} Within a sequence of nine
adenines in exon 14, a deletion (c.3637delA) was identified in 11
unrelated HA families and an insertion (c.3637insA) was detected in
two unrelated HA families. Thus, the single-nucleotide deletion/in-
sertion at this site represents a mutation hot spot within our cohort.
Additional frameshift mutations observed include a three-base dele-
tion (c.599_601delAAAC mutation) causing an in-frame amino acid de-
letion (p.Glu200del) within the A2 structural domain, and a single base
deletion at the end of exon 26 (c.7031delG), leading to the loss of the
termination codon and a 39 amino acid extension to the polypeptide
chain (p.Gly2344Alafs‘39). Future analysis is required to elucidate the
mechanistic details surrounding the impact these mutations have on
FVIII protein expression. Finally, 22 large rearrangements were iden-
tified using MLPA technology, including 16 gross deletions spanning
all exons, and six gross duplications spanning all exons, except exon
1. Two large deletions (c.4740+?_4933-?del, c.5054+?_5219-?del) be-
tween 150 and 200 bases in exon 14 were screened by NGS and ver-
ified by RT-PCR. Although their existence was carefully established,
the accurate break points remain unknown. Further research is neces-
sary to identify the exact break points and assess their pathogenicity.

In developed countries, replacement therapy for haemophilia is
an effective treatment option, and prenatal diagnosis resulting in
termination of pregnancy is not commonly practiced. Due to eco-
nomic pressure and social attitudes in developing countries, prenatal
diagnosis is urgently needed to prevent births of babies with haemo-
philic phenotypes. As a result of the prenatal diagnosis carried out
in this study, two women with prenatally diagnosed haemophilic
sons continued their pregnancy, while the other 101 women decided
to terminate their pregnancies. A follow-up was carried out and the
phenotypes of the newborns were consistent with the results of the
prenatal diagnosis.

5 | CONCLUSION

This study revealed a mutation spectrum of the F8 gene in a large
sample of HA patients in China. Ninety-one novel mutations were
reported, including 51 single-nucleotide substitutions, 33 frameshift
mutations, two gross deletions, three gross insertions and two com-
plex mutations. The mechanism by which the identified mutations af-
fect disease severity requires additional research in order to provide
a better understanding of the genotype-phenotype association in HA. The mutation profiles of HA obtained here will provide a useful complementary database for the detection and diagnosis of patients and carriers of HA in China. In addition, the prenatal diagnosis of 274 samples from 235 pregnant HA carriers was determined and verified in two cases where the pregnancies were carried to term. Our results demonstrate that potential HA carriers should define their status prior to pregnancy, so that timely prenatal diagnosis, if desired, is possible.

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CONFLICT OF INTERESTS
The authors stated that they had no interests which might be perceived as posing a conflict or bias.

AUTHOR CONTRIBUTIONS
YF performed experiments, analysed the data, prepared tables and figures and wrote the manuscript. Q-Q L, P-L S and NL interpreted the data, edited the manuscript. R-X G and X-D K designed the study, supervised the interpretation and statistical analysis of the data, edited the manuscript. All the authors approved the final version of the manuscript.

DATA AVAILABILITY STATEMENT
The data supporting the findings of this study are available within the article and its supplementary information files.

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SUPPORTING INFORMATION
Additional supporting information may be found online in the Supporting Information section.

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