Genomic architecture of fetal central nervous system anomalies using whole-genome sequencing

Ying Yang1,3, Sheng Zhao2,3, Guoqiang Sun2,3, Fang Chen1, Tongda Zhang1, Jieping Song2, Wenzhong Yang3, Lin Wang1, Nianji Zhan1, Xiaohong Yang2, Xia Zhu2, Bin Rao3, Zhenzhen Yin1, Jing Zhou1, Haisheng Yan1, Yushan Huang1, Jingyu Ye1, Hui Huang2, Chen Cheng2, Shida Zhu1, Jian Guo1, Xun Xu1 and Xinlin Chen1

Structural anomalies of the central nervous system (CNS) are one of the most common fetal anomalies found during prenatal imaging. However, the genomic architecture of prenatal imaging phenotypes has not yet been systematically studied in a large cohort. Patients diagnosed with fetal CNS anomalies were identified from medical records and images. Fetal samples were subjected to low-pass and deep whole-genome sequencing (WGS) for aneuploid, copy number variation (CNV), single-nucleotide variant (SNV, including insertions/deletions (indels)), and small CNV identification. The clinical significance of variants was interpreted based on a candidate gene list constructed from ultrasound phenotypes. In total, 162 fetuses with 11 common CNS anomalies were enrolled in this study. Primary diagnosis was achieved in 62 cases, with an overall diagnostic rate of 38.3%. Causative variants included 18 aneuploids, 17 CNVs, three small CNVs, and 24 SNVs. Among the 24 SNVs, 15 were novel mutations not reported previously. Furthermore, 29 key genes of diagnostic variants and critical genes of pathogenic CNVs were identified, including five recurrent genes: i.e., TUBA1A, KAT6B, CC2D2A, PDHA1, and NF1. Diagnostic variants were present in 34 (70.8%) out of 48 fetuses with both CNS and non-CNS malformations, and in 28 (24.6%) out of 114 fetuses with CNS anomalies only. Hypoplasia of the cerebellum (including the cerebellar vermis) and holoprosencephaly had the highest primary diagnosis yields (>70%), while only four (11.8%) out of 34 neural tube defects achieved genetic diagnosis. Compared with the control group, rare singleton loss-of-function variants (SLoFVs) were significantly accumulated in the patient cohort.

RESULTS
Demographic characteristics of cohort and prenatal imaging features
Detailed clinical information such as pregnancy history, gestation, prenatal ultrasound phenotypes, and post-mortem examinations of all fetuses were reviewed and are listed in Supplementary Table 1. The median maternal age of the cohort was 27. No statistical differences in age median or distribution were detected between the cohort and Chinese women who gave birth in 2015.

INTRODUCTION
Fetal central nervous system (CNS) anomalies are among the most common congenital abnormalities. For example, the prevalence of neural tube defects (NTDs) is estimated to be 18.6/10,000 live births globally. However, the incidence of intracranial malformations with an intact neural tube is not certain as most escape detection prenatally or at birth and only manifest in later life. Therefore, screening and diagnosing CNS anomalies as early as possible is essential to provide critical information for clinical decisions. Ultrasonography is the most important modality for evaluating fetal CNS defects. Fetal magnetic resonance imaging (MRI) can also provide additional information for fetal CNS assessment. However, prenatal diagnosis of CNS anomalies remains challenging due to ongoing growth and fetal brain development.

Genomic variations are important etiologies of fetal anomalies. Karyotyping and chromosomal microarray analysis (CMA) are effective at diagnosing chromosomal anomalies and copy number variations (CNVs). In current clinical practice, exome sequencing (ES) is usually applied after uninformative CMA results to detect single-gene mutations on exons. This strategy may be economical, but it overlooks the fact that pathogenic genetic variation can differ considerably among congenital diseases. Furthermore, time is very limited for accurate fetal diagnosis. Therefore, a one-for-all genetic testing modality is necessary. Whole-genome sequencing (WGS) with an average depth of 30–40X can detect CNVs, single-gene mutations, and structural variations such as intragenic CNVs and intron mutations. Various studies have called for an expansion in diagnostic utility for infants and children for a wide spectrum of disorders. However, although the cost of WGS is decreasing rapidly, it is still expensive compared to CMA and ES. Therefore, the genomic background of imaging phenotypes could achieve a balance between cost and efficiency. Although hundreds of genetic syndromes involving the CNS have been studied, their prenatal imaging manifestations remain poorly described. Furthermore, genomic studies of large cohorts with fetal CNS malformations are lacking. In this study, the genomic landscapes of 162 unselected fetuses were explored to demonstrate the distribution of genomic variations and their characteristics in major types of ultrasonographic-detected fetal CNS anomalies, including multifactorial diseases such as NTDs and hydrocephalus, to gain a better understanding of CNS anomalies. The diagnostic power of WGS for fetal CNS ultrasound anomalies was evaluated, thus providing a solid foundation for flexible choice in prenatal genetic screening and diagnosis.
(determined from survey data from the National Bureau of Statistics of China)\textsuperscript{12}. The chromosomal sex ratio of the fetuses was 1:1 (81/81), suggesting no sex differences in the cohort. More than 60% of the final ultrasound diagnoses were obtained after 28 weeks. In total, 11 typical CNS anomalies were identified in the current cohort, as shown in Supplementary Fig. 1. Fetuses may have had more than one type of CNS or non-CNS malformation.

Hydrocephalus and NTDs were the most frequent phenotypes in the cohort, each occurring in 34 fetuses. In addition, 114 fetuses had anomalies in the CNS only, while 48 had both CNS and non-CNS abnormalities (not including the typical facial anomalies of holoprosencephaly or clubfoot of Chiari II malformation). Autopsy and/or prenatal MRI data were available for 83 fetuses, with additional structural malformations not diagnosed by prenatal ultrasound found in eight cases. This included two cerebral cortical malformations found by MRI, i.e., periventricular heterotopia and parietal frontal dysplasia, in addition to the aplasia of the corpus callosum (ACC) and arachnoid cysts identified by prenatal ultrasound. Other findings from the autopsy included abnormalities in the fingers and toes in three fetuses, vascular malformations in two fetuses, and right iliosacral joint malformation in one fetus.

**Sequencing and genetic diagnosis by WGS**

Two-stage sequencing was applied, with 26 cases subjected to low-pass WGS only and 136 cases sequenced to an average depth of 41.9 ± 0.4-fold coverage for diagnosis and further analysis. Three fetuses failed CNV calling after both low-pass and deep WGS due to turbulence of read depth across the whole genome, although SNV analysis was not affected. Following low-pass WGS, 19 aneuploids and 342 CNVs (>100 kb) were identified. Deep WGS identified an average of 3,925,317 ± 2403 single-nucleotide polymorphisms (SNPs) and 938,371 ± 2860 indels for each sample. Small CNVs (ranging from 50 bp to 100 kb) beyond the detection limit of mainstream CMA were found in 102 samples without primary diagnosis by low-pass WGS or SNV analysis. Four samples did not pass the quality control test for small CNV analysis. On average, 2866 ± 40 small CNVs were identified in each sample, including 1203 ± 18 gene-containing CNVs and 89 ± 3 exon-containing CNVs. The numbers of CNVs containing genes and exons were linearly dependent on total number of small CNVs (Supplementary Fig. 2). Only small CNVs containing exons with a frequency lower than 0.01 in our in-house database were assessed by the clinical review panel. As a result, an average of 10.8 ± 0.9 small CNVs were assessed for each sample.

Overall, the primary diagnosis was achieved in 62 cases (diagnosis rate 38.3%), as shown in Fig. 1. In total, 18 aneuploids, 21 CNVs, and three small CNVs were diagnosed (Table 1). Pathogenicity scores and supporting references are listed in Supplementary Table 4. Furthermore, as shown in Table 2, 26 pathogenic or likely pathogenic SNVs were found, which may explain the fetal phenotypes. The pathogenicity criteria are listed in Supplementary Table 5. Among them, 16 are novel mutations that have not been reported previously, seven are known pathogenic variants, and three are listed in the Single-Nucleotide Polymorphism database (dbsNP) but without clear clinical significance. A possible mosaic 47,XXX was found in P1133, which showed Chiari II malformation. However, there was no evidence indicating a correlation between 47XXX and Chiari II malformation. The eight pathogenic or likely pathogenic variants classified as a carrier, incidental, or secondary are listed in Supplementary Table 2.

The primary diagnostic yield of WGS varied significantly among the different ultrasound-defined CNS anomalies. As shown by the polyline in Fig. 2, hypoplasia of the cerebellum (including the cerebellar vermis) and holoprosencephaly had the highest primary diagnosis rates (>70%), followed by aplasia/hypoplasia of the corpus callosum, Dandy-Walker variants, intracranial cysts, and ventriculomegaly, with primary diagnosis yields of ~50%. Primary diagnosis yields for hydrocephalus, microcephaly, and undiagnosed abnormal echoes were between 20 and 30%. Unsurprisingly, primary diagnoses of destructive lesions and NTDs only reached ~10%. Diagnostic variants were present in 34 (70.8%) of the 48 fetuses with both CNS and non-CNS anomalies, and were present in 28 (24.6%) of the 114 fetuses with CNS anomalies only.

There were 13 cases of isolated brain anomalies, including isolated ACC, cystic lesions, and ventriculomegaly, with six identified by primary genetic diagnosis. According to the detection limits of the variant size of common clinical genetic testing tools, such as karyotyping, CMA, and exome sequencing, the diagnostic variants were divided into three levels: i.e., chromosomal anomalies >5 Mb, CNVs between 100 kb–5 Mb, and single-gene variants, including SNVs and small CNVs. As indicated in Fig. 2, chromosomal anomalies and single-gene variants showed the greatest contribution to primary diagnosis in this cohort. In cases with only CNS anomalies, although the overall diagnosis rate was 24.6%,
15 (53.4%) out of 28 diagnosed cases were attributed to single-gene variants. Therefore, ES and deep WGS, which can detect single-gene variants, should be considered after uninformative clinical karyotyping for these types of anomalies.

As shown in Fig. 2a, among the 29 genes of diagnostic variants (26 SNVs and three established critical genes of diagnostic CNVs), five were found in more than one fetus (i.e., TUBA1A, KAT6B, CC2D2A, PDHA1, and NF1). Based on the OMIM database, 70% of these key 29 genes are related to autosomal dominant diseases and 15% are related to autosomal recessive diseases. The remaining 15% are X-linked or X-linked dominant, as shown in Fig. 2b. Protein-protein interaction analysis of these genes using the STRING database (http://string-db.org) showed a greater degree of interconnectivity than expected by chance (R = 1.75 × 10−10), and significant enrichment in brain development, forebrain development, and anatomical structure morphogenesis (Supplementary Fig. 3).

**Rare singleton deleterious variant enrichment**

In total, SNVs in 136 cases were screened for singleton deleterious variants. Compared with the control group, singleton deleterious missense variants with different MAFs (0, 0.001) were not accumulated in the cohort (Fig. 3a, b). However, rare singleton loss-of-function variants (SLoFVs) with MAFs of 0 or <0.001 were significantly more common in the cohort than in the control group, as shown in Fig. 3c, d. The median number of rare SLoFVs (MAF ≤ 0.001) was 13.5 and 9 in the cases and controls, respectively, with P = 2.2 × 10−16 by the two-sided Wilcoxon test.

These results suggest that the accumulation of rare SLoFVs may be related to fetal CNS anomalies and should be considered to improve diagnostic yield.

**Genomic architecture of NTDs**

Despite being a common CNS abnormality both clinically and in this cohort, NTDs were found with a relatively low diagnosis rate, which is likely related to their complex etiologies, including various genetic and environmental factors. Even so, pathogenic or likely pathogenic variants in four NTD cases were identified, including Trisomy 18, 7q36.1q36.3 deletion, 4q31.3q32.1 deletion, and homozygous nonsense c.4333C > T(p.Arg1445*) in CC2D2A causing Meckel Gruber syndrome in P1279.

The non-canonical Wnt/planar cell polarity (PCP) signaling pathway is implicated in the pathogenesis of NTDs, and 13 genes in the PCP pathway are involved in human NTDs. Rare singleton deleterious variants in these 13 PCP genes (listed in Supplementary Table 3) were identified in two NTD cases, with none found in the control group. The two variants were NM_014246.1(CELS1R1): c.7673G > A(p.Arg2558His) in P135 and NM_002336.2(LRP6): c.1252T > C(p.Tyr418His) in P382. In addition, known pathogenic missense mutation c.1744G > A(p.Gly582Ser) in COL3A1, which has been reported to cause Ehlers-Danlos syndrome IV, was found in P194 with a heterozygous missense variant with unknown significance.

The second NTD case, P866 was diagnosed with exencephaly at 14 weeks, and a heterozygous missense variant with unknown significance in COL3A1 c.542C > G(p.Pro181Leu) was also identified. Allele frequency of this variant in ExAC_EAS was 0.0002 and
multiple lines of computational evidence supported a deleterious effect. Moreover, we found a canonical splice site mutation NM_000264.3:c.2757_2758delCT (p.Phe919Leufs*39) in PTCH1 was found, suggesting basal cell nevus syndrome rather than TS. Basal cell nevus syndrome is characterized by multiple nevoid basal cell epitheliomas and broad phenotypes, including cardiac fibroma and intracranial calcification22. In patient P954, a complex-4 160-bp deletion (2: 228230706-228234866) in the TM4SF20 gene was found, which consisted of two microdeletions separated by a 100-bp gap. Prenatal sonography of the fetus showed a widened left lateral ventricle and abnormal hypoechoic signals around the bilateral anterior horns. This intragenic ancestral deletion is reported to be segregated with early language delay disorders and cerebral white matter hypointensity (WMH) in 15 unrelated families predominantly from Southeast Asian populations23. In that study, one severely affected patient, born prematurely at 33-weeks of gestation, presented gross enlargement of the third and lateral ventricles with near-complete loss of periventricular white matter, while other patients showed milder but varying degrees of WMH. In the present study, variants of NF1 were found in two cases, i.e., P114 and P884. Ultrasonography revealed that P114 had hydrocephalus and clubfoot, whereas P884 was found to have enlarged lateral and third ventricles, a missing septum pellucidum, and a cleft palate. These findings were suspected to be tuberous sclerosis (TS) but ultrasound and MRI manifestation of the intracranial lesions were atypical. WGS identified no potential deleterious variants in TSC1 and TSC2. Instead, a frameshift mutation NM_000264.3:c.2757_2758delCT (p.Phe919Leufs*39) in PTCH1 was found, suggesting basal cell nevus syndrome rather than TS. Basal cell nevus syndrome is characterized by multiple nevoid basal cell epitheliomas and broad phenotypes, including cardiac fibroma and intracranial calcification22. In patient P954, a

Table 2. Diagnostic SNVs identified by WGS.

| No. | Gene     | HGVSnom         | Zygosity | Classification | Related disease and inheritance |
|-----|----------|-----------------|----------|----------------|---------------------------------|
| 49  | KAT6B    | c.3747del(Gly1251Glufs*21) | Het P    | Genitopatellar syndrome, AD |
| 432 | STIL     | c.3835 C>T(p.Arg1279Cys)  | Het LP   | Microcephaly 7, primary, AR |
|     | STIL     | c.2344_2347delTC(G.p.Leu782Thrfs*2) | Het P    |                                   |
| 211 | CC2D2A   | c.3829 T>C(p.Cys1277Arg)  | Het LP   | Joubert syndrome 9, AR |
|     | CC2D2A   | c.3874 G>T(p.Asp1292Tyr)   | Het LP   |                                   |
| 234 | KAT6B    | c.3660dup(p.Arg1221*1)    | Het P    | Genitopatellar syndrome, AD |
| 246 | TUBA1A   | c.748 G>T(p.Val250Phe)    | Het LP   | Lissencephaly 3, AD |
| 273 | GC2      | c.1125_1135delGCCTCTCTCTG(p.Alad379Glyfs*109) | Het LP   | Lymphatic malformation 3, AD |
| 348 | DNM1     | c.345_346delAG(p.Glu116Lysfs*6)  | Het LP   | Encephalopathy, AD |
| 357 | FGFR3    | c.1948A>G(p.Lys650Glu)    | Het P    | Thalassemic dysplasia, AD |
| 431 | TUBA1A   | c.614 A>T(p.Asp205Val)    | Het LP   | Lissencephaly 3, AD |
| 464 | PDHA1    | 3c.923_929delAGGAAGT(p.Ser312Valfs*1) | Het LP   | Pyruvate dehydrogenase E1 deficiency, XLD |
| 479 | GLI2     | c.94dup(p.Ala32Glyfs*34)   | Het LP   | Culler-Jones syndrome |
| 558 | PTPN11   | c.1403 C>T(p.Thr468Met)   | Het P    | Noonan syndrome 1, AD |
| 627 | NSD1     | c.5177 C>T(p.Pro1726Leu)  | Het LP   | Sotos syndrome, AD |
| 648 | FOXY1    | c.171_180delCCCGCGGCCG(p.Pro60Argfs*129) | Het LP   | Rett syndrome, congenital variant, AD |
| 730 | FANC    | c.1330-1G>A           | Homo LP  | Fanconi anemia, complementation group C, AR |
| 733 | SIX3     | c.339 G>A             | Het P    | Holoprosencephaly 2, AD |
| 836 | SOX2     | c.480 C>G              | Het P    | Optic nerve hypoplasia and abnormalities of CNS, AD |
| 903 | OFD1     | c.1103_1106delGTAT(p.Ile369Lysfs*18) | Het LP   | Orofaciodigital syndrome I, XLD |
| 924 | ARX      | c.1074-1G>A           | Het LP   | Lissencephaly, XL2 |
| 942 | NID1     | c.1786C>T(p.Arg596)*    | Het LP   | Dandy-Walker malformation, AD |
| 993 | PDHA1    | 3c.933_936delAGAT(p.Ser312Argfs*13) | Het P    | Pyruvate dehydrogenase E1 deficiency, XLD |
| 1278 | CC2D2A   | c.4333 C>T(p.Arg1445*) | Homo P   | Meckel syndrome 6, AR |
| 222 | PTC1     | 3c.2757_2758delCT(p.Phe919Leufs*39) | Het LP   | Basal cell nevus syndrome, AD |
| 114 | NF1      | c.1742dup(p.Leu581Phefs*6) | Het P    | Neurofibromatosis, type I, AD |

Imaging manifestations as phenotypes of molecular diagnosis

Patient phenotypes are extremely useful in narrowing candidate genes in untargeted sequencing such as ES and WGS21. Sequencing results are also of great significance for clarifying prenatal sonographic diagnosis. For example, P222 showed ventriculomegaly, hyperechoic lesions in the periventricular zone, and cardiac space-occupying lesions (suspected rhombomymom) accompanied by pericardial and pleural effusion, as seen in Fig. 4a–d. These findings were suspected to be tuberous sclerosis (TS) but ultrasound and MRI manifestation of the intracranial lesions were atypical. WGS identified no potential deleterious variants in TSC1 and TSC2. Instead, a frameshift mutation NM_000264.3:c.2757_2758delCT (p.Phe919Leufs*39) in PTCH1 was found, suggesting basal cell nevus syndrome rather than TS. Basal cell nevus syndrome is characterized by multiple nevoid basal cell epitheliomas and broad phenotypes, including cardiac fibroma and intracranial calcification22. In patient P954, a
In this cohort of pregnancies with fetal CNS anomalies, a primary genetic diagnosis was achieved in 38.3% (62/162) of cases based on WGS. Although commonly used CMA plus ES was not applied here for direct comparison with WGS, several previous studies on diagnostic yields of genetic testing at different resolution levels were reviewed and those with relatively large sample sizes are summarized in Table 3. At the microscopic level, we found that the low-pass WGS diagnostic rate was close to that of karyotyping, although balanced translocation and triploid analyses were not applicable using our WGS approach. Among the cases without microscopic genetic diagnosis, low-pass WGS found additional causative CNVs in 2.3% of cases at the sub-microscopic level.

As shown in Table 3, diagnostic SNVs were found in 14.8% of the cohort, which increased to an 18.9% diagnostic rate after uninformative low-pass WGS. However, ES diagnostic yields of fetal anomalies show considerable variation. For example, in a prospective cohort study of 243 maternal-fetal samples, Petrovski et al. reported an over-diagnosis rate of 10% among fetuses with prenatal structural abnormalities based on trio WES, with diagnostic genetic variation in the CNS subgroup of 22% (11/49). In another large prenatal ES study of 610 nuclear families, Lord et al. reported a diagnostic yield of 4.3% in 69 fetuses with brain anomalies. Fetuses with abnormal karyotypes or relevant CNVs were excluded in both studies. These variations among studies may be due to different sample sizes, fetal phenotype compositions, or criteria of variant classification and patient exclusion. In the current study, the fetuses were studied retrospectively, rather than selected based on previous genetic results or genetic etiology. This was because the main purpose of our research was not only to evaluate the diagnostic capabilities of WGS, but also to demonstrate the genomic architecture of common abnormal ultrasound manifestations, and thus ensure that appropriate genetic testing tools are applied in prenatal settings and screenings.
It is worth noting that intragenic CNVs and their possible causal relationships with fetal anomalies were systematically evaluated in the current study. Although several genetic testing panels designed for specific diseases may involve probes or primers for detecting intragenic CNVs, they have not been used in a wide range of disease-related genes. Their application scope is even more limited prenatally as fetal abnormalities are difficult to diagnose differentially and the genetic background of fetal structural anomalies is highly heterogeneous. Although WES has become a primary strategy for sequencing in patients with suspected genetic diseases, CNV detection remains challenging due to its technological characteristics. Compared with CNVs identified using deep WGS data from the same sample, common algorithms of CNVs calling using WES data suffer from limited power. However, intragenic CNVs may account for a substantial proportion of pathogenic variants. Truty et al. investigated the prevalence and properties of intragenic CNVs in >143,000 individuals referred for genetic testing and found a 10% prevalence of intragenic CNVs among individuals with a positive test result and high frequencies in neurological diseases. In this study, intragenic CNV analysis identified three possible pathogenic intragenic CNVs, resulting in an additional diagnosis rate of 2.9%. In addition, we identified other endogenous CNVs encompassing internal exons and predicted that they would adversely affect the transcriptional reading frame of genes with loss-of-function mechanisms. However, we found no conclusive evidence that they were related to abnormalities in the fetal CNS. Therefore, additional WGS studies are required to establish prenatal ultrasound manifestations with intragenic CNVs.

The HPO database can be used to select candidate genes. However, prenatal ultrasound results of the brain may be non-specific or evolving, and minor abnormalities may be missed or undiagnosed. For example, although experienced experts can detect signs of abnormal cortical development through advanced neurosonography and MRI, these anomalies are difficult to confirm mid-trimester. In the current study, using WGS, enlargement of the lateral ventricle and subarachnoid space was confirmed to be lissencephaly (e.g., in P431 and P796). Therefore, to enhance genetic diagnosis of abnormalities in the fetal CNS, describing abnormal ultrasound findings may be more useful than performing disease diagnosis.

An increased burden of rare deleterious variants in genes, pathways, and genomes has been found in neurological and developmental disorders, such as schizophrenia, epilepsy, and autism spectrum disorder. In this study, rare singleton deleterious variants, including predicted missense and loss-of-function variants with different MAF frequencies (i.e., <0.01, 0.001, 0), were examined in the 136 cases and 200 controls. Compared with the control group, only rare SLoFVs were statistically enriched in the cohort. Chen et al. has reported the accumulation of rare SLoFVs in NTDs. In our study, despite this trend, the increase in SLoFVs in NTDs was not significant, which may be due to the small NTD sample size in our study.

Fig. 3 Accumulation of rare singleton deleterious variants in fetal CNS cohort. Distribution of rare singleton deleterious missense variants of a MAF = 0; b MAF ≤ 0.01. Accumulation of rare SLoFVs of c MAF = 0, with median of 8 in cases (solid line) and 6 in controls (dashed line) with \( P = 1.7 \times 10^{-9} \) by two-sided Wilcoxon test and d MAF ≤ 0.01, with median of 13.5 in cases and 9 in controls with \( P = 2.2 \times 10^{-16} \) by two-sided Wilcoxon test. SLoFVs singleton loss-of-function variants.
Though WGS has an absolute advantage in variant detection, there has long been concern that WGS may require more fetal DNA, longer turnaround time, and higher cost\(^8\). These concerns were not warranted. The required DNA depends on the library construction protocols, not sequencing itself. Usually, 1 μg of genomic DNA is used as the standard protocol for testing multiple products across popular platforms\(^9,10\), with some kits requiring even less DNA. The TruSeq Exome Library Prep protocol (November 2015) uses 100 ng of starting DNA with Covaris fragmentation. The MGI Easy Universal DNA Library Prep Set requires only 0.5–50 ng if fetal DNA is extremely limited. Furthermore, WGS does not need additional DNA for CNV analysis, because both low-pass and deep WGS only require a single WGS sequencing library. In regard to turnaround time, it usually takes 2–15 weeks to obtain and interpret ES results\(^7\). In contrast, the WGS turnaround time in our study was 2–3 weeks per sample for interpretation of chromosomal anomalies, CNVs, SNVs, and small CNVs, and another week for Sanger validation. Thus, for routine protocols, WGS is as fast as ES, and if the extra time required for CMA is considered as a prerequisite for ES, WGS is even faster. In addition, on the BGI platform, the sequencing costs of WGS are lower than those of CMA plus ES, given that CMA costs about US$600. In view of the decline in the cost of human genome sequencing, the price difference between WGS and WES is now about US$600. For example, Illumina introduced US$600 genome sequencing with the launch of its NovaSeq™ 6000 v1.5 kit in 2020\(^11\). BGI currently offers US$500 genome sequencing with its T7 platform and has introduced US$100 genome sequencing with its new DNBSSEQ Tx platform and CoolMPS sequencing kits\(^12\).

Although WGS is a suitable method for identifying fetal CNS abnormalities, our approach has some limitations. Firstly, triploids were not analyzed in this study. Furthermore, due to the lack of large-scale databases, it was difficult to determine the clinical significance of variants in introns and noncoding areas. Even though we did find some variants in introns that were previously reported as likely pathogenic, we considered that they were more likely to be benign due to their high frequency in the control database. As a continuation of this preliminary study on abnormalities in the CNS, we are now studying more cases of fetal ultrasound abnormalities in each anatomical system, which should hopefully further prove the potential power of WGS in genetic screening.

**METHODS**

**Patient identification, ultrasound examination, and sample collection**

Patients were found by a multi-level referral system of prenatal screening and diagnosis between 2015 and 2017. This referral system was launched by the Maternal and Child Health Hospital of Hubei Province (MCHH, China) and includes 156 prenatal screening units and 32 diagnostic centers across Hubei and several districts in middle and southwestern China. All screening units and diagnostic centers received training from the MCHH following the practice guidelines of the International Society of Obstetrics & Gynecology (ISUOG)\(^52\). If fetal structural anomalies were detected during routine ultrasound scans at any of these units or centers, patients were transferred to the MCHH for systematic prenatal ultrasound by at least two chief physicians to obtain a final diagnosis. MRI was further provided upon patient/family acceptance. Those patients who donated invasive testing samples, abortion samples, or birth samples were invited to participate in a congenital defect study. Here, patients diagnosed with fetal CNS anomalies were identified from the congenital defect study by reviewing medical records and images. In total, 162 patients who provided a fetal sample and written informed consent were included (161 with umbilical cord tissue samples collected after termination of pregnancy, and one amniotic fluid sample). Demographic information was asked of the pregnant women, but they could refuse to answer. WGS was performed after pregnancy outcome (i.e., abortion or live birth), and thus outcome was not influenced by the WGS results. This project followed Chinese state and local regulations related to biological and medical research and was approved by the MCHH and BGI Ethics Committee (BGI-IRB17039).

**Whole-genome sequencing**

Genomic DNA from fetal tissue was extracted using the salting-out method, as described in Miller et al\(^13\). To be simplified, approximately of 200 mg tissue was diced and homogenated with 2 ml of lysis buffer(10 mM Tris, 400 mM NaCl and 2 mM Na2EDTA). Then the tissue lysates were incubated at 65°C with 0.2 ml of 10% SDS and 0.5 ml of a protease K solution (1 mg protease K in 1% SDS and 2 mM Na2EDTA) for 30 min. After incubation, 2 ml of 6 M NaCl was added and vigorously shaken several times, followed by centrifugation at 12,000 rpm for 5 min. The supernatant
### Table 3. Comparison of diagnostic yields for variants at different resolutions.

| Resolution          | Publications | Present study |
|---------------------|-------------|---------------|
|                      | CNS total   | CNS only      |
|                      | CNS + other | CNS only      |
| Microscopic level    | 18.4% (37/201) | 4.3% (21/48) |
| Balanced translocation, triploid | 9.6% (11/114) | NA |
| Submicroscopic level | 2% (4/201)  | 1.9% (2/103)  |
| CNA                  | 6.0% (19/317) | 6.0% (19/317) |
| Intragenic CNV (50 bp – 100 kb) | 3.4% (9/263) | NA |
| NA not applicable    | 11.9% (12/103) | 3.4% (9/263) |

- **CNS**: Common Nervous System
- **CNA**: Copy Number Alteration
- **CNV**: Copy Number Variation
- **SNV**: Single Nucleotide Variation

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**Variant calling and annotation**

Chromosomal anomalies, including aneuploids and rare CNVs, were called based on the pipeline developed by Dong et al.55. Deep sequencing data were aligned to the human reference genome GRCh37/hg19 and SNVs were called and filtered using the Edico Genome DRAGEN Bio-IT Platform66. Small CNVs were called using the SpeedSeq SV pipeline (v.0.0.3a)67. Small CNVs and SNVs were annotated using bcfanno (v1.4) (https://github.com/shiquan/bcfanno) with frequencies from several public databases (ExAC68, GnomAD69, and G100060) and an in-house population database containing 790 samples sequenced to ~40X depth using the same sequencing and analysis platforms.

**Primary diagnosis**

Variants were interpreted by a clinical review panel consisting of geneticists, senior sonographers, MRI radiologists, and obstetrician-gynecologists to decide the clinical significance and relevance of the fetal imaging phenotypes.

First, aneuploids and CNVs were manually classified by geneticists into five categories: i.e., pathogenic, likely pathogenic, uncertain significance (VUS), likely benign, and benign, as per the American College of Medical Genetics and Genomics (ACMG) guidelines for the evaluation of CNV pathogenicity: pathogenic ≥0.99, likely pathogenic 0.90–0.9861, Pathogenic or likely pathogenic CNVs were further evaluated if they showed evidence related to fetal imaging manifestations. Relevant pathogenic or likely pathogenic CNVs were designated as primary diagnostic variants.

The SNVs were filtered before manual interpretation. First, SNVs with minor allele frequencies (MAF) >0.01 in the public and in-house databases or in introns and noncoding areas were filtered out. Imaging manifestations for each fetal sample were transformed to related Human Phenotype Ontology (HPO) terms62. Genes related to HPO terms in the HPO database formed a fetus-specific phenotype list. SNVs not found on any gene or not on genes in the candidate gene list were filtered out. The remaining SNVs underwent the same diagnostic assessment as CNVs but the classification criteria followed the ACMG guidelines for SNV interpretation63.

If primary diagnostic SNVs were not identified, then the interpretation processes of CNVs were applied to small CNVs to determine primary diagnosis.

**Incidental and secondary findings**

Variants previously classed as pathogenic/likely pathogenic in the ClinVar Archive (https://www.ncbi.nlm.nih.gov/clinvar/) or as protein-truncating variants affecting a known disease gene via a loss-of-function mechanism but lacking evidence to support its association with prenatal phenotypes were classified as incidental findings. Secondary diagnostic findings were defined as incidental findings based on the ACMG recommendation list64.

**Rare singleton deleterious variant analysis**

The SNVs identified by deep WGS were filtered to retain high-quality variants only. The filtering criteria were read depth ≥20, altered allelic read depth ≥3, and variants ≤5 bp. Deleterious variants included missense variants predicted to be damaged based on the following prediction algorithms: SIFT65 and CADD score >2066, M-CAP >0.02567, and REVEL >0.368, as well as loss-of-function variants, including nonsense, frameshift, canonical splice sites, and stop gain/loss. Singleton variants were those identified only once in the cohort. The number of singleton deleterious variants with MAFs from 0 to 0.001 in the public databases were calculated and compared in cases and controls. The case group consisted of 136 samples in the cohort where deep WGS data were available, and the...
control group consisted of 200 randomly selected individuals from the in-house population database mentioned above.

Validation
Primary pathogenic or likely pathogenic SNVs were validated by Sanger sequencing. Microduplications were validated by quantitative polymerase chain reaction (qPCR) and microdeletions were validated by PCR and agarose gel electrophoresis. Primers were listed in Supplementary Table 6.

Reporting summary
Further information on research design is available in the Nature Research Reporting Summary linked to this article.

DATA AVAILABILITY
Data are uploaded to NCBI with accession number PRJNA798107. Variation data can be accessed in dbSNP with the link https://www.ncbi.nlm.nih.gov/SNP/snp_viewTable.cgi?handle=YY_MCH. Data are also publicly available in the China National GeneBank Database (CNGB) Nucleotide Sequence Archive (Accession Number: CNP0002431).

CODE AVAILABILITY
All analyses in this study were performed using Edico Genome v2.4.1, SpeedSeq 5V pipeline (v.0.03a4; bcftool v1.4), R version 3.5.2, Python 3.6, Custom R and Python scripts for the singleton variant analyses are available upon request.

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REFERENCES
1. Blencowe, H., Kancherla, V., Moorthie, S., Darlison, M. W. & Modell, B. Estimates of global and regional prevalence of neural tube defects for 2015: a systematic analysis. Ann. N. Y. Acad. Sci. 1414, 31–46 (2016).
2. International Society of Ultrasound in, O. & Gynecology Education, C. Sonographic examination of the fetal central nervous system: guidelines for performing the ‘basic examination’ and the ‘fetal neurosonogram’. Ultrasound Obstet. Gynecol. 26, 1110–1114 (2006).
3. Van den Veyver, I. B. Prenatally diagnosed developmental abnormalities of the fetal central nervous system and genetic syndromes: a practical review. Prenat. Diagn. 39, 666–678 (2019).
4. Wapner, R. J. et al. Chromosomal microarray versus karyotyping for prenatal diagnosis. N. Engl. J. Med. 367, 2175–2184 (2012).
5. Paladini, D. et al. Accuracy of neurosonography and MRI in clinical management of fetuses referred with central nervous system abnormalities. Ultrasound Obstet. Gynecol. 44, 188–196 (2014).
6. Van den Veyver, I. B. Prenatally diagnosed developmental abnormalities of the central nervous system and genetic syndromes: a practical review. Prenat. Diagn. 39, 666–678 (2019).
7. Wetmanowski, H., Szychta, P., Blochowiak, K., Jundzill, A. & Czajkowski, R. Basal cell nevus syndrome (Gorlin-Goltz syndrome); genetic predisposition, clinical picture and treatment. Postepy Dermatol. Alergol. 34, 381–387 (2017).
8. Liszewski, W. et al. TM4SF20 ancestral deletion and susceptibility to a pediatric disorder of early language delay and cerebral white matter hyperintensities. Am. J. Hum. Genet. 93, 197–210 (2013).
9. Lord, J. et al. Prenatal exome sequencing analysis in fetal structural anomalies detected by ultrasound. J. Ultrasound 182, 210–216 (2017).
10. Li, Y., Anderson, L. A., Ginns, E. I. & Devlin, J. J. Cost-effectiveness of karyotyping, chromosomal microarray analysis, and targeted next-generation sequencing of patients with unexplained global developmental delay or intellectual disability. Mol. Diagn. Ther. 22, 129–138 (2018).
11. Tsiplova, K. et al. A microcosting and cost-consequence analysis of clinical genomic testing strategies in autism spectrum disorder. Genet. Med. 19, 1268–1275 (2017).
12. Schwarze, K., Buchanan, J., Taylor, J. C. & Wordsworth, S. Are whole-exome and whole-genome sequencing approaches cost-effective? A systematic review of the literature. Genet. Med. 20, 1122–1130 (2018).
13. van Nimwegen, K. J. et al. Is the $1000 genome as near as we think? A cost analysis of next-generation sequencing. Clin. Chem. 62, 1458–1464 (2016).
14. Phillips, K. A., Fletcher, M. J. & Ladabaum, U. Is the “$1000 Genome” really $1000? understanding the full benefits and costs of genomic sequencing. Technol. Health Care 23, 373–379 (2015).
15. Hayden, E. C. Technology: The $1,000 genome. Nature 507, 294–295 (2014).
16. Normand, E. A. et al. Clinical exome sequencing for fetuses with ultrasound abnormalities and a suspected Mendelian disorder. Genome Med 10, 74 (2018).
17. Swaminathan, R. et al. Clinical exome sequencing reports: current informatics practices and future opportunities. J. Am. Med. Inform. Assoc. 24, 1184–1191 (2017).
18. Meng, L. et al. Use of exome sequencing for infants in intensive care units: ascertainment of severe single-gene disorders and effect on medical management. JAMA Pediatr. 171, e173438 (2017).
19. Wang, H. et al. Clinical utility of 24-h rapid trio-exome sequencing for critically ill infants. NPJ Genom. Med. 5, 20 (2020).
20. Sun, L. et al. Prenatal diagnosis of central nervous system abnormalities by high-resolution chromosomal microarray analysis. BioMed. Res. Int. 2015, 626379 (2015).
21. Fu, F. et al. Whole exome sequencing as a diagnostic adjunct to clinical testing in fetuses with structural abnormalities. Ultrasound Obstet. Gynecol. 51, 493–502 (2018).
22. Shaffer, L. G. et al. Detection rates of clinically significant genomic alterations by microarray analysis for specific anomalies detected by ultrasound. Prenat. Diagn. 32, 986–995 (2012).
23. Daniel, A. et al. Prospective ranking of the sonographic markers for aneuploidy: data of 2143 prenatal cytogenetic diagnoses referred for abnormalities on ultrasound. Aust. N. Z. J. Obstet. Gynaecol. 43, 16–26 (2003).
24. Tan, R. et al. An evaluation of copy number variation detection tools from whole-exome sequencing data. Hum. Mutat. 35, 899–907 (2014).
44. Truty, R. et al. Prevalence and properties of intragenic copy-number variation in Mendelian disease genes. Genet. Med. 21, 114–123 (2019).
45. Purcell, S. M. et al. A polygenic burden of rare disruptive mutations in schizophrenia. Nature 506, 185–190 (2014).
46. Leu, C. et al. Genome-wide polygenic burden of rare deleterious variants in sudden unexpected death in epilepsy. ElifeMedicine 2, 1063–1070 (2015).
47. Ji, J., Shimony, J., Gao, F., McKinstry, R. C. & Gutmann, D. H. Optic nerve tortuosity in children with neurofibromatosis type 1. Pediatr. Radiol. 43, 1336–1343 (2013).
48. Chen, Z. et al. Threshold for neural tube defect risk by accumulated singleton loss-of-function variants. Cell Res. 28, 1039–1041 (2018).
49. Chilamakuri, C. S. et al. Performance comparison of four exome capture systems for deep sequencing. BMC Genomics 15, 449 (2014).
50. Illumina, I. Illumina Announces the NovaSeq800 v1.5 Reagent Kit c with the $600 Genome, https://www.illumina.com/company/news-center/press-releases/2020/9c48adff-5b78-4e18-81e6-7c7b83d79f.html (2020).
51. LeMieux, J. MGI Delivers the $100 Genome at AGBT Conference, https://www.genengnews.com/news/mgi-delivers-the-100-genome-at-agbt-conference/ (2020).
52. Salomon, L. J. et al. Practice guidelines for performance of the routine mid-trimester fetal ultrasound scan. Ultrasound Obstet. Gynecol. 37, 116–126 (2011).
53. Miller, S. A., Dykes, D. D. & Polesky, H. F. A simple salting out procedure for extracting DNA from human nucleated cells. Nucleic Acids Res. 16, 1215 (1988).
54. Huang, J. et al. A reference human genome dataset of the BGISEQ-500 sequencer. Gigascience 6, 1–9 (2017).
55. Dong, Z. et al. Low-pass whole-genome sequencing in clinical cytogenetics: a validated approach. Genet. Med. 18, 940–948 (2016).
56. Miller, N. A. et al. A 26-hour system of highly sensitive whole genome sequencing for emergency management of genetic diseases. Genome Med. 7, 100 (2015).
57. Chiang, C. et al. SpeedSeq: ultra-fast personal genome analysis and interpretation. Nat. Methods 12, 966–968 (2015).
58. Leik, M. et al. Analysis of protein-coding genetic variation in 60,706 humans. Nature 536, 285–291 (2016).
59. Karczewski, K. J. et al. The mutational constraint spectrum quantified from variation in 141,456 humans. Nature 581, 434–443 (2020).
60. Genomes Project, C. et al. A global reference for human genetic variation. Nature 526, 68–74 (2015).
61. Riggs, E. R. et al. Technical standards for the interpretation and reporting of constitutional copy-number variants: a joint consensus recommendation of the American College of Medical Genetics and Genomics (ACMG) and the Clinical Genome Resource (ClinGen). Genet. Med. 22, 245–257 (2020).
62. Robinson, P. N. et al. The Human Phenotype Ontology: a tool for annotating and analyzing human hereditary disease. Am. J. Hum. Genet. 83, 610–615 (2008).
63. Richards, S. et al. Standards and guidelines for the interpretation of sequence variants: a joint consensus recommendation of the American College of Medical Genetics and Genomics and the Association for Molecular Pathology. Genet. Med. 17, 405–424 (2015).
64. Kalia, L. S. et al. Recommendations for reporting of secondary findings in clinical exome and genome sequencing, 2016 update (ACMG SF v2.0): a policy statement of the American College of Medical Genetics and Genomics. Genet. Med. 19, 249–255 (2017).
65. Ng, P. C. & Henikoff, S. SIFT: predicting amino acid changes that affect protein function. Nucleic Acids Res. 31, 3812–3814 (2003).
66. Rentzsch, P., Witten, D., Cooper, G. M., Shendure, J. & Kircher, M. CADD: predicting the deleteriousness of variants throughout the human genome. Nucleic Acids Res. 47, D686–D694 (2019).
67. Jagadeesh, K. A. et al. M-CAP eliminates a majority of variants of uncertain significance in clinical exomes at high sensitivity. Nat. Genet. 48, 1581–1586 (2016).
68. Ioannidis, N. M. et al. REVEL: an ensemble method for predicting the pathogenicity of rare missense variants. Am. J. Hum. Genet. 99, 877–885 (2016).

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AUTHOR CONTRIBUTIONS

F.C., S.Z., J.G., X.C., and X.X. designed the study. Y.Y., T.Z., and Y.H. analyzed the WGS data and the clinical review panel (Y.Y., S.Z., G.S., J.S., W.Y., L.W., X.Y., X.Z., B.R., J.Z., H.H., C.C., and X.C.) reviewed the images and clinical information and interpreted the variants. S.Z., X.Y., C.W., X.Z., H.H., and C.C. enrolled the patients and collected the samples. Z.Y. and H.Y. performed variant validation experiments. Y.Y. and X.C. wrote the manuscript. Y.Y., T.Z., N.Z., and J.Y. produced the figures and tables.

COMPETING INTERESTS

The authors declare no competing interests.

ADDITIONAL INFORMATION

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Correspondence and requests for materials should be addressed to Jian Guo, Xun Xu or Xinlin Chen.

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