Mate-pair low-pass genome sequencing for prenatal diagnosis of fetuses with ultrasound anomalies: abridged secondary publication

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KEY MESSAGES

1. DNA from 400 fetuses with ultrasound anomalies was examined using chromosomal microarray analysis (CMA) and low-pass mate-pair genome sequencing (GS) to identify clinically significant copy-number variants (CNVs), structural variants, and regions with absence of heterozygosity.
2. CMA and low-pass mate-pair GS reported diagnostic yields of 8.5% and 10.3%, respectively.
3. Low-pass mate-pair GS additionally identified cryptic CNVs (n=4) and mosaic CNVs (n=2), and reclassified 12 (60%) of 20 variants of uncertain significance identified by CMA.
4. Low-pass mate-pair GS classified 14 structural variants as variants of uncertain significance due to the unavailability of cultured cells for RNA sequencing or inconclusive RNA sequencing results in the submitted samples.
5. Low-pass mate-pair GS can serve as an alternative for the confirmation of fetal ultrasound anomalies.

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Introduction

In China, the rate of birth defects is estimated to be 5.6%. Ultrasonography and follow-up invasive prenatal diagnosis can provide prognostic information for the option of termination of pregnancy, as well as planning for future pregnancies including prenatal diagnosis and preimplantation genetic testing.

Next-generation sequencing, including low-pass genome sequencing (GS), has been used for prenatal copy-number variant (CNV) analysis with enhanced detection resolution and improved sensitivity for identifying mosaicism, compared with chromosomal microarray analysis (CMA).[1,2] Next-generation sequencing is recommended for germline structural variant detection by the American College of Medical Genetics and Genomics.[3] In our pilot studies, low-pass mate-pair GS (4-fold) enabled a comprehensive investigation of CNVs, structural variants, and regions with absence of heterozygosity.[4] We applied this method in a prospective cohort of 400 fetuses with ultrasound anomalies, along with CMA, to evaluate detection performance.

Methods

Pregnant women who underwent invasive genome testing for fetal ultrasound anomalies were recruited. DNA was extracted, and CMA was performed. Additionally, 500 ng of genomic DNA was fragmented into 3-to-8-kb fragments. Mate-pair library construction was performed and sequenced to >4-fold read depth.[4] CNVs, structural variants, and regions with absence of heterozygosity were identified using our previously reported method[5] and then validated by breakpoint-junction-specific polymerase chain reaction and Sanger sequencing, as well as quantitative polymerase chain reaction.

RNA sequencing (RNA-seq) with enrichment of messenger RNA was performed from cultured cells of chorionic villi or amniotic fluid, generating >10 Gb of data per sample. Paired reads were aligned to the human reference genome (hg19) using STAR. Expression levels (in transcripts per million) of candidate genes were compared with those of our in-house gestation-matched controls.

Results

Among 400 fetuses with ultrasound anomalies, CMA yielded a diagnosis in 34 (8.5%) cases with aneuploidies, pathogenic/likely pathogenic CNVs, and suspected parental consanguinity, whereas low-pass mate-pair GS yielded a diagnosis in 41 (10.3%) cases (Table). Both methods consistently identified 14 cases with aneuploidies; however, low-pass mate-pair GS additionally identified a case (with a negative CMA finding) of mosaic trisomy 12 at a 20% mosaic level, which was confirmed by karyotyping (12/80 cells).

TABLE. Detection yields provided by chromosomal microarray analysis and low-pass mate-pair genome sequencing

Classification	No. of cases detected				
	Chromosomal microarray analysis	Low-pass mate-pair genome sequencing	Low-pass mate-pair genome sequencing		
			Copy-number variants / aneuploidies	Structural variants	Regions with absence of heterozygosity
Pathogenic/likely pathogenic	34	41†	37*	5*	3
Variant of uncertain significance	20	20	6	14	0

* Four cases with pathogenic/likely pathogenic copy-number variants were involved in translocation (n=3) and insertion (n=1).
† Seven cases had additional pathogenic findings identified by low-pass mate-pair genome sequencing, including one mosaic aneuploidy, one mosaic deletion, four pathogenic cryptic deletions, and one *de novo* insertion.

Both methods consistently identified 17 cases with pathogenic/likely pathogenic CNVs; additionally, low-pass mate-pair GS identified six other cases of pathogenic/likely pathogenic CNVs. Three of the cases were affected by homozygous Southeast Asian-type thalassaemia;[1] a mosaic 9Mb 15q terminal deletion at a 20% level (Fig 1) was detected in a fetus with increased nuchal translucency (NT) and generalised hydrops (22B2742), which had been missed by CMA. Furthermore, in case 22B0083 with a ventricular septal defect, low-pass mate-pair GS identified a 35-kb intragenic deletion involving the second exon of *RBFOX2*. Given that *de novo* loss-of-function mutations or deletions encompassing *RBFOX2* have been found in congenital heart disease probands,[6,7] this deletion was considered likely pathogenic. Finally, case 22C1583 was referred (increased NT, positive Down syndrome screening, and left ventriculomegaly) for CMA, which reported a *de novo* duplication of 3.5 Mb, whereas low-pass GS indicated an ins(2;13)(q14.2;q31.3) insertion involving this duplication (Fig 2). Haploinsufficient *GLI2* is located downstream of the insertion site, and an over 2-fold reduction of *GLI2* expression was observed compared with the control.

Low-pass mate-pair GS also identified the exact genomic locations and orientations of pathogenic/likely pathogenic deletions. Three cases with terminal deletions and duplications occurring simultaneously were attributed to parental translocations. In case 23B0659 with hydrocephaly and a ventricular septal defect, two interstitial deletions in 14q were reported by CMA, but only a smaller deletion was confirmed in the father (Fig 3). Low-pass mate-pair GS indicated a complex insertion involving chromosomes 1, 14, and 22 in the father, in which chromosome 14 was fragmented into eight segments. Segments 3 and 7 of chromosome 14 were inserted into chromosome 1, whereas segments 2 and 4 were inserted into chromosome 22; segment 6 was lost. The fetus inherited only the derivative chromosome 14, which explained the discrepancies between deletions identified in the fetus and those identified in the father.

CMA reported 15 duplications as variants of uncertain significance in 15 cases due to the involvement of known disease-causing gene(s) through a loss-of-function mechanism at the CNV boundary. Of these, 11 (73.3%) were defined as forward tandem duplications by low-pass mate-pair GS; they were unlikely to lead to disease via a loss-of-function mechanism because genes involved at the CNV boundaries remained intact. Overall, among 20 cases with variants of uncertain significance reported by CMA, 11 were reclassified as likely benign and one as likely pathogenic, resulting in a 60% revision of classification for variants of uncertain significance.

Low-pass mate-pair GS also identified six CNVs as variants of uncertain significance. In case 23C0031 with increased NT and a prenatal skin fold of 4.07 mm, low-pass mate-pair GS identified a 12-kb intragenic heterozygous deletion in *DCBLD2*, the deletion of which causes cardiomyopathy[8] in an autosomal recessive manner. A reduction of RNA expression and aberrant RNA splicing were observed, warranting investigation. Further studies are needed to determine whether an additional point mutation arose through a compound heterozygosity mechanism.

Low-pass mate-pair GS identified four translocations (one balanced), 28 inversions, and 26 insertions with or without cryptic duplications/deletions. In case 22C1007 with increased NT and positive Down syndrome screening (CMA negative), a *de novo* balanced translocation t(3;6)(p14.1;q16.1) was identified and then confirmed by karyotyping; however, RNA-seq could not be performed due to failure of cell re-culture. Concerning inversions, sizes varied from 12 kb to 2.6 Mb. None was classified as pathogenic/likely pathogenic because no haploinsufficient disease-causing gene was directly disrupted or no conclusive evidence from RNA-seq was available. With respect to structural rearrangements, low-pass mate-pair GS identified four cases with pathogenic findings (three unbalanced translocations and one insertion) and 14 cases with variants of uncertain significance.

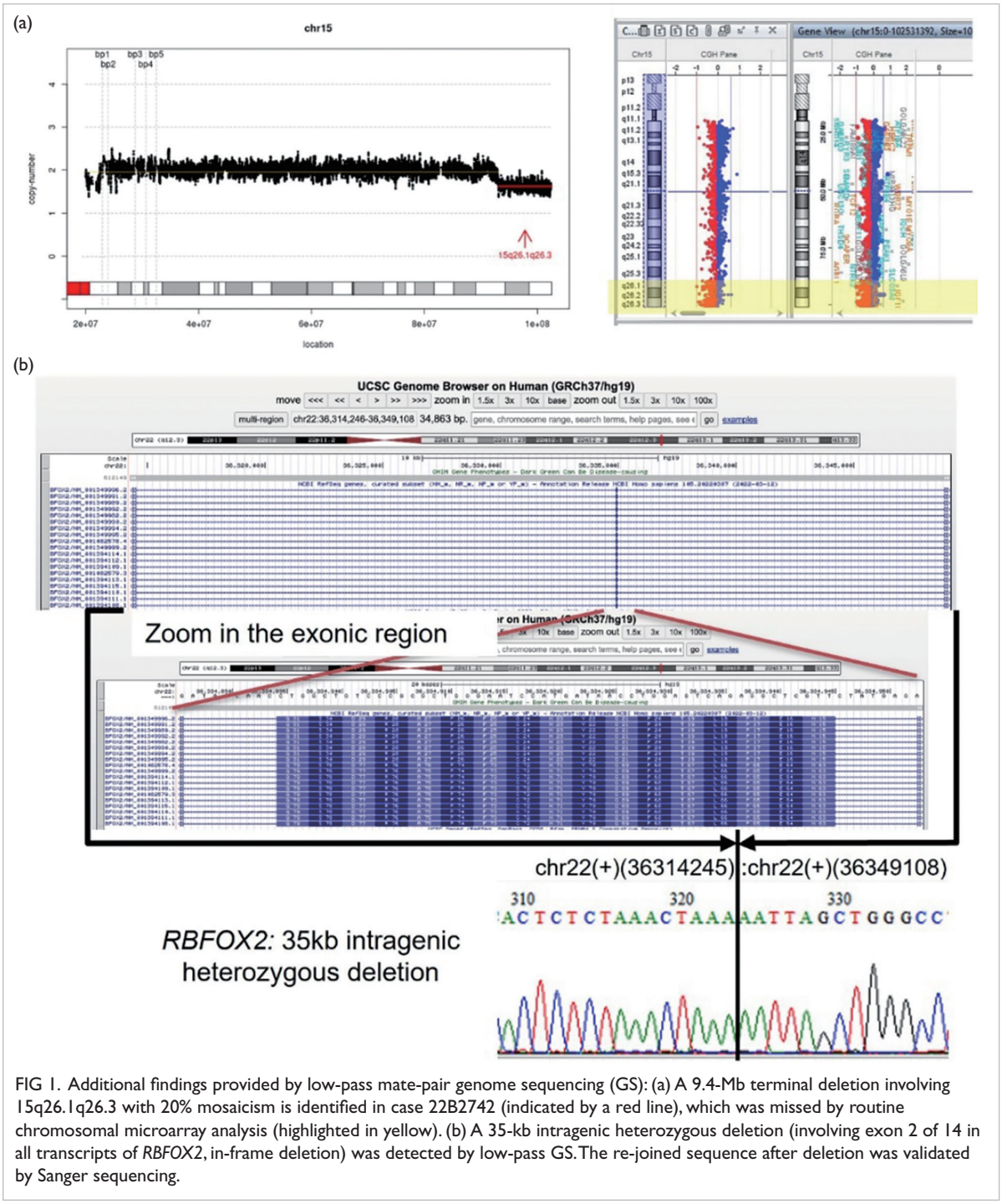

FIG 1. Additional findings provided by low-pass mate-pair genome sequencing (GS): (a) A 9.4-Mb terminal deletion involving 15q26.1q26.3 with 20% mosaicism is identified in case 22B2742 (indicated by a red line), which was missed by routine chromosomal microarray analysis (highlighted in yellow). (b) A 35-kb intragenic heterozygous deletion (involving exon 2 of 14 in all transcripts of *RBFOX2*, in-frame deletion) was detected by low-pass GS. The re-joined sequence after deletion was validated by Sanger sequencing.

Absence of heterozygosity analysis indicated that three (0.8%) cases potentially involved parental consanguinity, warranting further investigation into homozygous point mutations that contributed to the fetal anomalies.

The average turnaround time was 9 days (including 3.5 days for sequencing and automated data analysis without hands-on processing) for low-pass mate-pair GS and 5 days for CMA. The reagent costs were HK$1700 for low-pass mate-pair GS and HK$1720 for CMA.

Discussion

We prospectively investigated detection performance of low-pass mate-pair GS and CMA in 400 fetuses with ultrasound anomalies. Low-pass mate-pair GS provided an additional 1.8% diagnostic yield compared with CMA (10.3% vs 8.5%); it also revised CNV classification in 60% (12/20) of cases with variants of uncertain significance identified by CMA.

Low-pass mate-pair GS demonstrated its

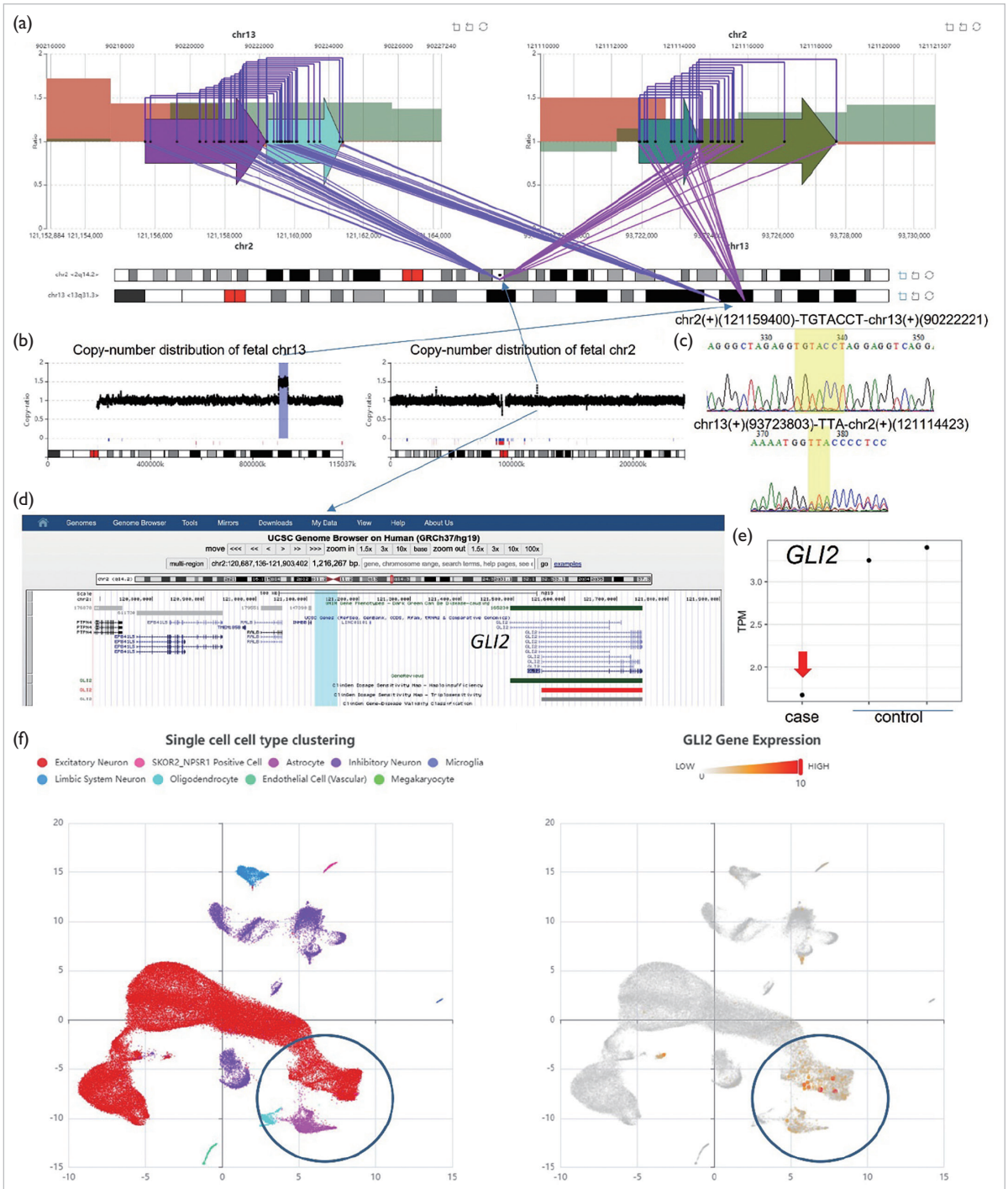

FIG 2. A *de novo* insertion identified by low-pass mate-pair genome sequencing: (a) and (b) Chimeric read-pair and copy-ratio analysis revealed an insertion ins(2;13)(q14.2;q31.3). (c) Breakpoint-junction-specific polymerase chain reaction and Sanger sequencing confirmed the rearrangement. (d) Haploinsufficient *GLI2* is located downstream of the insertion site. (e) An >2-fold reduction of expression in the case compared with gestational-week-matched controls was observed. (f) Single-cell RNA sequencing indicates that *GLI2* is highly expressed in astrocytes and excitatory neurons.

advantage in detecting small CNVs, balanced translocations, and inversions; it revealed a high prevalence of rare CNVs and structural variants in the population, highlighting the need for a large population-based dataset to aid variant interpretation. Additionally, low-pass mate-pair GS detected and delineated the composition of genomic changes involving CNVs, such as insertions and complex structural rearrangements, resulting in revisions to clinical management for families. Our findings suggest that low-pass mate-pair GS can serve as an alternative for the confirmation of fetal ultrasound anomalies, with potential extension to other indications.

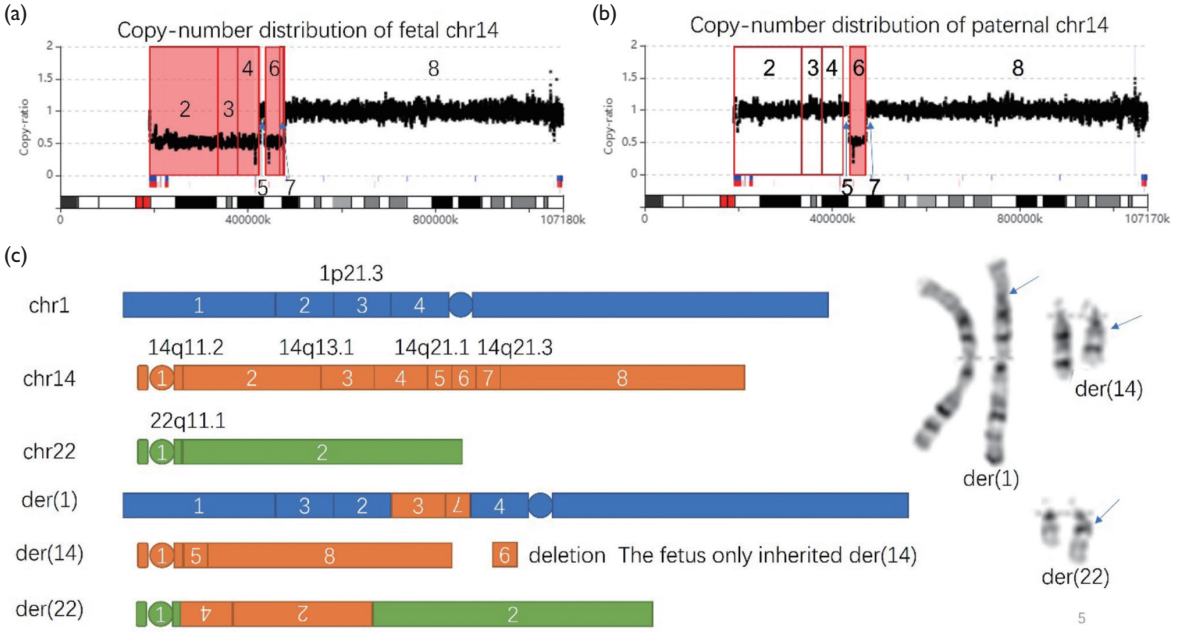

FIG 3. Genomic composition revealed by low-pass mate-pair genome sequencing: Copy-number distribution of chromosome 14 in the (a) fetus (23B0659) and (b) father. Two interstitial heterozygous deletions are identified in the fetus, but only one smaller deletion is identified in the father (segment 6). (c) A complex rearrangement involving chromosomes 1, 14, and 22 is shown in the father, but the fetus inherits only the derivative chromosome 14. (d) Karyotyping analysis of the father confirms the finding. Each arrow indicates the location of abnormalities in the derivative chromosome.

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Disclosure

The results of this research have been previously published in:

1. Zhou Z, Tan C, Chau MHK, et al. TEDD: a database of temporal gene expression patterns during multiple developmental periods in human and model organisms. Nucleic Acids Res 2023;51:D1168-D1178.
2. Li K, Zhao Y, Chau MHK, et al. Low-pass genome sequencing-based detection of paternity: validation in clinical cytogenetics. Genes (Basel) 2023;14:1357.
3. Qian J, Wang H, Liang H, et al. Mate-pair sequencing enables identification and delineation of balanced and unbalanced structural variants in prenatal cytogenomic diagnostics. Clin Chem 2025;71:155-68.

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References

1. Wang H, Dong Z, Zhang R, et al. Low-pass genome sequencing versus chromosomal microarray analysis: implementation in prenatal diagnosis. Genet Med 2020;22:500-10.
2. Chaubey A, Shenoy S, Mathur A, et al. Low-pass genome sequencing: validation and diagnostic utility from 409 clinical cases of low-pass genome sequencing for the detection of copy number variants to replace constitutional microarray. J Mol Diagn 2020;22:823-40.
3. Raca G, Astbury C, Behlmann A, et al. Points to consider in the detection of germline structural variants using next-generation sequencing: a statement of the American College of Medical Genetics and Genomics (ACMG). Genet Med 2023;25:100316.
4. Dong Z, Zhao X, Li Q, et al. Development of coupling controlled polymerizations by adapter-ligation in mate-pair sequencing for detection of various genomic variants in one single assay. DNA Res 2019;26:313-25.
5. Dong Z, Chau MHK, Zhang Y, et al. Deciphering the complexity of simple chromosomal insertions by genome sequencing. Hum Genet 2021;140:361-80.
6. Homsy J, Zaidi S, Shen Y, et al. De novo mutations in congenital heart disease with neurodevelopmental and other congenital anomalies. Science 2015;350:1262-6.
7. Glessner JT, Bick AG, Ito K, et al. Increased frequency of de novo copy number variants in congenital heart disease by integrative analysis of single nucleotide polymorphism array and exome sequence data. Circ Res 2014;115:884-96.
8. Alhamoudi KM, Barhoumi T, Al-Eidi H, et al. A homozygous nonsense mutation in DCBLD2 is a candidate cause of developmental delay, dysmorphic features and restrictive cardiomyopathy. Sci Rep 2021;1:12861.