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Development of cytogenomics for prenatal diagnosis: from chromosomes to single nucleotides: a review

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Prenatal diagnosis encompasses traditional cytogenetics and molecular-based techniques. In the new era of genomics, challenge to prenatal diagnosis has led to revised diagnostic strategies. In this review, we discuss the application of chromosomal microarray and a new prenatal diagnosis workflow in the public setting in Hong Kong. Using this prenatal diagnosis workflow, up to 40% of fetuses with structural anomalies can be identified with an underlying genetic aetiology, leaving the majority of cases undiagnosed. With the advancement of next generation sequencing, we are able to tackle the challenge of investigating chromosomal changes to single nucleotide variant level. Therefore, we also discuss whole exome sequencing, whole genome sequencing, and long-read sequencing, as well as their limitations and prenatal applications. This DNA-based technology should be evaluated for prenatal clinical application in Hong Kong.

Keywords: Prenatal diagnosis; Whole exome sequencing; Whole genome sequencing

Introduction

Conventional G-banded karyotyping with a resolution of 5 to 10 Mb was the gold standard for detecting numerical and structural chromosomal abnormalities in prenatal diagnosis. It has a turnaround time of about 2 weeks, because it requires cell culture, metaphase preparation, and karyotyping by trained cytogeneticists. It is therefore mostly superseded by chromosomal microarray (CMA), which can examine DNA copy number variations (CNVs) at an increased resolution and detect microdeletion and micro-duplication on top of gross chromosomal imbalances. CMA can achieve higher diagnostic yield in both prenatal and postnatal settings. Since June 2019, CMA has been the first-line test for prenatal diagnosis in public hospitals in Hong Kong. Nonetheless, advancement in genomic analysis by next generation sequencing (NGS) [also known as massively parallel sequencing] and challenge to prenatal diagnosis have led to revised diagnostic strategies.

A definitive cytogenomic and genetic prenatal diagnosis by conventional cytogenetics and molecular-based techniques (including CMA and NGS) enables more informed choices and counselling of parents regarding prognosis, and hence empower parents in making pregnancy decisions. It provides reassurance of continuation of the pregnancy when the prognosis is good, and an option of termination of pregnancy when the prognosis is poor. Accurate and rapid cytogenomic and genetic diagnosis facilitates targeted in utero treatment and postnatal management, informs reproductive risk of future pregnancy, and has implications for other family members. In this review, we discuss the application of CMA, whole exome sequencing (WES), and whole genome sequencing (WGS) in prenatal diagnosis (Table).

Prenatal diagnosis workflow with CMA

CMA detects gain and loss of genomic regions by hybridization of fluorescently labelled test DNA from a patient (fetal sample) onto probe targets with known genomic coordinates, which are usually fixed on a glass slide. Depending on the type of CMA platform, there are oligonucleotide probes, single-nucleotide-polymorphism (SNP) probes, and a combination of two for detecting chromosomal abnormalities. Both oligonucleotide-based CMA and SNP-based CMA can determine CNVs, but only the latter can genotype SNPs on DNA target. The genotype information of the SNPs enables detection of maternal

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Table. Comparison of cytogenomic technologies

Molecular technology	Resolution	Detection of chromosomal change	Run time (turnaround time)	Throughput per test	Prenatal use in Hong Kong
Chromosomal microarray	100-200 kb	Copy number variants	2-3 days (7 working days)	1-8 samples per chip (depending on platform)	Yes
Whole exome sequencing	1 bp	Variants in exon	3-4 days (3-4 weeks)	6-12 samples (depending on the read-depth and gene coverage) per run on a medium throughput by next generation sequencing platform	Yes
Whole genome sequencing (low-coverage)	50-100 kb	Copy number variants	2-3 days (7 working days)	16-48 samples (depending on the read-depth and coverage) per run on a medium throughout by next generation sequencing platform	Yes
Long-reads sequencing	~10 bp accuracy	Structural variants and breakpoint mapping	Hours to 2 days (unknown)	Various (depending on purpose and region of interest)	No (yes for preimplantation genetic testing on chromosomal structural rearrangement)

cell contamination in the fetal sample, triploidy, and copy number neutral changes, namely absence of heterozygosity, uniparental isodisomy, and segmental iso/heterodisomy.

The limitations of CMA include inability to detect balanced structural rearrangement of chromosomes, low level mosaicism (sensitivity level is platform specific and ranges from 20% to 30%), polyploidy (except for triploidy by SNP-based CMA), CNVs not represented on the array design (such as supernumerary marker chromosomes that are of centromeric and heterochromatic origin where no probe can be designed from these repetitive sequence regions), and uniparental heterodisomy (unless trio analysis of SNP-based CMA is performed). Chorionic villus specimens with abnormal or mosaic findings should be interpreted with caution as there is a possibility of confined placental mosaicism, which should be excluded by confirmatory testing on amniotic fluid sample.

Chromosomal imbalances may suggest structural rearrangement. Unbalanced translocations can usually be inferred from having terminal deletion of one chromosome together with terminal duplication of another chromosome. Unbalanced translocations can be confirmed by karyotyping and/or fluorescence in-situ hybridization. Both of which are valuable tools and cannot be replaced by CMA alone in the study of structural chromosomal imbalances such as ring chromosome, marker chromosomes, isochromosomes, isodicentric chromosomes, and unbalanced translocations. Their corresponding quantitative gain or loss of chromosomal DNA can only be reflected in CMA results.

CMA is commonly used (in place of karyotyping) for prenatal diagnosis as supported by major professional societies in different countries[1-5]. Systematic reviews have shown an increased diagnostic yield of CMA of 3.5% to 10% for fetuses with ultrasound abnormality and normal karyotype, while the detection of variants of uncertain clinical significance remains low at around 1% to 2%[6-9]. Studies have demonstrated the clinical utilities of CMA[10,11], supporting its use as an adjunct diagnostic tool in prenatal cases with fetal ultrasound abnormalities[12-14]. It has been shown to be a cost-effective diagnostic test in pregnancies with fetal ultrasound anomalies[15,16]. A multicentre study in UK on array comparative genomic hybridisation in prenatal diagnosis of fetal anomalies concluded that CMA was a robust, acceptable, and probably cost-effective method to detect more clinically significant chromosomal imbalances in anomalous fetuses[17]. In Hong Kong, CMA has been accepted as a part of prenatal diagnosis to improve the prenatal care[18-21] and to investigate the underlying causes of fetal abnormalities[7, 22-33] that cannot be achieved by conventional cytogenetics alone.

Since June 2019, a new prenatal diagnostic workflow has been implemented in public hospitals in Hong Kong (Figure 1). It integrates CMA as a first-line test with quantitative fluorescent polymerase chain reaction

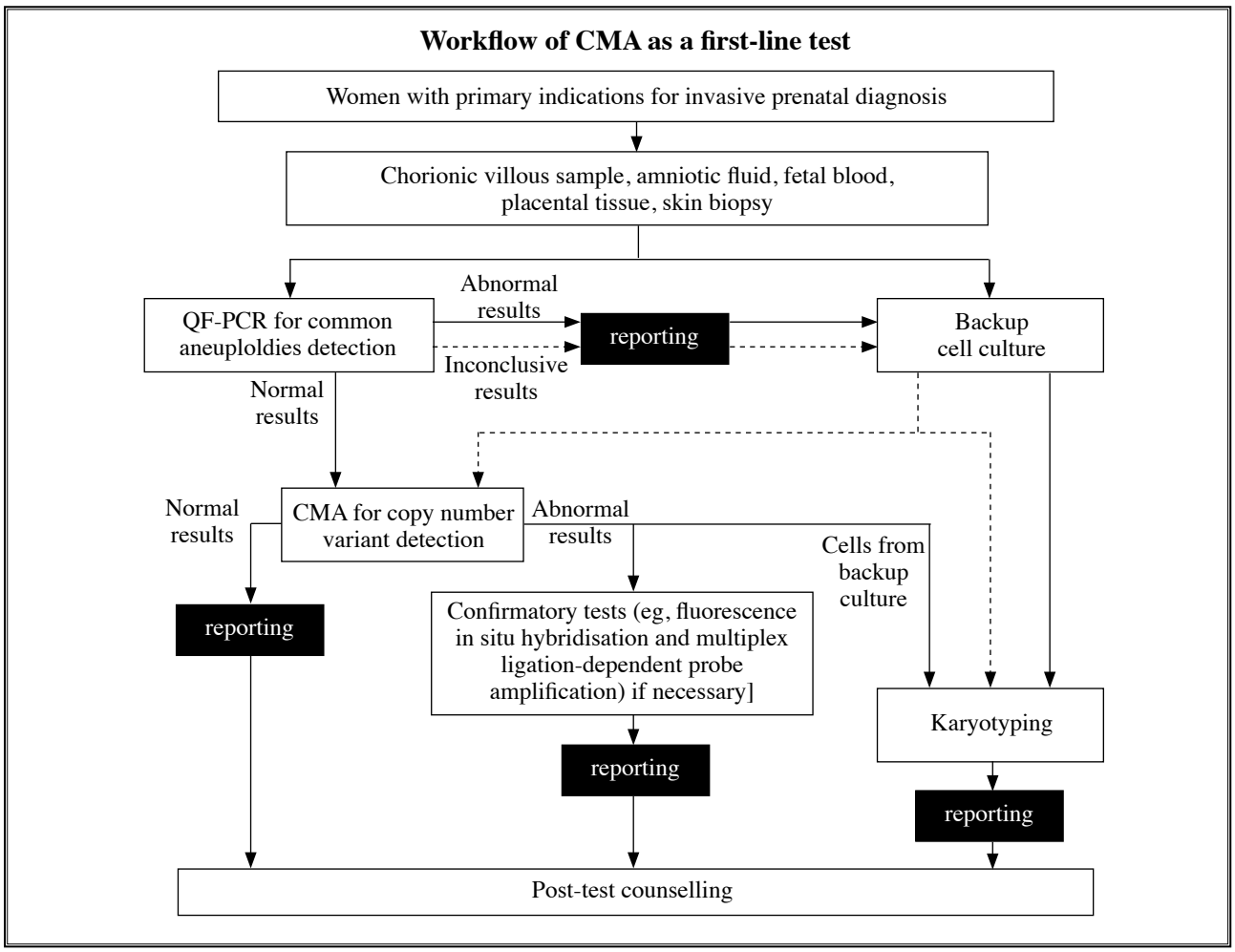

Figure 1. Workflow of chromosomal microarray (CMA) as a first-line test in prenatal diagnosis. Abnormal quantitative fluorescent polymerase chain reaction (QF-PCR) results include trisomies 13, 18, and 21, monosomy X, and triploidy. Inconclusive QF-PCR results indicate unable to conclude normal number of chromosomes 13, 18, 21 and inconclusive result for sex chromosomes. Dotted arrows indicate workflow for samples with inconclusive QF-PCR results.

(QF-PCR) for rapid common aneuploidies detection and conventional G-banded karyotyping. It is offered free to pregnant women with positive Down syndrome screening (including positive non-invasive prenatal test), fetal nuchal translucency ≥3.5 mm, structural abnormalities detected on ultrasound examination, and family history of chromosomal or genetic disorder. This workflow is also offered to women with second trimester miscarriage and stillbirth. These tests are performed by two accredited laboratories: the Prenatal Diagnostic Laboratory at Tsan Yuk Hospital using the Affymetrix CytoScan 750k SNP array and the Prenatal Genetic Diagnosis Centre at the Chinese University of Hong Kong using Fetal DNA Chip. Genetic counselling support is provided by the two clinical teams for complicated cases.

Regarding the new workflow, DNA extracted from fetal sample is subjected to rapid aneuploidies detection by QF-PCR while backup cell culture is set up. If QF-PCR shows normal results, CMA is performed. If QF-PCR shows abnormal results (trisomy 13, 18, 21, monosomy X, and triploidy), conventional karyotyping is performed. For samples with QF-PCR results showing XXX, XXY, and XYY, both CMA and conventional karyotyping are performed, as sex chromosome aneuploidy is unlikely to explain the ultrasound anomaly. CMA is performed using cultured or uncultured cells to rule out submicroscopic CNVs for samples with inconclusive QF-PCR that subsequently shows normal karyotyping results. In fetal samples with maternal cell contamination or inadequate amount of extracted DNA, CMA is performed on cultured cells after QF-PCR testing. Parental CMA is performed to clarify the inheritance of the CNVs detected in the fetal sample as indicated. G-banded karyotyping is performed

for cases with abnormal CMA to confirm the structural rearrangement and to inform future reproductive risk. Further testing such as uniparental disomy testing by short tandem repeat markers to rule out heterodisomy is arranged after discussion with referring doctor if it is clinically indicated.

For CNV interpretation, a 3-tier classification (benign, uncertain clinical significance, and pathogenic) is generally adopted in our laboratory instead of 5-tier (benign, likely benign, uncertain clinical significance, likely pathogenic, and pathogenic), as suggested by the American College of Medical Genetics and Genomics guideline[34]. It does not affect pathogenic variant classification and impact on the diagnostic yield[7]. Interpretation of CNV is more challenging in the prenatal setting than in the postnatal setting, because of the limited phenotype information from ultrasound examination. The clinical significance of CNV depends on its size, gene content, evidence on haploinsufficiency or triplosensitivity, inheritance of the CNV, any previous reports, and relevance between the disrupted gene and phenotype. In general, whole genome CMA enables detection of CNV at size of 100-200 kb on the backbone and at smaller sizes on disease-focused regions.

In accordance to the Royal College of Pathologists 2015 recommendation[35], certain low penetrance neuro-susceptibility CNVs are not reported in the public hospital setting, including proximal 1q21.1 duplications (overlapping RBMBA gene), 15q11.2 BP1-BP2 deletions or duplications (overlapping NIPA1 gene), 15q13.1q13.3 duplications, 16p13.11 deletions or duplications (overlapping MYH11 gene), 16p12.2 deletions (overlapping CDR2 gene), Xp22.31 duplications (overlapping STS gene), and Xp22.33 deletions (overlapping SHOX gene). They have no strong evidence of linking to potential phenotypes on the basis of genes involved for the pregnancy (future child) or have no clinically actionable consequence for that child or family in the future.

Next generation sequencing for CNV analysis

NGS enables analysis of nucleotides variation (using WES) and study of CNVs[36]. Compared with hybridisation technology in CMA, NGS generates sequencing reads that are mapped on chromosomes and quantitatively counted and segmented into region of an equal copy number. These features of NGS are used to developed low-pass (or low-coverage) WGS for CNV analysis. The degree of read depth of this low-pass WGS is approximately an average of 0.25× to 1× with respect to the whole human genome[37-39], meaning that a given nucleotide in a human genome is read once or less than that of an average, as not the whole genome is covered and sequenced. Low-pass WGS is most beneficial in terms of cost per sample, turnaround time, and sensitivity and resolution in CNV detection. Depending on the workflow, 6 to 28 million single end reads of 35 to 51 bp generated from each sample suffice for CNV analysis[36,37,40]. Such NGS-based analysis for CNV detection is referred to as CNV-seq[36,41] or low-pass (or low-coverage) WGS/NGS[37,40].

The main advantage of NGS-based CNV analysis is the ability to adjust platform resolution by in silico manipulation of window size, which can be performed in data processing. This is not possible for CMA platform as its genomic resolution is fixed by the probe density and coordinate although the number of probes and the size of CNV can be defined in data analysis. The NGS-based method can adjust the resolution by altering the number of samples processed within the batch: fewer samples in a batch increase read-count per sample, hence increasing the resolution of imbalances to be detected. The NGS-based method requires relatively low amount of starting genetic material of 100 to 200 ng[36,37], depending on the sequencing platform and protocol. Low-pass NGS-based CNV analysis shares some of the limitations of CMA. It cannot detect polyploidy (except for 69,XXY)[40] and balanced structural rearrangement, unless by increasing sequencing read-depth, which in turn increases the cost per sample. At this low level of read-depth, it cannot detect uniparental disomy, compared with SNP-based CMA. In order to be cost-efficient, samples multiplexing (≥20 samples) is necessary.

Interpretation of CNV detected by low-pass NGS follows the same rules for CMA. Low-pass NGS is a reliable and robust alternative for CNV analysis with shorter turnaround time, higher resolution, capable of detecting lower level of mosaicism (by scaling up the sequencing depth), and improved detection of CNV, compared with CMA. Its clinical utility in prenatal setting has been demonstrated in prospective studies[36,42-44]. A large-scale prospective study in Mainland China involving 3429 women with amniocentesis reported detection of 2.83% pathogenic/likely pathogenic CNV, and 1.43% of variants of uncertain significance[43]. This led to expert recommendation in Mainland China to offer CNV-sequencing as first-line test for prenatal diagnosis under a confined context[45]. As large population scale projects such as the 100 K Genome Project are being conducted, more

data will be generated for NGS-based CNV interpretation, and hence NGS-based CNV analysis is likely to become a first-line test for prenatal diagnosis in the near future.

Whole exome sequencing

The human genome consists of about 3 billion base-pairs, and only 1% to 2% of DNA sequences encode for protein. Exomes refer to genome regions that contain exons, and it is estimated that 85% to 90% of all disease-causing mutations reside in the exome. WES is a type of NGS that focuses on gene exons. Basic workflow of WES starting from DNA extraction and library preparation to massively parallel sequencing on a sequencing instrument can be accomplished in <3-4 days. It is then followed by bioinformatics analysis of sequencing data, result interpretation, literature search, and if necessary, final result verification and inheritance analysis (when trio WES is not performed) using Sanger DNA sequencing. At the moment, the turnaround time of WES for prenatal diagnosis is around 3-4 weeks. In brief, DNA is extracted and fragmented into shorter pieces (200-400 bp) and ligated with adaptors for clonal amplification during sequencing reaction. To select and enrich for exonic regions, capture probes (short oligos that can hybridise to target DNA) are used. In commercially available exome capture kit, the total number of capture probes range from a few hundred thousands to millions to ensure broad and specific coverage of the exome. Once the target DNA is enriched and amplified, it becomes the 'library' for subsequent massively parallel sequencing to produce millions of short sequencing reads. For WES, an average of 100× read depth for proband or a lower threshold of average read depth of 70× for trios analysis is reliable to detect the single nucleotide change[46]. If a lower depth of coverage is obtained, Sanger sequencing should be performed for confirmation.

In prenatal diagnosis, trio WES (of parents and fetus) enables different inheritance analysis models, including de novo, autosomal recessive, autosomal dominant, X-linked recessive inheritance, mitochondrial, and imprinted gene variations (Figure 2). Advantages of trio WES with respect to the efficiency of variant detection and interpretation have been reported[47-49]. Targeted analysis of a gene panel is also plausible for a genetically heterogeneous condition with a clear clinical diagnosis. It has the advantage of focusing on known variants and genes related to the disease of interest, such as Noonan syndrome and skeletal dysplasia. Disease panels usually cover several to tens of genes; thus, the sequencing cost and result interpretation are not as demanding as WES[50].

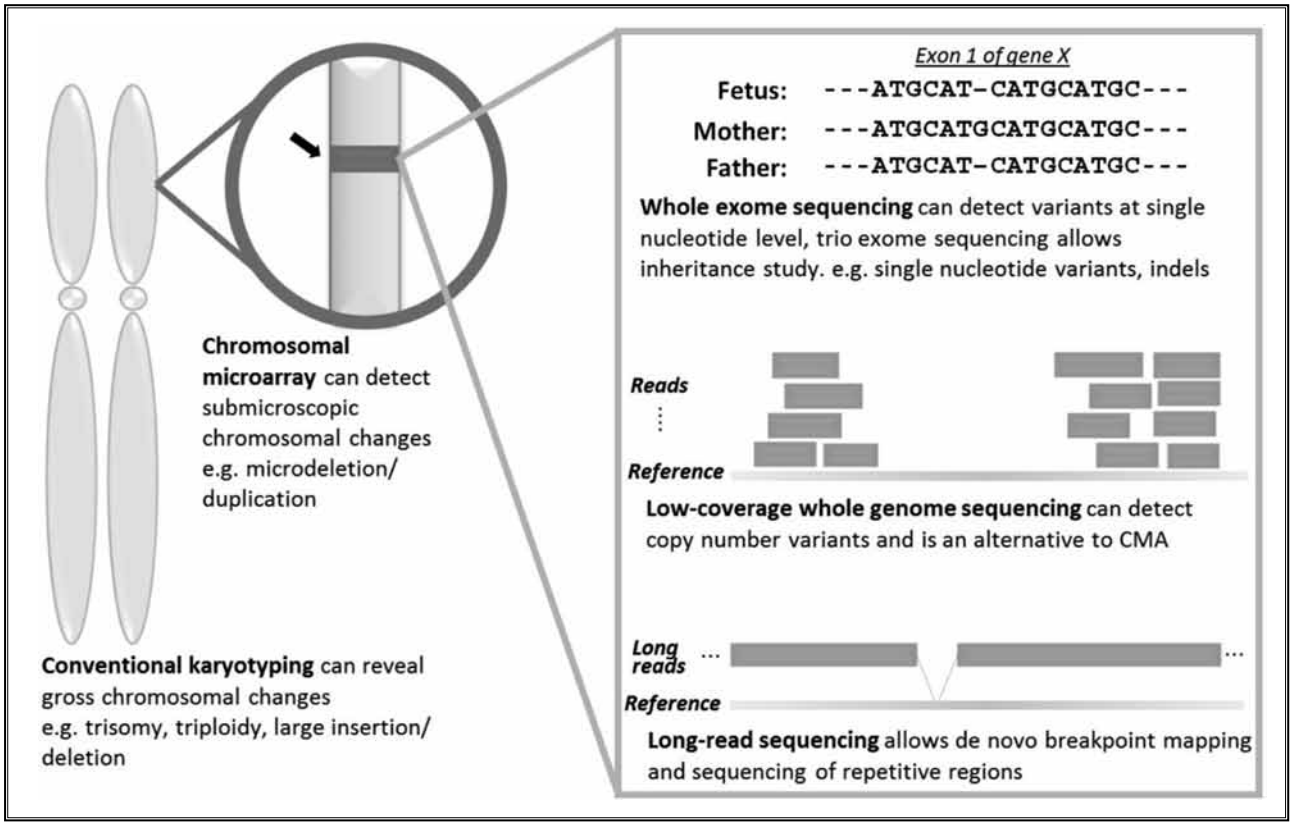

Figure 2. Advances in DNA-based technology in enhancing prenatal molecular diagnosis

Interpretation of WES findings varies among different laboratories and relies on multidisciplinary expertise from clinical scientist, geneticist, and clinicians. Classification of variants is based on the American College of Medical Genetics guidelines[51], and interpretation of the variant is highly evidence-based with reference to the literature, database, and matching clinical phenotypes. Challenges remain in understanding and reporting variants of uncertain clinical significance in the prenatal setting.

WES is mainly applied for prenatal diagnosis of monogenic disorders in fetuses with structural abnormalities. The PAGE study[52] in the UK analysing 610 trios reported an increased diagnostic yield of 8.5% of pathogenic variants and an additional 3.9% variants of uncertain significance that have potential clinical usefulness after exclusion of aneuploidy and large CNVs. Fetuses with multisystem or skeletal anomalies had the highest diagnostic yield of 15.4%. A study in US examining 234 consecutive fetuses using a similar approach reported diagnostic variants in overall 10.3% of fetuses[53]. Fetuses with multiorgan system involvement, skeletal, lymphatic or effusion, central nervous system, and renal anomalies had the highest diagnostic yield of 16% to 24%. Our recent study showed that WES could identify pathogenic variants in 9.1% and variants of uncertain clinical significance in 18.2% of fetuses with structural congenital anomalies that showed normal results in CMA and karyotyping[54]. The diagnostic yield for pathogenic variants in our study was consistent with that in the above studies[52,53].

However, there are limitations to its routine application, including requirement of rapid pipeline for analysis and a multidisciplinary team for timely interpretation of results preferably before 24 weeks' gestation, which is the legal limit of termination of pregnancy in Hong Kong. Other limitations include incomplete coverage of some genomic regions that are difficult to be enriched by the capture method and incomplete prenatal phenotyping by ultrasound examination alone as genotype-phenotype correlation can be weak. In addition, ethical issues include how to obtain adequate informed consent and reporting of incidental or secondary findings in parents' or fetus. Trios analysis may reveal non-paternity and consanguinity. There is also a possibility of reclassification of variants necessitating re-analysis or re-contact of patients. The position statement of the International Society of Prenatal Diagnosis states that diagnostic sequencing should best be offered for evaluation of fetuses under a research setting or in consultation with expert genetic professionals. Other points to consider include[55]: (1) trio approach is preferred for timeliness of result interpretation and pathogenicity assessment; (2) there is limited genotype-phenotype correlation in the fetal period and thus uncertainty on variant interpretation in the prenatal setting; and (3) involvement of a multidisciplinary team with expertise in both clinical and laboratory aspects for informed consent, pre and post-test counselling, and variant interpretation.

Whole genome sequencing for structural variant and chromosomal breakpoint discovery

Large structural variants (up to mega base-pair level) such as deletion, insertion, balanced or unbalanced translocations are studied by CMA (or traditionally by karyotyping). However, CMA is not capable of identifying chromosomal breakpoint at the nucleotide level. Several techniques have been developed to map chromosome breakpoints to the kilo base-pair (kb) level[56-62]. However, these techniques are time-consuming, expensive, and do not provide enough information of the breakpoint-linked SNPs for haplotyping analysis[63]. The advent of third-generation long-read sequencing has improved the definition of structural variants and their breakpoints, and there is growing interest in exploring the landscape of structural variants in the germline of a large number of genomes[64].

Third-generation long-read sequencing, or single molecule sequencing, refers to sequencing a DNA molecule continuously up to 80 kb (Figure 2). By mapping the long sequencing reads to the reference genome, large chromosomal changes can be detected, and their precise locations can be pinpointed to determine if any genes are involved. In highly repetitive regions of the genomes or GC-rich loci, long-read sequencing is feasible with a low error rate. Popular long-read sequencing platforms include single-molecule real-time technology by Pacific Biosciences and Oxford Nanopore sequencing technologies[65,66].

There are reports on single-molecule real-time long-read sequencing in detecting AGG interruptions in females with a FMR1 premutation for fragile X syndrome. The single-molecule real-time platform is the only technology so far that can separate the two repeats derived from different X-chromosomes, and hence is superior to PCR-based assays[67,68]. Long-read sequencing by Nanopore sequencing technologies on preimplantation genetic testing on chromosomal structural rearrangement can distinguish the balanced reciprocal translocation carrier embryos from

the euploid non-carrier embryos for transfer[63]. Library preparation for Nanopore sequencing can be completed in 90 minutes. The long-read sequencing results enable high-resolution breakpoint mapping, which can narrow down the region of interest for verification through Sanger sequencing, if necessary. There appears to be a lack of literature on using long-read sequencing and WGS on the study of structural variants and chromosomal breakpoints in the prenatal setting. A modified WGS 'jumping library' approach (a 13-day sequence and analysis pipeline) for genetic diagnosis of CHARGE syndrome in a single prenatal sample showed a direct disruption of CHD7 gene caused by a translocation breakpoint at 8q12.2[61]. It is technically feasible to generate whole fetal genome from cellular and cell-free DNA from amniotic fluid sample[69]. However, the high cost, turnaround time, and interpretation of high number of variant calls make such WGS difficult to be applied for clinical use in the near future.

Conclusion

Since June 2019, a new prenatal diagnostic workflow has been implemented in Hong Kong public hospitals, integrating CMA, QF-PCR, and conventional G-banded karyotyping. The next challenges are to accumulate experience in WES in terms of diagnostic workflow and result interpretation, and pre- and post-test counselling. Multidisciplinary input from clinical geneticists, obstetricians, clinical scientists, and genetic counsellors facilitates interpretation and understanding of WES results. However, application of long-read sequencing and WGS for structural variant and breakpoint analysis is limited, owing to the high cost and a lack of validated data analysis tools. Their potential utility in prenatal diagnosis remains to be explored.

Declaration

The authors have no conflicts of interest to disclose.

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