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Genomic Testing for Prenatal Clinical Evaluation of Congenital Anomalies

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Abstract

Congenital anomalies occur in about 2–3% of liveborn and 20% of stillborn infants. They constitute a serious public health and epidemiological problem. The etiology of congenital anomalies is complex; they can result from genetic factors, environmental factors, or a combination of both. It is estimated that genetic factors represent an important cause of congenital anomalies and may be due to different genetic mechanisms: aneuploidies, deletions and duplications of DNA segments, and single gene disorders. Due to the genetic complexity, the targeted prenatal genetic diagnostics of congenital anomalies is usually problematic and challenging. In recent years new diagnostic algorithms for prenatal genetic testing are being developed with the advent of new genomic technologies, like molecular karyotyping and next-generation sequencing. These technologies offer testing options that exceed conventional karyotyping and targeted molecular genetic testing with better diagnostic yield. In this chapter, an overview of the conventional genetic diagnostic approach and the use of new genomic technologies in the diagnostic algorithm of prenatally detected congenital anomalies are discussed.

Keywords: congenital anomalies, epidemiology, etiology, conventional karyotyping, molecular karyotyping, next-generation sequencing, prenatal diagnostics

1. Introduction

Congenital anomalies (CAs) occur in about 2–3% of liveborn and 20% of stillborn infants. They are an important cause of neonatal mortality, children morbidity, and long-term disability and...
so constitute a serious public health and epidemiological problem [1]. A significant proportion of CAs is detected before birth by routine ultrasound examination and other screening techniques [2]. Prenatal identification of CAs can have significant emotional and psychological consequences for parents and affected babies [2]. Therefore, it is imperative to make the right diagnosis as soon and as accurate as possible. Genetic testing in prenatal period is an area with very sensitive and ethically challenging situations [3]. Determining a genetic diagnosis prenatally permits parents to make informative reproductive decisions and to be counseled about possible fetal outcomes [4]. It adds important information for current pregnancy in the terms of full phenotype beyond ultrasonographically detected abnormalities on the one hand and postnatal prognosis on the other [3]. Consequently, it is important to use appropriate genetic testing approach to obtain a specific diagnosis.

In recent years new diagnostic algorithms for prenatal genetic testing are being developed with the advent of new genomic technologies, like molecular karyotyping (comparative genomic hybridization) and next-generation sequencing (NGS). These technologies offer testing options that exceed conventional karyotyping of the fetus and provide better diagnostic yield. Despite the evidence of important additional diagnostic yield of new technologies in the etiology of CAs, they have not been systematically implemented in clinical prenatal diagnostic algorithms in several national healthcare systems.

2. The epidemiology and etiology of CA

A CA is defined as any structural anomaly present at birth. Major CAs are anomalies that have medical, surgical, or cosmetic significance and occur in about 2–3% of liveborn and 20% of stillborn infants [1]. Thus, CAs are more prevalent that many chronic childhood diseases, such as autism, pediatric cancers, and type 1 diabetes, are an important cause of neonatal mortality, children morbidity, and long-term disability [1, 5]. Therefore, they represent an important public health and epidemiological problem.

CAs can be isolated or present in a characteristic pattern affecting one or more organ systems. The overall prevalence of most major CAs does not vary much across ethnic groups [6, 7]. However, the risk for different types of anomalies is variable and may be related to genetic susceptibilities and also to cultural and social differences that can influence exposures (e.g., neural tube defects due to a dietary deficiency of folic acid) [6, 7]. The prevalence of most major birth defects over time has remained constant, but some have shown a significant increase such as gastroschisis [6, 7].

The etiology of CAs is complex. CAs can be the result of genetic factors, environmental factors, or a combination of both [8, 9], although the underlying etiology often remains unknown. It is estimated that genetic factors represent an important cause of CAs and may result from different genetic mechanisms: the most common are aneuploidies, deletions, and duplications of DNA segments (collectively known as CNV), and single gene disorders [8, 9]. Some disorders have an
epigenetic basis; genes can be silenced or activated by modifications that may depend on the parent of origin or other influences [4].

With the traditional diagnostic approach using conventional karyotyping and direct molecular genetic testing, the etiology of CAs remains undiagnosed in 65–70%, including cases with multifactorial or polygenic etiology (e.g., isolated neural tube defects or cleft palate), 15–25% is thought to be genetic or genomic—chromosomal in 10–15% and monogenic in 10%—and 10% is thought to be due to environmental factors [4]. Although most CAs are isolated and sporadic; the genetic contribution has long been recognized, and specific genes involved are increasingly being identified. However, the majority of isolated CAs are thought to be caused by a complex interplay of genetic and environmental factors and follow the so-called multifactorial or polygenic inheritance [4, 8, 9]. On the other hand, multiple CAs are often part of a syndrome, of chromosomal or monogenic etiology.

In the prenatal settings, the frequency of chromosomal abnormalities depends on many factors: the gestational age, type of the anomaly, the number of anomalies, and the combination of anomalies identified [10]. In retrospective series, chromosomal abnormalities were found in 2–18% of cases when isolated and in 18–35% when multiple CAs were prenatally detected on ultrasound [10, 11]. Chromosomal abnormalities are more common in spontaneous abortions (50%) than in stillbirths (6–13%) [12].

Due to the frequency, morbidity and lethality CAs pose an important public healthcare problem. For the planning of preventive healthcare measures, it is very important to determine the epidemiology and etiology of CAs.

The following chapters give an overview of the conventional genetic diagnostic approach and the use of new genomic technologies in the prenatal genetic diagnosis of CAs.

3. Conventional genetic diagnostic approach

Currently fetal karyotyping and targeted genetic testing are still most commonly used in the genetic diagnostic evaluation of high-risk pregnancies including morphologically abnormal fetus detected by ultrasound examination and positive result of the screening test or due to parental chromosome rearrangement or genetic disorder with a known pathogenic variant. Despite the recent shift of genetic diagnostics toward genomic approach, the conventional diagnostic approach encompassing the karyotyping and targeted molecular genetic testing is worth noting.

3.1. Karyotyping

To identify possible genetic causes underlying ultrasonographically detected CAs or positive result of the screening test (nuchal translucency, combined screening test, triple/quadruple hormone test), a full chromosome analysis has been widely used and regarded as the gold
standard from the late 1990s to about 2010. A diagnostic yield of classical karyotype is more than 18% in fetuses presenting with isolated or multiple CAs [10].

As chromosome analysis is subjective and experience-dependent [13], its insufficiencies have been complemented by fluorescent in situ hybridization (FISH) analysis, where a DNA-specific fluorescent probe is hybridized to the complementary sequence in a cell preparation. In contrast to conventional chromosome analysis, FISH can be used to study cultured or direct cell preparations (metaphase/interphase FISH). FISH allows for the detection of repetitive regions like satellites in the acrocentric chromosome or variable length of pericentromeric heterochromatin not covered by genomic methods [13]. Currently, FISH analysis is a valuable tool for the identification of the origin of the marker chromosome composed of heterochromatin [14] and complex chromosome rearrangements and mechanism of the chromosome rearrangement [15, 16]. Locus-specific fluorescent probes detect subtelomere and interstitial submicroscopic chromosomal rearrangements associated with clinically recognizable phenotypes with diagnostic yield of 3–6% for chromosome abnormalities [17]. However, screening with FISH for tandem duplications seems to be of limited value.

FISH is a more targeted approach, because it requires prior knowledge of chromosome region of interest but has limited utility as a first-tier investigation [18].

Chromosome analysis may also be considered as a quantitative method, which can accurately detect the proportion of the mosaicism. As many as 0.16% of cases with low-level yet clinically significant chromosome mosaicism would be undetected by array CGH method [18]. In addition, in mosaic cases with two abnormal cell lines resulting in a no-net gain or loss (i.e., 45,X/47, XXX) array, CGH would return to normal result [18].

Different types of mosaicism can be found in prenatal diagnostics, like confined placental mosaicism (CPM), true fetal mosaicism, and clonal expansion because of in vitro cultivation [20]. CPM is found in about 1–2% of chorionic villi samples, and certain chromosome trisomies are typically found, like trisomy of chromosome 2, 7, or 16 [19].

Over the last decades, chromosome analysis has been the cornerstone in prenatal genetic diagnosis. In fetuses with multiple CA, there is a chance of more than 18% to detect a chromosomal abnormality [11], while a chance for a chromosomal aberration in cases with an isolated CA is not well determined [20, 21].

There are some pros to why fetal karyotyping remains in the everyday genetic practice. The chromosome analysis assesses the number (aneuploidies) and the structure of chromosomes (chromosome rearrangements) in a single assay (i.e., free trisomy 21 versus unbalanced Robertsonian translocation involving chromosome 21 or balanced reciprocal translocations) [18].

However, because of its low resolution, the need for cell cultivation, which is time-consuming and artifact prone, and the inability to detect complex abnormalities, the chromosome analysis is placed behind other high-throughput genomic investigations [19].

Currently, karyotyping remains the investigation of choice for low-risk pregnancies with normal fetal morphology, like advanced maternal age with increased risk for trisomy 21 [22].
3.2. Targeted molecular genetic testing

As mentioned above, about 10% of CAs is thought to be associated with monogenic disease [4]. Ultrasound examination can detect many fetal structural abnormalities, from the early anatomic survey in the first trimester to morphology and biometry in the second trimester and monitoring of the fetal growth in the third trimester. In addition to ultrasound, fetal magnetic resonance is now widely used to improve imaging of the central nervous system structures [23].

The conventional genetic approach using targeted molecular methods, like Sanger or PCR, is useful (enables) in the diagnostics of cases with ultrasonographically well-defined phenotypes associated with specific diagnostic hypothesis in genetically homogenous CA (e.g., TAR, achondroplasia) and is the method of choice for prenatal testing in cases of familial monogenic condition with known pathogenic variant [24]. In cases with poorly defined phenotype and genetically heterogeneous CAs (many genes responsible for the same phenotype), this approach rarely warrants the diagnosis.

While the conventional genetic diagnostic approach is time-consuming, labor-intensive, and with limited diagnostic yield, the new genomic approaches and technologies, like molecular karyotyping and next-generation sequencing, offer new possibilities to establish specific prenatal genetic diagnosis in high-risk pregnancies.

3.3. Molecular karyotyping

Growing knowledge and important technical evolution in the last two decades enabled us, in the clinical context, to detect and interpret smaller and smaller genomic imbalances. The classical karyotyping has been replaced first by comparative genomic hybridization and soon thereafter with array-based CGH (aCGH). It is becoming widely applied in the prenatal setting, where it is recommended by many professional societies for routine prenatal diagnostic testing in fetuses with ultrasound anomalies [25].

The comparative genomic hybridization using microarrays (aCGH) is based on competitive hybridization of short segments of whole genome DNA to preprepared probes (short sequences of DNA), spotted on a glass slide in a precise grid (microarray). The DNA of a patient and reference sample DNA are both digested with restriction enzymes to generate short fragments and after that labeled with two different fluorescent dyes. Both patient and reference DNAs are combined and hybridized to the same microarray, thereby competing for the same probes. A specialized scanner measures signal intensities and dedicated software links signals to specific genomic regions (Figure 1). When there is a deletion in the patient, we see it as a predominance of reference DNA in that genomic region. As a result, a relative log ratio of patient’s signal compared to reference signal gives a curve with negative values. Despite enabling the detection of progressively smaller genomic imbalances, one needs to be aware of the limitations—the technique will not detect low-level mosaicism, triploidy, balanced translocations, and point mutations.
After performing several large prospective and retrospective studies, it is estimated that this technique offers a 5–10% increase in detection of clinically relevant copy number variation in fetuses with ultrasound anomalies (compared to conventional karyotyping) [3, 26–28].

In the first years, the technology has been used only in fetuses with multiple congenital anomalies where the yield of pathogenic CNVs was reported to be up to 20%. With the broader use, gained experience, and numerous data on normal variation, molecular karyotyping has been introduced in different prenatal situations. It is also used in the cases of isolated congenital anomalies, increased nuchal translucency only, or positive maternal serum screening. In some countries, all prenatal genetic testing is performed with molecular karyotyping, if the invasive approach has been employed [29]. Namely, a systematic review and meta-analysis of 17 evaluated studies demonstrated additional 5% of clinically relevant CNVs over conventional karyotype in the group of fetuses with isolated increased nuchal translucency NT > 3.5 mm. Even more, a copy number abnormality was identified in 1.7% of fetuses with a normal ultrasonographic examination result with an indication for invasive testing being advanced maternal age or positive aneuploidy screening test [28, 30].

Last but not least, molecular karyotyping has two additional benefits over classical karyotype. As it does not need dividing cells, it can be performed directly from the prenatal sample,
speeding up the whole process and giving results in a few days. Even more, only a small amount of DNA is needed, and therefore, it can be easily performed even on those samples with an insufficient amount of material.

The abovementioned added diagnostic yield of microarrays over conventional karyotyping provides evidence that molecular karyotyping should be used as a method of choice for the analysis of potential genetic causes of fetal congenital anomalies. Consistent with this is the ACOG committee opinion which states that molecular karyotyping is recommended instead of a conventional karyotype when there is one or more ultrasonographically identified CAs in the fetus [31].

However, there are still some limitations in prenatal settings, such as the possibility of detecting variants of unknown significance (VOUS), CNV in susceptibility loci, and secondary findings.

The identification of a variant of unknown significance (VOUS) still occurs in 0.3–1% of prenatal cases, despite the wide use of the technology and diverse population data from pre- and postnatal testing. The percentage depends on the resolution and type of the platform used [28, 32]. Currently, there are no guidelines on how to deal with VOUS findings in the prenatal settings. Different practices exist between laboratories. Parental samples can be obtained at the same time as the prenatal sample, so that they are accessible if there is a need to test the origin of certain VOUS identified in the fetus. On the other hand, they can be collected later in those cases that need additional testing. The management of pregnancy is significantly influenced by knowing if specific CNV is de novo or inherited. Some laboratories report all VOUS findings; others only report such CNV when it occurs de novo and taking into account the size and location of the identified CNV.

Yet again, different approaches can be employed when discussing detection and reporting of secondary findings and CNV in susceptibility loci—some laboratories report all such findings, whereas others have specific national or internal guidelines and lists of specific variants that are reported and those that are not reported in prenatal settings [29, 33]. It is important to emphasize the need for informative pretest genetic counseling, where these situations are discussed with future parents.

Generally, it is well accepted that CNVs in susceptibility loci with higher penetrance are reported as such in the context of prenatal genetic testing. A clear difference between such findings and other known fully penetrant microdeletion/microduplication syndromes must be presented to the pregnant couple.

### 3.4. Next-generation sequencing

Although microarray analysis has increased the diagnostic yield in comparison to conventional karyotyping, a considerable proportion of fetuses with multiple CAs have a normal karyotype and also a normal microarray result and thus remain without a definitive diagnosis. Determining the cause of CAs in those cases is, during the prenatal period, usually very challenging and frustrating. Genetic testing can be a long process, and the quick turnaround
required for prenatal testing limits this process. Additionally, there is often an incomplete presentation of characteristic phenotypes. So targeted gene sequencing is limited by poorly defined phenotype, genetic heterogeneity, and a limited time period during pregnancy.

When CAs are associated with genetic changes in multiple genes, then sequencing the panel of genes using next-generation sequencing (NGS) should be considered a method of choice. The next-generation sequencing approach is based on parallel sequencing of multiple DNA fragments in a single reaction. This enables high-throughput sequencing of large segments of human genome in a cost-efficient manner (Figure 2).

While this approach is widely used in postnatal settings, its use is more limited prenatally, for reasons similar to that of Sanger sequencing. The limitation of gene panel (gene targets)-based approaches is in their dependence on correct diagnostic hypothesis and the long time to reach a diagnosis in case appropriate panel is not selected or cannot be selected due to a nonspecific clinical presentation. These issues can be addressed by using either mendeliome sequencing or whole exome sequencing, which use NGS to sequence the coding exons of genes associated with Mendelian diseases or all genes in the human genome, respectively. Mendeliome and exome sequencing are achieved by capturing exonic sequences using exon-specific probes. In

![Figure 2. Two causative variants are shown identified by next-generation sequencing (clinical exome sequencing) in a fetus with Joubert syndrome. Part A depicts variant Cys615Arg and part B depicts variant Arg441Cys, both in TMEM67 gene. Both variants have previously been reported as pathogenic, and segregation analysis has shown them to be present in the compound heterozygous form, clarifying the cause in this case.](image)
this way, exonic sequences of the human genome are enriched in the sample, making it possible to focus sequencing on those regions. The principle benefit of this approach is that rather than performing multiple separate gene panel tests for identification of monogenic causes of CAs, a single genetic test is performed, and then results for any gene panel can be inspected depending on the observed clinical signs and symptoms [34]. This makes such an approach significantly faster and without the need for cascading numerous laboratory tests in the case of negative results. Such an approach is also robust in cases with nonspecific clinical presentation without a clear diagnostic hypothesis and in cases that were misdiagnosed.

Current reports have consistently shown the benefit of using exome sequencing in the diagnosis of fetal CA. The current evidence suggests that a genomic abnormality may be identified in up to 20–30% of fetuses with multiple CA and with normal standard genetic results. While initial reports have shown a modest added benefit of exome sequencing in multiple CAs [35], later studies showed considerably higher yields. In a study by Drury et al., exome sequencing could resolve additional 21% cases of pregnancies with multiple congenital anomalies, abnormal ultrasound findings, and a normal microarray result [36]. Even higher diagnostic yields were reported in more selected series of cases—a recent report has shown a positive yield of 47% in fetuses with high suspicion of an underlying genetic disorder and a negative microarray and/or targeted tests [37]. Similarly, Alamillo and colleagues reported a relatively high positive yield in patients with prenatal ultrasound anomalies [38]. These cases illustrate the potentially important role of this new technology in the routine prenatal diagnostics of CAs.

Several studies have now shown that exome sequencing can also be used to detect structural variants and a variety of other pathologic variants apart from simple single-nucleotide variants [39]. This property makes exome sequencing an efficient test for structural and point variation. Despite this, molecular karyotyping is still considered the method of choice in prenatal diagnostics, predominantly because of lower costs and well-established evidence of sensitivity and specificity in the prenatal setting. With the reduction of the price of exome sequencing and with increasing evidence supporting its sensitivity, we expect that next-generation sequencing will ultimately be used for detection of structural and point mutations in a single test. Exome sequencing is, however, a method of choice in multiple CAs cases with normal results of molecular karyotyping and strong clinical suspicion of a monogenic etiology. Accordingly, American College of Medical Genetics has released a policy statement suggesting that WES can be used in the clinical assessment of “a fetus with a likely genetic disorder in which specific genetic tests, including targeted sequencing tests, available for that phenotype have failed to arrive at a diagnosis.” However, the statement stresses the limitations of the use of this technology in the prenatal setting, including long turnaround times and high rates of false positives, false negatives, and VOUS [40].

Despite several benefits outlined above, there are additional challenges associated with NGS-based approaches, particularly in the prenatal diagnostic practice. Exome sequencing is a demanding diagnostic test, requiring a complex set of laboratory, bioinformatic, and interpretative steps before a clinical report may be issued. For this reason, its turnaround time usually ranges from several weeks to several months. To address this issue, there is an incentive to offer urgent exome sequencing service and thus offer provision of clinical reports within less
than a month’s time. This significantly increases the utility of this test in prenatal diagnosis. Sequencing of parental samples along with the fetal sample may also be used to facilitate the timely interpretation of the sequencing results. Furthermore, we believe that limiting the set of reported variants to known and clear pathogenicity is also an option in order to limit the complexity of the analysis only to clinically actionable and conclusive results. Furthermore, as in other genome-wide analysis approaches, NGS-based diagnostics inherently raise the issues of incidental findings and variants of uncertain clinical significance. Due to limited time and other specificities in prenatal diagnostics, several approaches should be employed to address these issues. These include (1) limiting the gene target to genes with overlapping clinical symptoms and signs and (2) limiting the reported variants to definitely pathogenic and likely pathogenic variant classes. Furthermore, efficient collaboration within a multidisciplinary team is often crucial in clarifying the clinical relevance of identified variants. Opting for this approach, it is possible to utilize diagnostic advantages of NGS-based approaches while reducing the chance of encountering uncertain and unsolicited findings. Nevertheless, extensive genetic counseling should be offered to patients while stressing the possibility of identification of VUS findings and incidental findings.

4. Proposed workflow

The goal of prenatal genetic testing in fetuses with CAs is to determine if there is a genetic etiology and consequently enabling well-informed genetic counseling to the parents about the prognosis, reproductive options, obstetric and pediatric management, and recurrence risks.

Different approaches of prenatal genetic diagnostics are used. Despite known evidence of important additional diagnostic yield of new genomic technologies to the etiology of CAs, most countries still use the traditional genetic diagnostic approach. Hereinafter we present a diagnostic workflow that is currently in use at our institution (Figure 3). It incorporates the use of new genomic technologies and is focused on the expected diagnostic yield and limited diagnostic time frame.

When fetal CAs are detected on an ultrasound examination, we offer an invasive procedure for diagnostic genetic testing.

In cases when specific chromosomal disorder (e.g., double bubble and trisomy 21) or monogenic syndrome is strongly suspected on the initial evaluation and single gene testing is straightforward (e.g., achondroplasia), we exclude common aneuploidies (trisomy 13, 18, and 21, and aneuploidies of sex chromosomes) first, using quantitative fluorescence-polymerase chain reaction (QF-PCR), and proceed with single gene testing, respectively.

When the specific clinical diagnosis is not apparent, we use a genomic approach for the detection of genetic etiology of CAs. Because aneuploidies represent the commonest genetic etiology of CAs, we first opt for QF-PCR to exclude the aneuploidies mentioned above.
If the results are normal we proceed with molecular karyotyping instead of a conventional karyotype as studies showed that this approach allows for the highest diagnostic yield. We use this approach whether the anomaly appears to be isolated or multiple anomalies are detected.

In fetuses with normal results of molecular karyotyping, with multiple CAs, and a strong clinical indication for monogenic etiology, we proceed with WES; our approach involves sequencing the fetus as well as the biological parents (so-called trio sequencing), which increases the diagnostic yield by filtering out thousands of uninformative genomic variants as well as shortens the analysis turnaround time (less than 3 weeks at our institution).

In cases when CAs are lethal or have unfavorable prognosis, the parents often decide to terminate the pregnancy, but for the purpose of genetic counseling, it is still important to obtain the diagnosis. Accordingly, we shift the diagnostic process after the termination of the pregnancy. Thorough dysmorphological and pathohistological evaluation may give additional information on the specific phenotype and thus enables a more direct diagnostic approach in the aborted fetus. Otherwise, we use the diagnostic approach similar to the approach during the pregnancy.

Different medical specialists are involved in the process of prenatal diagnosis of CAs. The role of a clinical geneticist in the whole pathway of genetic diagnostics of a pregnancy with CAs in a close collaboration with other medical specialists (obstetricians, surgeons, radiologists, pathologists, etc.) and a multidisciplinary approach is undisputed due to all the complexities of prenatal diagnostics of CAs, their clinical presentation and phenotype evaluation, choice of the right genetic testing strategies, interpretation of genetic testing results, and their communication to patients and families.

**Figure 3.** Diagnostic algorithm for the prenatal genetic diagnostics of CAs. CAs, congenital anomalies; QF-PCR, quantitative fluorescence-polymerase chain reaction; NGS, next-generation sequencing.
5. Conclusions

With the advent of new genetic genomic technologies in the prenatal settings, the diagnostic yield in the etiology of CAs can be significantly improved. This has important consequences for the patients, as it enables the identification of the cause of CAs and, consequently, their prevention, as well as understanding the genetic epidemiology of CAs and designing optimal professional and cost-effective diagnostic algorithms for the diagnostics of CAs.

With the implementation of new genomic technologies in the diagnostic algorithm, approximately 50% of the genetic etiology of prenatally detected CAs can be explained. Therefore, we suggest a timely implementation of these technologies in prenatal diagnostics of CAs.

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References


