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Low-pass genome sequencing reveals associations between copy number variations and fetal ultrasonographic anomalies and soft markers in a cohort of 43,721 fetuses

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Abstract

Objective To systematically explore the association between pathogenic/likely pathogenic copy number variations (pCNV) and ultrasonographic anomalies and soft markers. Design Retrospective cohort study. Setting Data were obtained from multiple centers in china. Population or Sample Fetuses performed low-pass genome sequencing and ultrasonography between 2016 and 2020. Method The yields of pCNV under various ultrasonographic indications were compared with that of fetuses with no identifiable anomalies. In addition, the ultrasonographic characteristics of aneuploidy and pCNV were described in comparison with those of fetuses without chromosomal aberrations. Main Outcome Measures Yields of aneuploidy and pCNV in different ultrasonographic indications. Results Ten of the 12 ultrasonographic anomalies had significantly higher yield of pCNV, except for fetal hydrops and abnormal amniotic fluid, of which the gastrointestinal, facial, respiratory systems, and abdominal wall defect were rarely reported. Similarly, five of the 12 soft markers had significantly higher yield of pCNV, with single umbilical artery being rarely reported. Furthermore, this study reported that four duplications/deletions were associated with novel ultrasonographic findings. Conclusions Based on specific ultrasonographic phenotypes, prenatal genetic testing could be considered in a tailored fashion. Keywords Low-pass genome sequencing; ultrasonographic anomaly; soft marker; copy number variations; aneuploidy; prenatal diagnosis Tweetable abstract Fetuses with structural anomalies and specific soft markers are recommended for copy number variations analysis

INTRODUCTION

Ultrasonographic anomalies and soft markers are common indications for prenatal chromosomal analysis.[1] Standard karyotyping and chromosomal microarray (CMA) have become the primary diagnostic tools for fetuses with growth disorders and congenital anomalies. Recently, low-pass genome sequencing (low-pass GS) with enhanced resolution and high throughput has emerged as an alternative to CMA for genetic testing.[2, 3] It has been applied to genetic diagnoses in prenatal, miscarriage, and postnatal cases,[4-6] and was reported to have a 1.7%-3.4% improvement in additional yield compared with routine CMA.[2, 6] Furthermore, low-pass GS has received attention due to its shorter turnaround time, reduced DNA requirements, lower technical repetition rate and lower cost.[4]

The yields of aneuploidy and likely pathogenic/pathogenic copy number variations (pCNV) vary with different ultrasonographic findings. Previous studies showed that the yield of pCNV was 6%-7% in ultrasonography anomalous fetuses with a normal karyotype,[7, 8] and 0.4%-2% in fetuses without anomalies.[9-11] Cardiovascular, genitourinary, skeletal, and central nervous system defects were reported to be most commonly associated with chromosomal aberrations.[12-18]Therefore, the American College of Obstetricians and

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Gynecologists (ACOG) and the Society for Maternal-Fetal Medicine (SMFM) recommend CMA as a first-tier test in the diagnostic evaluation of fetal structural abnormalities for fetuses undergoing prenatal diagnosis.[19] Additionally, previous studies have demonstrated that aneuploidy and pCNV were frequently presented in specific soft markers, such as increased nuchal translucency, ventriculomegaly, and thickened nuchal fold.[20-23] In particular, the SMFM recommends CMA for fetuses with ventriculomegaly.[24] However, ultrasonographic anomalies and soft markers comprise diverse subtypes, which may have significant differences in the yield of aneuploidy and pCNV.[25-27]

Therefore, it is crucial to systematically explore the correlation between various ultrasonographic anomalies and soft markers and aneuploidy/ pCNV. In this study, we comprehensively analyzed the yield of aneuploidy and pCNV in 12 types of ultrasonographic anomalies and soft markers based on a large cohort of 43,721 fetuses to provide data support for the risk assessment of aneuploidy/pCNV underlying different ultrasonographic findings. For each aneuploidy/pCNV, we compared the ultrasonographic characteristics of fetuses with and without chromosomal aberrations to elucidate the association of specific genomic alterations with specific ultrasonographic anomalies.

MATERIALS AND METHODS

Sample collection and ethical approval

Pregnancies were recruited from multiple centers in China for chorionic-villus sampling, amniocentesis or percutaneous umbilical blood sampling, between January 2016 and October 2020. Each participant was subjected to low-pass GS and ultrasonography. Soft markers were categorized into 12 isolated types, while ultrasonographic anomalies were categorized into 12 isolated types based on Human Phenotype Ontology (HPO) terms and the site of anomalies detected by ultrasonography. Fetuses satisfying any of the following conditions were included in the study: 1) advanced maternal age (women aged [?] 35 years) with normal ultrasonographic results, normal non-invasive prenatal screening results, normal Down syndrome biochemical screening tests, and no family/personal history of chromosomal abnormality; 2) ultrasonographic anomalies (including anomalies in cardiovascular, genitourinary, central nervous, gastrointestinal, skeletal, facial, or respiratory system, cystic hygroma, abnormal amniotic fluid, fetal growth restriction, fetal hydrops, and abdominal wall defect); 3) ultrasonographic soft markers (including increased nuchal translucency, choroid plexus cysts, absent or hypoplastic nasal bone, echogenic intracardiac focus, mild ventriculomegaly, single umbilical artery, mild pyelectasis, aberrant right subclavian artery, echogenic bowel, short femur/humerus length, thickened nuchal fold and enlarged cisterna). In total, 43,721 samples were included in this study. The cohort was divided into three groups by gestational week: First trimester (11w-13w+6d), Second trimester (14w-27w+6d), and Third trimester (28w-). Although there was some overlap between soft markers and anomalies in all trimesters, it had little influence on the result obtained. This study was approved by the Ethics Committee of Center for Medical Genetics, Central South University, Hunan, China. The ethics application reference numbers were 2015031002 (approval date: 2016.1-2019.12) and 2019-1-23 (approval date: 2019.05-2024.05). All subjects signed a written informed consent form for genetic investigation of pregnancy.

Low-pass genome sequencing

Genomic DNA was extracted from prenatal specimens according to the manufacturer's protocol. An STR-based semi quantitative PCR assay was used to check for maternal DNA contamination from this procedure. Samples with >10% contamination were excluded from the study. Finally, 200 ng of genomic DNA was randomly fragmented, and DNA libraries were constructed by end-repaired, A-tailed, and adaptor ligation.[28]

Low-pass GS was performed as previously described, with a mean coverage of 0.06X.[5, 28, 29] Mapped reads were allocated to 20-kilobase (kb) bin sizes with 5-kb sliding to identify CNVs. CNV profiles of each chromosome were represented as log2 of the mean sequencing reads of each sequencing bin along the chromosome. Any two CNVs with [?] 60% reciprocal overlap were identified as the same. Publicly available genomic databases including 1000 genomes, DGV (http://dgv.tcag.ca/dgv/app/home), OMIM (https://www.omim.org/),

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DECIPHER (https://decipher.sanger.ac.uk/), ClinVar (https://www.ncbi.nlm.nih.gov/clinvar/), UCSC (http://genome.ucsc.edu/), and PubMed (https://pubmed.ncbi.nlm.nih.gov/) were used as reference CNV sources. The pathogenicity of identified CNVs was assessed based on the American College of Medical Genetics (ACMG) guidelines.[30] Only germline aneuploidy and pCNV were considered in further analyses.

Statistical analysis

Results and graphics were completed using R software (version 3.6.3). Previous studies have indicated that the yield of pCNV is unrelated to maternal age.[31] Therefore, 12730 fetuses with maternal age [?] 35 years and normal ultrasonography, defined as "fetuses with no identifiable anomalies", were used as a control cohort for comparison of the yield of pCNV for fetuses with abnormal ultrasonographic findings. The association between ultrasonographic findings and pCNV were performed by Binomial test and Fisher's exact test with Bonferroni correction. $P < 0.05$ was considered statistically significant.

RESULTS

Demographics of fetuses

A total of 43,721 pregnancies with low-pass GS and ultrasonography from 29 provinces in China were included in this study (Figure S1). The mean maternal age was 32.65±6.10 years, and the mean gestational week (at the time of low-pass GS) was 21.52±4.49 weeks. Aneuploidy yields were 6.60% (878/13,312) , 7.24% (1,280/17,679), and 2.52% (321/12,730) of fetuses with ultrasonographic anomalies, soft markers, and no identifiable anomalies, whereas the pCNV yields were 6.24% (831/13,312) , 3.64% (644/17,679), and (1.94%, 247/12,730), respectively (Table 1). The most common aneuploidy in fetuses was trisomy 21 (3.09%, 1,350/43,721), followed by trisomy 18 (1.11%, N=486/43,721) and 45,X syndrome (0.52%, 229/43,721), while the most common pCNV in fetuses was 22q11.21 deletion (0.27%, 119/43,721), followed by 15q11.2 deletion (0.24%, 105/43,721) and Xp22.31 deletion (0.22%, 96/43,721).

The yield of aneuploidy in the first trimester of pregnancies with ultrasonographic anomalies and soft markers was ˜9 (333/736 vs 501/10,148) and ˜3 times (873/6,583 vs 391/9,775) higher than that in the second trimester, respectively (Table 1). Additionally, The yield of pCNV of each trimester was significantly higher than control group. There was no obvious trend for the yields between different trimesters (Table S1).

Yields of aneuploidy and correlation with ultrasonographic findings

Among all isolated ultrasonographic anomalies, the fetal hydrops had the highest yield of aneuploidy (34.85%), followed by cystic hygroma (24.76%), and abdominal wall defect (13.71%) (Table 2). Among all isolated soft markers, the increased nuchal translucency had the highest yield of aneuploidy (12.43%), followed by absent or hypoplastic nasal bone (6.63%) and thickened nuchal fold (5.47%)(Table 3).

We observed that the yields of aneuploidy increased with the number of anomalies or soft markers. When fetuses presented with multiple ultrasonographic anomalies or soft markers, the yields of aneuploidy increased from 5.05% (597/11,818) to 28.83% (79/274) and 6.98% (1,107/15,856) to 16.81% (20/119), respectively. Specifically, the yield of aneuploidy in fetuses with both fetal hydrops and cystic hygroma was up to 64.94% (50/77) (Table S2). The yield of aneuploidies in fetuses with increased nuchal translucency and absent or hypoplastic nasal bone was as high as 61.63% (53/86) (Table S3).

Overall, three aneuploidies, including trisomy 18, 45,X syndrome, and trisomy 13,

were significantly associated with ultrasonographic anomalies, while trisomy 21 was significantly associated with soft markers (Figure S2A). Specifically, trisomy 21 significantly associated with soft markers such as absent or hypoplastic nasal bone, increased nuchal translucency, and thickened nuchal fold, as well as with

ultrasonographic anomalies, including cystic hygroma and fetal hydrops (Figure S2B). Trisomy 18 was significantly associated with soft markers and ultrasonographic anomalies, presenting in three types of soft markers (single umbilical artery, increased

nuchal translucency, and choroid plexus cysts) and ultrasonographic anomalies (fetal

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hydrops, cystic hygroma, and abdominal wall defect). Both 47,XXY and 47,XYY syndromes tended to occur with normal ultrasonographic findings and showed no significant association with soft markers and ultrasonographic anomalies. In addition, 45,X syndrome was significantly associated with increased nuchal translucency, fetal hydrops, and cystic hygroma, while trisomy 13 was significantly associated with abdominal wall defect and increased nuchal translucency (Figure S2B).

Comparison of the yields of pCNV in fetuses with ultrasonographic anomalies and soft markers with control group

The yields of pCNV for fetuses with ultrasonographic anomalies and soft markers were 3.37 times and 1.91 times higher than in fetuses with no identifiable anomalies, respectively. The yield of pCNV was significantly higher in 10 types of ultrasonographic anomalies (P<0.05, OR=1.63-2.63), of which the yield in six types of ultrasonographic anomalies was over 6%, including abdominal wall defect (7.43%, P=0.00066, OR=4.06, 95%CI [2.11,7.14]), cardiovascular system (7.07%), skeletal system (6.72%), central nervous system (6.63%), fetal growth restriction (6.43%), and respiratory system (6.04%). Abnormal amniotic fluid and fetal hydrops showed no significant differences in the yield of pCNV (Table 2). Furthermore, we observed that the yield of pCNV increased with the number of ultrasonographic anomalies. When fetuses presented with multiple ultrasonographic anomalies, the yields of pCNV increased from 5.68% (671/11,818) to 14.96% (41/274). Specifically, the yield of pCNV in fetuses with genitourinary and central nervous systems was 33.33% (6/18) (Table S2).

The yield of pCNV was significantly higher in thickened nuchal fold (5.84%, P=0.0017, OR=3.13, 95% CI [1.76,5.19]), mild ventriculomegaly (4.40%, P<0.0001, OR=2.33, 95% CI [1.71,2.30]), increased nuchal translucency (4.10%, P<0.0001, OR=2.16, 95% CI [1.90,2.45]), single umbilical artery (3.67%, P=0.022, OR=1.93, 95% CI [1.27,2.81]), and absent or hypoplastic nasal bone (3.21%, P=0.0034, OR=1.68, 95% CI [1.27,2.16]), and no significantly difference was observed in seven soft markers (Table 3). Similar to the ultrasonographic anomalies, the yield of pCNV were increased with the number of soft markers. When fetuses had multiple soft markers, the yield of pCNV increased from 3.53% (560/15,856) to 5.88% (7/119). Notably, only fetuses with increased nuchal translucency and single umbilical artery showed a significantly higher yield of pCNV (14.71%, P=0.017, OR=8.71, 95% CI [2.63,22.77]) (Table S3).

The correlation between pCNV and ultrasonographic findings

Nine pCNV were significantly associated with ultrasonographic anomalies, and no pCNV was significantly associated with soft markers (Figure 1A). As expected, 22q11.21, 15q11.2, and 16q11.2 deletions were significantly associated with cardiovascular system, increased nuchal translucency, and skeletal system, respectively (Figure 1B). The 17q12 deletion was significantly associated with genitourinary system and abnormal amniotic fluid. Fetuses with 7q11.23 and 18p11.31-p11.21 deletions were significantly associated with cardiovascular system and increased nuchal translucency, respectively. All fetuses with 5p15.33-p14.2, 7q35-q36.3, and 13q31.1-q34 deletions, and 11q23.3-q25 and 9p24.3-p13.3 duplication, showed significant association with central nervous system. Fetuses with 17p11.2 deletion were significantly associated with two soft markers, including enlarged cisterna magna and mild ventriculomegaly. Fetuses with 1q43-q44 deletion were associated with cardiovascular system and central nervous system, and fetuses with 11q24.2-q25 deletion were associated with cardiovascular system. Consistent with previous studies,[32, 33] 4p16.3, 4p16.3-p15.2, and 4p16.3-p16.1 deletions were significantly associated with fetal growth restriction. Interestingly, this is the first study to report that 4p16.3-p16.1, 13q33.3-q34, and 3p26.3-p26.1 deletions were significantly associated with genitourinary system, respiratory system, and abdominal wall defect, respectively. Furthermore, we firstly reported that 3q25.2-q29 duplication was associated with cystic hygroma and abdominal wall defect (Figure 1B).

Discussion

Main findings

Based on 43,721 fetuses recruited, this study had three main principle findings. First, the yields of pCNV

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detected by low pass GS in fetuses with ultrasonographic anomalies and soft markers were 6.24% and 3.64%, respectively, consistent with previous results obtained by CMA.[7, 23] Second, ten of the 12 ultrasonographic anomalies had significantly higher yields of pCNV, except for fetal hydrops and abnormal amniotic fluid, of which the gastrointestinal, facial, respiratory systems and abdominal wall defect are rarely reported. Similarly, five of the 12 soft markers had significantly higher yields of pCNV, with single umbilical artery being rarely reported. Third, 51 significant pCNV-ultrasonography associations were observed and described in this study, of which 4p16.3-p16.1 deletion, 3p26.3-p26.1, 13q33.3-q34 deletions and 3q25.2-q29 duplication were firstly reported to be associated with genitourinary system, abdominal wall defect, respiratory system, and cystic hygroma and abdominal wall defect, respectively.

Strengths and Limitations

A major strength of our study is the large sample size including 43,721 fetuses with low-pass GS and ultrasonography. Most previous studies explored the yield of pCNV in fetuses with ultrasonographic phenotype by using CMA frequently in small cohorts. Secondly, all anatomical system anomalies were included rather than several common systems only, which makes our study more representative and generalizable. In addition, the contribution of aneuploidy/pCNV to fetuses with ultrasonographic abnormalities was evaluated by comparing with the group without identifiable anomalies, providing a powerful and objective guidance for prenatal genetic diagnosis.

This study had some limitations. There was no further ultrasonographic imaging

follow-up, which might have overestimated the contribution of chromosomal aberrations in fetuses with normal ultrasonographic findings. Also, postnatal outcome

data were unavailable to verify prenatal ultrasonographic anomalies. Furthermore, this study only considered chromosomal-level variations, and lacked gene-level variations. We expected future study could combine whole exome sequencing and cytogenetic methods to improve the identification of genetic disorder in fetuses with ultrasonographic anomalies.[34-36] Meanwhile, due to lack of fetuses with normal ultrasonography and maternal age <35 years, no comparison was performed for the yield of aneuploidy between fetuses with abnormal ultrasonographic findings and control group. Finally, some overlap may occur between soft markers and anomaly groups, as reports of increased nuchal translucency, thickened nuchal fold and cystic hygroma from different centers and at different gestational weeks, may present within the same group. Despite our best efforts to classify the ultrasonographic anomalies, an international uniform classification system is still lacking. Therefore, we advocate implementation of such a system to facilitate the comparability of cohorts.

Interpretation

The yields of aneuploidy and pCNV varied among the anatomical systems affected. Our results support that CNV analysis should be performed in fetuses with structural anomalies,[19] especially for fetuses with cystic hygroma, fetal hydrops, and abdominal wall defect, as the yield of aneuploidy/pCNV in these three anomalies was 21.14%-38.64%. Among 12 isolated ultrasonographic anomalies, this study discovered several well-known pCNV-associated organ systems (cardiovascular, genitourinary, skeletal and central nervous systems),[15, 25] and provided sufficient evidence for rarely reported associations between abdominal wall defect, facial and respiratory systems, and fetal growth restriction and pCNV (Table S4). Notably, we firstly revealed that isolated abdominal wall defect was strongly associated with pCNV, in addition to its known association with aneuploidy.[37] The yield of pCNV in fetuses with abnormal amniotic fluid and fetal hydrops showed no significant difference from that in fetuses with no identifiable anomalies, indicating that pCNV may be not the main genetic cause of these two anomalies. Notably, the yield of aneuploidy for fetal hydrops was up to 34.85%, especially when fetal hydrops combined with cystic hygroma, the rate was as high as 64.94%. Therefore, quantitative fluorescence polymerase chain reaction (QF-PCR) may be recommended for these fetuses before CNV analysis.

Previous studies have reported the benefits of CNV analysis in the etiological diagnosis of fetuses with isolated increased nuchal translucency and mild ventriculomegaly, with pCNV yields of approximately 2.5-5.0% and

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2.0-4.4% [20, 21, 23, 38, 39] , respectively (Table S5). The yields in our study was 4.10% and 4.40%, respectively, both significantly higher than those in fetuses with no identifiable anomalies, which support CNV analysis for these two soft markers. Although the SMFM recommended that karyotyping or CNV analysis for thickened nuchal fold should be based on clinical conditions and patients preferences[40], our results showed that it was worth performing CNV analysis, as fetuses with thickened nuchal fold had the highest yield of pCNV and showed significantly higher yield in pCNV. However, we could not replicate the previous associations between aberrant right subclavian and short femur/humerus length and pCNV.[23] Our study represents the one of the largest CNV analysis thus far. Based on our results, we suggest careful prenatal decision-making for fetuses with these two soft markers. Interestingly, we found that single umbilical artery was significantly associated with pCNV, contrary to a previous study of 126 fetuses.[23] This difference may be explained by the sample size, which requires replicated cohorts for further verification.

We replicated previous well-known pCNV-phenotype associations such as 22q11.2 deletion[41] and 7q11.23 deletion[42] with cardiovascular defect, 17q12 deletion with genitourinary system defect [43], and 4p16 deletion with fetal growth restriction [44]. Simultaneously, skeletal system abnormalities were strongly associated with 16p11.2 deletion, which was rarely reported in previous studies. Except for the known association with fetal growth restriction, 4p16.3-p16.1 deletion showed a novel association with genitourinary system. Additionally, this study firstly observed that 3p26.3-p26.1 and 13q33.3-q34 deletions and 3q25.2-q29 duplication were associated with abdominal wall defect, respiratory system, and cystic hygroma and abdominal wall defect, respectively, expanding the phenotypic spectrum of such unusual pCNV. Moreover, our findings show that fetuses with 22q11.21 deletion had a lower percentage of choroid plexus cysts than fetuses without chromosomal aberrations, suggesting that choroid plexus cysts were less likely to occur in fetuses with 22q11.21 deletion.

Conclusion

In summary, our study comprehensively elucidates the association of 12 ultrasonographic anomalies and 12 soft markers with pCNV in comparison to fetuses with no identifiable anomalies, providing reliable reference for appropriate genetic counselling of fetuses with different ultrasonographic findings. We recommend CNV analysis for fetuses with structural anomalies and specific soft markers, such as increased nuchal translucency, mild ventriculomegaly, absent or hypoplastic nasal bone, single umbilical artery, and thickened nuchal fold. Furthermore, we expect that, this large dataset could be used in machine learning for artificial intelligence-based assessment of fetal genetic risk in the future.

Declaration of interests

The authors declare no competing interests.

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Author contributions

Lijuan Pan: Conceptualization, Resources, Data curation, Writing-Original Draft, Investigation; Yi Zhang: Methodology, Software, Formal analysis, Resources, Data curation, Writing-Original Draft curation, Visualization; Desheng Liang: Conceptualization, Writing-Review & Editing; Jing Yuan: Resources; Jue Wang: Resources; Yinchen Shen: Resources; Junjie Lu: Resources; Aihua Xia: Resources; Jinchen Li: Conceptualization, Methodology, Writing-Review & Editing; Lingqian Wu: Conceptualization, Writing-Review & Editing, Supervision, Project administration, Funding acquisition. The authors report no conflict of interest.

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Web Sources

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Table 1. Summary of the yield of aneuploidy/pCNV in the study.

Group	**Group**	**N (%)**	**Aneuploidy (%)**	**pCNV (%)**
Ultrasonographic anomalies (N=133,12)	**Gestational week**	**Gestational week**	**Gestational week**	**Gestational week**
	First trimester	736 (5.53%)	333 (45.24%)	39 (5.30%)
	Second trimester	10,148 (76.23%)	501 (4.94%)	648 (6.39%)
	Third trimester	2,428 (18.24%)	44 (1.81%)	144 (5.93%)
	Num of ultrasonographic anomalies	**Num of ultrasonographic anomalies**	**Num of ultrasonographic anomalies**	**Num of ultrasonographic anomalies**

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Group	Group	N (%)	Aneuploidy (%)	pCNV (%)
	1	11,818 (88.78%)	597 (5.05%)	671 (5.68%)
	2	1,220 (9.16%)	202 (16.56%)	119 (9.75%)
	[?] 3	274 (2.06%)	79 (28.83%)	41 (14.96%)
	Total	13,312 (100%)	878 (6.60%)	831 (6.24%)
Soft markers (N=17,679)	**Gestational week**	**Gestational week**	**Gestational week**	**Gestational week**
	First trimester	6,583 (37.24%)	873 (13.26%)	274 (4.16%)
	Second trimester	9,775 (55.29%)	391 (4.00%)	326 (3.34%)
	Third trimester	1,321 (7.47%)	16 (1.21%)	44 (3.33%)
	Num of soft markers	**Num of soft markers**	**Num of soft markers**	**Num of soft markers**
	1	15,856 (89.69%)	1,107 (6.98%)	560 (3.53%)
	2	1,704 (9.64%)	153 (8.98%)	77 (4.52%)
	[?] 3	119 (0.67%)	20 (16.81%)	7 (5.88%)
	Total	17,679 (100%)	1,280 (7.24%)	644 (3.64%)
Fetuses with no anomalies (N=12,730)	Fetuses with no anomalies (N=12,730)	12,730 (100%)	321 (2.52%)	247 (1.94%)

The cohort in the study was divided into three group: fetuses with ultrasonographic anomalies, fetuses with soft markers, and fetuses with no anomalies (normal ultrasonography and maternal age [?] 35 years). Gestational week was divided into three groups: First trimester (11w-13w+6d), Second trimester (14w-27w+6d), and Third trimester (28w-). pCNV: pathogenic/likely pathogenic copy number variations.

Table 2. The yield of low-pass sequencing in fetuses with detailed ultrasonographic anomaly.

Ultrasonographic anomaly	Ultrasonographic anomaly	N	Aneuploidy (%)		pC
				N (%)	P
1	cardiovascular system	3,620 (27.19%)	207 (5.72%)	256 (7.07%)	< 0
	genitourinary system	1,743 (13.09%)	19 (1.09%)	73 (4.19%)	< 0
	abnormal amniotic fluid	1,057 (7.94%)	24 (2.27%)	35 (3.31%)	0.05
	central nervous system	980 (7.36%)	31 (3.16%)	65 (6.63%)	< 0
	fetal growth restriction	856 (6.43%)	16 (1.87%)	55 (6.43%)	< 0
	gastrointestinal system	820 (6.16%)	30 (3.66%)	32 (3.90%)	**0.0**
	skeletal system	774 (5.81%)	28 (3.62%)	52 (6.72%)	< 0
	facial system	616 (4.63%)	14 (2.27%)	30 (4.87%)	< 0
	respiratory system	497 (3.73%)	9 (1.81%)	30 (6.04%)	< 0
	cystic hygroma	416 (3.13%)	103 (24.76%)	20 (4.81%)	**0.0**
	fetal hydrops	264 (1.98%)	92 (34.85%)	10 (3.79%)	0.62
	abdominal wall defect	175 (1.31%)	24 (13.71%)	13 (7.43%)	**0.0**
	Total	11,818 (88.78%)	597 (5.05%)	671 (5.68%)	< 0
2	2	1,220 (9.16%)	202 (16.56%)	119 (9.75%)	< 0
3	[?] 3	274 (2.06%)	79 (28.83%)	41 (14.96%)	< 0

Ultrasonographic anomalies were divided in three groups: 1 (fetuses with one ultrasonographic anomaly), 2 (fetuses with two ultrasonographic anomalies), and [?] 3 (fetuses with more than two ultrasonographic anomalies). For group 1, ultrasonographic anomalies were further divided into 12 isolated anomalies. Each subgroup was compared with fetuses with no identifiable anomalies and maternal age [?] 35 years. pCNV:

pathogenic/likely pathogenic copy number variations. OR: odds ratio; CI: confidence interval. Binomial test with bonferroni correction was to compare yields between groups. Bold values denote statistically significant at the P value < 0.05 level.

Table 3. The yield of low-pass sequencing in fetuses with detailed soft marker.

Soft marker	Soft marker	N (%)	N (%)	Aneuploidy (%)
1	increased nuchal translucency	increased nuchal translucency	6,188 (35.00%)	769 (12.43%)
	choroid plexus cysts	choroid plexus cysts	2,101 (11.88%)	70 (3.33%)
	absent or hypoplastic nasal bone	absent or hypoplastic nasal bone	1,901 (10.75%)	126 (6.63%)
	echogenic intracardiac focus	echogenic intracardiac focus	1,278 (7.23%)	32 (2.50%)
	mild ventriculomegaly	mild ventriculomegaly	1,113 (6.30%)	39 (3.50%)
	single umbilical artery	single umbilical artery	762 (4.31%)	19 (2.49%)
	mild pyelectasis	mild pyelectasis	729 (4.12%)	10 (1.37%)
	aberrant right subclavian artery	aberrant right subclavian artery	475 (2.67%)	6 (1.26%)
	echogenic bowel	echogenic bowel	424 (2.40%)	5 (1.18%)
	short femur/humerus length	short femur/humerus length	404 (2.29%)	11 (2.72%)
	thickened nuchal fold	thickened nuchal fold	274 (1.55%)	15 (5.47%)
	enlarged cisterna magna	enlarged cisterna magna	207 (1.17%)	5 (2.42%)
	Total	Total	15,856 (89.69%)	1,107 (6.98%)
2	2	2	1,704 (9.64%)	153 (8.98%)
3	[?] 3	[?] 3	119 (0.67%)	20 (16.81%)

Soft markers were divided in three groups: 1 (fetuses with one soft marker), 2 (fetuses with two soft markers), and [?] 3 (fetuses with more than two soft markers). For group 1, soft markers were further divided into 12 isolated soft markers. Each subgroup was compared with fetuses with no identifiable anomalies and maternal age [?] 35 years. pCNV: pathogenic/likely pathogenic copy number variations. OR: odds ratio; CI: confidence interval. Binomial test with bonferroni correction was to compare yields between groups. Bold values denote statistically significant at the P value < 0.05 level.

Figure Legend

Figure 1. Association analysis between pCNV and ultrasonographic findings.

A. Association analysis between pathogenic/likely pathogenic copy number variations (pCNV) and ultrasonographic findings; B. Association analysis between pCNV and detailed ultrasonographic findings. Each pCNV was compared with fetuses without chromosomal aberrations. Values in parentheses indicate the number of samples with pCNV. The circle size represents values of odds ratio. a: chr9_138406667_141020000_del; b: chr9_140400001_141020000_del. The different colors represent P-values. Gray represents P-value [?] 0.05. Fisher's test with Bonferroni correction was used to compare yields between groups. P-value < 0.05 was statistically significant. OR: odds ratio

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