Copy number variants and fetal structural abnormalities in stillborn fetuses: a secondary analysis of the Stillbirth Collaborative Research Network study

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Abstract

Objective To examine the association of placental and fetal DNA copy number variants (CNVs) with fetal structural malformations (FSMs) in stillborn fetuses. Design A secondary analysis of stillbirth cases in the Stillbirth Collaborative Research Network (SCRN) study. Setting Multicenter, 59 hospitals in 5 geographic regions in the USA. Population 384 stillbirth cases of the SCRN study (2006-2008). Methods FSMs were grouped by anatomic system and specific malformation type (e.g., central nervous system, thoracic, cardiac, gastrointestinal, skeletal, umbilical cord and craniofacial defects). Single-nucleotide polymorphism array detected CNVs of at least 500kb. CNVs were classified into two groups: normal, defined as no CNVs>500kb or benign CNVs, and abnormal, defined as pathogenic or variants of unknown clinical significance. Main outcome measures The proportions of abnormal CNVs and normal CNVs were compared between stillbirth cases with and without FSMs using the Wald Chi-squared test. Results The proportion of stillbirth cases with any FSMs was higher among those with abnormal CNVs compared with those with normal CNVs (46.7% vs. 19.6%; p-value<0.001). The most common organ system-specific FSMs associated with abnormal CNVs were cardiac defects, followed by craniofacial and skeletal defects. A pathogenic deletion of 1q21.1 involving 46 genes (e.g., CHD1L) and a duplication of 21q22.13 involving 4 genes (SIM2, CLDN14, CHAF1B, HLCS) were associated with a skeletal and cardiac defect, respectively. Conclusion Specific CNVs involving several genes were associated with FSMs in stillborn fetuses. The findings warrant further investigation and may inform counseling and care surrounding pregnancies affected by FSMs at risk for stillbirth.

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Abstract

Objective

To examine the association of placental and fetal DNA copy number variants (CNVs) with fetal structural malformations (FSMs) in stillborn fetuses.

Design

A secondary analysis of stillbirth cases in the Stillbirth Collaborative Research Network (SCRN) study.

Setting

Multicenter, 59 hospitals in 5 geographic regions in the USA.

Population

384 stillbirth cases of the SCRN study (2006-2008).

Methods

FSMs were grouped by anatomic system and specific malformation type (e.g., central nervous system, thoracic, cardiac, gastrointestinal, skeletal, umbilical cord and craniofacial defects). Single-nucleotide polymorphism array detected CNVs of at least 500kb. CNVs were classified into two groups: normal, defined as no CNVs>500kb or benign CNVs, and abnormal, defined as pathogenic or variants of unknown clinical significance.

Main outcome measures

The proportions of abnormal CNVs and normal CNVs were compared between stillbirth cases with and without FSMs using the Wald Chi-squared test.

Results

The proportion of stillbirth cases with any FSMs was higher among those with abnormal CNVs compared with those with normal CNVs (46.7% vs. 19.6%; p-value<0.001). The most common organ system-specific FSMs associated with abnormal CNVs were cardiac defects, followed by craniofacial and skeletal defects. A pathogenic deletion of 1q21.1 involving 46 genes (e.g., *CHD1L*) and a duplication of 21q22.13 involving 4 genes (*SIM2* , *CLDN14* , *CHAF1B* ,*HLCS*) were associated with a skeletal and cardiac defect, respectively.

Conclusion

Specific CNVs involving several genes were associated with FSMs in stillborn fetuses. The findings warrant further investigation and may inform counseling and care surrounding pregnancies affected by FSMs at risk for stillbirth.

Keywords

Copy number variants; fetal; structural malformation; stillbirth

Running title

Copy number variants and fetal anomalies.

Tweetable abstract

Abnormal copy number changes in stillborn DNA are associated with fetal structural malformations.

Introduction

Fetal anomalies, defined as single or multiple structural malformations or functional changes, occur in 2–3% of fetuses and account for 20-30% of perinatal mortality.[1] In 9.5% and 13.7% stillbirth cases, respectively, fetal structural malformations and genetic abnormalities (e.g. aneuploidy) were identified as potential causes of death.[2-5] Other forms of genetic abnormalities such as copy number variants (CNVs) occurring as deletion or duplication of genomic material $>$ 1000 base pairs in length, can influence phenotype and cause disease by disrupting genes.[6, 7]

Recent studies showed associations between pathogenic CNV deletions and duplications with stillbirth cases and anomalous live-born fetuses.[4, 5] However, the relationship between CNVs and fetal structural malformations among stillbirth cases is uncertain. Routine ultrasound is utilized for detecting fetal structural malformation but remains limited in detecting organ system level malformations. For example, detection rate for all congenital cardiac malformations using ultrasound is only a 36 to 39%.[8]As chromosomal microarray assessments aid standard genetic testing for perinatal diagnoses,[9] identifying pathogenic CNVs associated with fetal structural malformations can guide the management and counseling of families at risk for stillbirth. As such, information about the likelihood of associated anomalies that are not apparent in the second trimester may inform important medical decisions.[8]

Interpreting the pathogenicity of CNVs, however, remains challenging,[6] as limited studies link relevant clinical information to genetic observations in a structured way. Following our recent work on CNVs and placental abnormalities,[10] we used our multicenter setting of stillbirth population to determine specific CNVs associated with fetal structural malformations. We hypothesized that duplicated or deleted pathogenic CNVs in fetal/placental genes are associated with fetal structural malformations. Determining specific pathogenic chromosomal abnormalities associated with fetal anomalies in stillbirth will improve databases that are essential for the interpretation of variants in diagnostic and research contexts.[11]

Methods

We conducted a secondary analysis of the Stillbirth Collaborative Research Network (SCRN) study, a racially/ethnically diverse, population-based case-control study of stillbirth with enrollment at the time of delivery. The SCRN study recruitment sites and study population details have been previously described.[4, 12] The study was supported by grant funding from the *Eunice Kennedy Shriver*National Institute of Child Health and Human Development: U10-HD045953 Brown University, Rhode Island; U10-HD045925 Emory University, Georgia; U10-HD045952 University of Texas Medical Branch at Galveston, Texas; U10-HDO45955 University of Texas Health Sciences Center at San Antonio, Texas; U10-HD045944 University of Utah Health Sciences Center, Utah; and U01-HD045954 RTI International, RTP. Secondary analysis of the primary research was supported in part by the National Center for Advancing Translational Sciences of the National Institutes of Health under Award Number 1UL01TR002538. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health. An advisory board reviewed the progress and safety of the study and written informed consent was obtained

from each participant. Study participants were not involved in the development of the research and a core outcome set has not been used in the research.

In the present analysis, we excluded cases with multifetal gestations and without perinatal postmortem examination data. Among eligible cases, we included those with and without major fetal anomalies as described previously.[13] Women were enrolled at the time of diagnosis of stillbirth, diagnosed as fetal death that occurred at 20 weeks' gestation or more. Gestational age was determined by the best clinical estimate from multiple sources including information from assisted reproductive technology, last menstrual period and/or obstetric ultrasound.[14] Women underwent a standardized maternal interview, medical record abstraction, biospecimen collection, an obstetric ultrasound exam, and postmortem examinations of the fetus and placenta. The standardized postmortem protocol ensured evaluation of stillbirth consistently across study geographic regions to best identify stillbirth where a fetal or placental condition caused or significantly contributed to the fetal death.[15] To avoid inadvertent exclusion of fetuses that may have been [?]20 weeks of gestation, fetal deaths occurring at 18-19 weeks and 6 days estimated gestational age with poor gestational dating criteria were included as stillbirth cases.[12, 13] Fetal autopsy and placental examinations of stillbirth cases were performed by perinatal pathologists using study-specific standardized protocols, that included centralized training.[15, 16]

Fetal structural malformations were prenatally diagnosed and grouped as major malformations by anatomic system and specific malformation type as described previously.[13] The groupings include cystic hygroma, central nervous system (open neural tube defect, anencephaly, hydranencephaly, hydrocephalus, holoprosencephaly, other) using a protocol for neuropathologic examination,[16] thoracic (congenital diaphragmatic hernia, cystic adenomatoid malformation, pulmonary sequestration, other), cardiac (anteroseptal defect, ventricular septal defect, atrioventricular canal defect, transposition of the great vessels, tetralogy of Fallot, other), gastrointestinal (gastroschisis, omphalocele, duodenal atresia, other), genitourinary (hydronephrosis/ureteropelvic junction obstruction, autosomal recessive polycystic kidney disease, multicystic/dysplastic kidney, posterior urethral valves, renal agenesis, other), skeletal (skeletal dysplasia, club feet, other), umbilical cord, craniofacial (cleft palate, other), hydrops fetalis, other and any anomalies. To further describe malformations, a 'write in' option was used, where sections were individually reviewed to ensure appropriate categorization. For example, 'craniofacial anomalies' category was added after data inspection revealed that these were not consistently categorized. In addition, a single diagnosis for 'hydrops' category was added, and when multiple abnormalities were noted and consistent with hydrops (i.e. ascites, pleural effusions, skin oedema). Other structural malformations were defined based on 'write in' option, where sections were individually reviewed to ensure that they were appropriately categorized. Multiple structural malformations (any malformation) were classified according to each system for which an abnormality was present.

Placental and stored/frozen fetal liver, fetal muscle and cord blood tissue biospecimens were collected from stillbirth cases. Sizes of placental biopsies varied, but they were as large as 1 cm^3.[16] In macerated fetuses, fresh samples were obtained from the placenta. DNA from placental biopsies was stored at -20°C for 2 to 5 years before microarray analysis, which was performed at a single laboratory (Columbia University Medical Center). DNA from stored frozen muscle and liver specimens was extracted immediately before microarray analysis and were used when placental DNA was unavailable for analysis. Fetal muscle, cord blood or fetal liver were used for chromosomal microarray assessment when placental DNA was unavailable (n=106 [19.9%]).

DNA Samples were analyzed using the Affymetrix Genome Wide Human single nucleotide polymorphism (SNP) Array 6.0 and the Chromosome Analysis Suite, version 1.0.1, and the NetAffx annotation database, version 28 for microarray analysis (Affymetrix). Data were aligned to the Human Genome release 18 (hg18). CNVs with [?]500 kb in size were detected using the SNP array. Analysis of the array data was conducted to determine aneuploidy, potential maternal-fetal contamination, and sex discordance. Classification of CNVs was based on the American College of Medical Genetics (ACMG) standards and guidelines for interpretation and reporting, with modifications as described previously.[4, 17] Due to improving resolution for determination of pathogenicity of CNVs, the number of novel structural variants is constantly increasing.[18] An

efficient computational analysis may be required to update pathogenicity score of manually defined variants of unknown clinical significance (VOUS) CNVs. Therefore, we implemented the latest ACMG guidelines[19]in high-throughput CNV analysis to classify and update pathogenicity of CNVs previously categorized as VOUS by using ClassifyCNV tool.[20] Using the ACMG guideline, we classified CNVs into two groups: abnormal CNVs, defined as pathogenic CNVs (including aneuploidy) or VOUS, and normal CNVs, defined as no CNVs $>$ 500 kb or benign CNVs.[21-23] As such, the abnormal CNVs and normal CNVs groups were compared in statistical analysis and only pathogenic CNVs (excluding aneuploidy, sex-chromosome and VOUS CNVs) were discussed.

We used the Wald Chi-squared test and a two-by-two table to compare the proportions of abnormal CNVs and normal CNVs between stillborn fetuses with and without fetal structural malformations. Other categorical measures were similarly compared between abnormal CNVs and normal CNVs. To compare continuous measures, ANOVA statistics was used. Data were analyzed with the use of statistical software programs: SAS version 9.4 (SAS Institute Inc), R and STATA version 15.0 (StataCorp), and ClassifyCNV tool.[20]

Results

Among women enrolled in the SCRN study, 384 stillbirth cases that had chromosomal microarray and fetal structural malformation assessments were included in the present analysis. Among 384 stillbirth cases, 58 (15.1%) had abnormal CNVs and 326 (84.9%) had normal CNVs. As previously described,[10] cases with abnormal CNVs were older, Hispanic, and with any fetal structural malformations in comparison to cases with normal CNVs. Similarly, cases with abnormal CNVs were not different in their proportions from those with normal CNVs in regard to other socio-economic factors, parity, fetal sex, maternal chronic hypertension, preeclampsia, diabetes and gestational diabetes.

The most common organ system-specific fetal structural malformations were cardiac defects, followed by craniofacial and skeletal defects, and hydrops. The proportion of cases with fetal structural malformations (any anomaly) was higher among those with abnormal CNVs compared with those with normal CNVs (46.7% vs. 19.6%; p-value$<$0.001;**Figure 1** and **Table S1**). We also found organ system-specific associations with abnormal CNVs. For example, the proportions of cases with cardiac, craniofacial and skeletal defects that had abnormal CNVs were higher than those that had normal CNVs (26.3% vs. 4.3%; p-value$<$0.001, 21.8% vs. 2.3%; p-value$<$0.001, and 19.2% vs. 4.0%; p=0.005, respectively). Hydrops, cystic hygroma and gastrointestinal defects were more common when abnormal CNVs were present, but in relatively smaller proportions (all p-values$<$0.05). Malformations categorized as 'other anomaly', umbilical cord abnormalities, central nervous system, thorax and genitourinary defects were not different in their proportions of abnormal CNVs in comparison to those in the normal CNVs group. The proportion of stillborn fetuses with normal CNVs that had any fetal structural malformation was lower than the proportion of those with normal CNVs and had no fetal structural malformation (70.3% vs 89.4%, p-value$<$0.001; **Table S2**). A pathogenic deletion of 1q21.1 involving 46 genes, including a known *CHD1L* gene, and a duplication of 21q22.13 involving 4 genes (*SIM2* , *CLDN14* ,*CHAF1B* , *HLCS*) were associated with a skeletal and cardiac defect, respectively (**Table 1**).

Discussion

Main Findings

In the present study, we found that abnormal CNVs are associated with several fetal structural malformations among stillborn fetuses. A pathogenic CNV deletion of 1q21.1 involving 46 genes (e.g.,*CHD1L*) and a duplication of 21q22.13 involving 4 genes (*SIM2* , *CLDN14* , *CHAF1B* , *HLCS*) were associated with a skeletal and cardiac defect, respectively.

Interpretation

In light of a previous large study that examined all fetuses,[5] our findings of CNVs in gene-rich regions that are associated with fetal cardiac and skeletal anomalies in stillborn fetuses is noteworthy. Donnelly *et. al.* showed that chromosomal microarray analysis remained most informative in particularly identifying fetuses

with cardiac and craniofacial anomalies, and most CNVs occurred in those with cardiac anomalies.[5] In general, fetuses with anomalies in more than one system had higher frequency of abnormal CNVs compared with fetuses without anomalies (13.0%; p-value<0.001), suggesting that chromosomal microarray analysis identifies anomalies dependent on the type and number of organ systems involved.

Cardiac abnormalities are among the most common congenital malformations, associated with stillbirth[13] and present in an estimated 0.8% of live births.[8]Skeletal defects involve a heterogeneous group of bone abnormalities resulting in abnormal growth and shape of the fetal skeleton.[24] Skeletal defects in the first trimester are associated with nuchal translucent, and in lethal dysplasias, bone shortening may be obvious as early as 11 weeks' gestation.[24] Among fetuses with perinatal diagnosis of ventricular septal defects, most common cardiac defects, chromosomal microarray was particularly effective in identifying pathogenic CNVs.[3] Thus, gene may potentially play a role in the etiology of fetal ventricular septal defects.

Deletion of 1q21.1 (4.0 Mb in size) and duplication of 21q22.13 (500 kb in size) CNVs were previously described as pathogenic abnormalities of stillbirth in the same study.[4] However, these CNVs were not described in the context of fetal skeletal or cardiac defects. Expression analyses of *CHD1L* (chromodomain helicase DNA binding protein 1 like), among 46 genes implicated in 1q21.1 region in our study, showed that it promotes neuronal differentiation of human embryonic cells. This suggested that *CHD1L* plays an important role in the nervous system development.[25] In addition, a pathogenic variant in *CHD1L* is also associated with short stature.[26] In the adult population, distal variant CNVs of 1q21.1 were reported to be associated with cerebral and cognitive alterations.[27] Furthermore, high prevalence of neurodevelopmental disorders are associated with CNVs at 1q21.[28] Specifically, the CNV microdeletion and microduplication at 1q21 were enriched in patients with schizophrenia, intellectual disabilities and autism spectrum disorder.[28-30] These studies highlight the pathophysiological mechanisms of CNVs in 1q21 in neurodevelopmental disorders.

Limitations and strengths

While our study has the potential to provide high-quality data of chromosomal abnormalities in fetuses suspected to have isolated cardiac and skeletal malformations, several limitations exist. First, microarray-based analyses may be limited to detect truly balanced rearrangements of chromosomes. The large chromosomal regions identified span several genes, and the Affy500K SNP Array is limited to detect genetic abnormalities at higher resolution, e.g., specific mutations in genes for clinical application. Up-to-date microarray platforms would be sensitive to detect CNVs at greater resolution (e.g., >150 kb), and with increased sequencing our knowledge of the pathogenicity of variants may change.[31] Next-generation sequencing of cases will improve diagnostic yield of genes in fetal structural malformations.[32] Further, due to lack of paternal DNA, we were not able to distinguish inherited from newly occurring CNVs in the placenta or fetus, which will be important future areas of investigation for identifying causative of genetic abnormalities. Finally, we have limited sample size to detect associations of CNVs with other fetal structural malformations. As resolution for determination of pathogenicity of CNVs is improved, CNVs that are thought to be benign may eventually be designated as pathogenic, increasing the sample size of stillborn fetuses identified as having abnormal CNVs in our study.

Several strengths of our study deserve mention. The population included in our study is geographically, ethnically and racially diverse, potentially ensuring the generalizability of our findings. Moreover, participants provided consent for a complete evaluation, including fetal standardized postmortem examination, placental pathological analysis, and maternal-fetal testing. These study design features provided careful phenotyping of stillbirth in our study, ensured accurate ascertainment of fetal structural malformations, and maximized the validity of the present analysis.

Conclusion

Among stillborn fetuses, specific CNVs involving several genes were associated with fetal structural malformations and warrant further investigation. These data improve genotype-phenotype databases, potentially informing more precise understanding of genetic etiologies of human developmental disorders.[6] The data may also inform counseling and care surrounding pregnancies affected by fetal structural malformations at

risk for stillbirth.

Disclosure of interests

Eunice Kennedy Shriver National Institute of Child Health and Human Development grants were received by the institutions listed. No other conflicts of interest are reported by authors. Completed disclosure of interests forms are available to view online as supporting information.

Contribution to authorship

RMS, UMR, HP and RLG had critical roles in the conception, planning and carrying out of the study. TW, AA, SLS, and VT played a critical role in data management and secondary data analysis. TW, RMS, SLS, UMR, HP, RLG, SD, AA, AZC, JMP, NRB and VT each played critical roles in conception, interpretation of the secondary data analysis and writing the manuscript. The University of Utah Institutional Review Board found this secondary analysis qualified for IRB exemption 5/9/2019 IRB#122488 due to the de-identified nature of the data.

Ethical approval

The study was approved by the institutional review board at each clinical site and the data coordinating center. An advisory board reviewed the progress and safety of the study and written informed consent was obtained from each participant.

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Data availability statement

The data that support the findings of this study are avail- able from the corresponding author upon reasonable request.

Table S1. Associations of placental/fetal CNVs with fetal structural malformations in stillbirths

Fetal Structural Malformations	abnormal CNVs	normal CNVs	P-value [a]
Total Stillbirth Cases	**58 (100)**	**326 (100)**	
Any anomaly	27 (46.7)	64 (19.6)	<.001
Cardiac defects	15 (26.3)	14 (4.3)	<.001
Craniofacial defects	13 (21.8)	8 (2.3)	<.001
Skeletal defects	11 (19.2)	13 (4.0)	0.005
Hydrops	11 (19.8)	12 (3.8)	0.004
Cystic Hygroma	10 (16.7)	4 (1.3)	0.003
Gastrointestinal defects	9 (15.8)	11 (3.3)	0.011
Genitourinary defects	6 (10.8)	11 (3.5)	0.102
Central Nervous System	5 (8.5)	10 (3.0)	0.169
Umbilical cord abnormalities	4 (7.1)	20 (6.0)	0.768
Thorax defects	4 (6.6)	6 (1.8)	0.152
Other anomaly	3 (5.3)	27 (8.2)	0.401

[a] P-values were based on a Wald chi-squared test.

Table 1. Specific pathogenic CNVs associated with fetal structural malformations in stillbirths

Structural Malformation	Type of CNV	Gestational Age at stillbirth	ISCN [a] Nomenclature
Skeletal defect	DEL	34w6d	arr 1q21.1(143,845,772-146,838,707)x1
Cardiac defect	DUP	22w6d	arr 21q22.13(36,685,848-37,185,921)x3

[a] International System for Human Cytogenetic Nomenclature

Table S2. Stillbirth cases by any fetal structural malformation status and abnormal CNVs vs. normal CNVs.

	Abnormal CNVs	Normal CNVs	Total
Any fetal structural malformation	27 (29.7%)	64 (71.4%)	91
No fetal structural malformation	31 (10.6%)	262 (89.4%)	293
P-value [a]	<0.001	<0.001	

[a] P-values were from Wald Chi-squared test that compared the proportions of fetuses with and without any fetal structural malformations among those with abnormal and normal CNVs.

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