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Prenatal Exome Sequencing in Anomalous Fetuses: New Opportunities and Challenges

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Abstract

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Conflict of Interest Declaration

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Purpose—We investigated the diagnostic and clinical performance of exome sequencing (ES) in fetuses with sonographic abnormalities with normal karyotype, microarray and, in some cases, normal gene specific sequencing.

Methods—ES was performed from DNA of 15 anomalous fetuses and from peripheral blood from their parents. Parents provided consent for the return of diagnostic results in the fetus, medically actionable findings in the parents, and identification as carrier couple for significant autosomal recessive conditions. We assessed perceptions and understanding of ES with mixed-methods in 15 mother-father dyads.

Results—In 7 (47%) of 15 fetuses, ES provided a diagnosis or possible diagnosis with identification of variants in the following genes: *COL1A1*, *MUSK*, *KCTD1*, *RTTN*, *TMEM67*, *PIEZO1*; and *DYNC2H1*. One additional case revealed a *de novo* nonsense mutation in a novel candidate gene (*MAP4K4)*. The perceived likelihood that ES would explain the results (5.2/10) was higher than the approximately 30% diagnostic yield discussed in pre-test counseling.

Conclusions—ES has diagnostic utility in a highly select population of fetuses where a genetic diagnosis was highly suspected. Challenges related to genetics literacy, and variant interpretation must be addressed by highly tailored pre- and post-test genetic counseling.

Keywords

prenatal; diagnosis; exome; ethics; counseling

INTRODUCTION

Congenital anomalies affect 2–4% of all infants and are responsible for 20% of perinatal deaths.[1] Currently, prenatal diagnosis begins with a positive serum or cell free DNA screen for aneuploidy. This is followed by targeted anatomical survey and diagnostic tests such as chorionic villus sampling or amniocentesis. Standard karyotype and microarray are obtained from chorionic villi or amniocytes, or if specific pathogenic variants are known in the parents, targeted sequencing is performed. While microarray increases diagnostic yield above standard karyotype alone, 80–90% of anomalous fetuses with a normal karyotype also have a normal microarray and thus remain without a definitive diagnosis.[2,3] Additional molecular genetic testing, either single gene or panels driven by phenotype may be performed if indicated and if a limited differential diagnosis suggests success for such targeted sequencing. Exome sequencing (ES), which provides sequence data from the exons (the coding regions) of known genes in the human genome, has proven to be a powerful diagnostic tool in adults and children with genetic disorders, such as birth defects and intellectual disability.[4,5] Compared to a 10% diagnostic rate using karyotype with microarray, ES has diagnostic rates of approximately 30% in a post-natal cohort of patients with birth defects.[4] The use of ES of fetal DNA obtained by amniocentesis has been reported in isolated cases.[6,7] Small case series reporting increased diagnostic utility of ES prenatally after a normal microarray have also been published showing diagnostic rates ranging from 10–57%.[8–10] Thus, ES appears to be a promising technique to fill the existing diagnostic gap for fetal diagnosis.

ES appears to be a promising technique because it has increased diagnostic capability when karyotype and microarray are normal and is less costly and more clinically applicable than whole genome sequencing. Our aim was to use ES to examine its utility for prenatal diagnosis in non-continuing (defined as pregnancy termination, intrauterine fetal demise, or neonatal death in the delivery room) pregnancies with multiple anomalies and normal results with standard prenatal genetic diagnostic tests (karyotype and microarray). Targeting this population for initial study focuses on those families with greatest need while avoiding some of the ethical complexities of communicating risk or study findings in on-going pregnancies. Additionally, because of the unique challenges related to implementation of exome sequencing prenatally, we sought to understand maternal perspectives, expectations, and understanding of fetal genetic results obtained by exome sequencing.

MATERIALS AND METHODS

Mother-father-fetus trios in pregnancies complicated by a fetus with multiple congenital anomalies were identified from the University of North Carolina at Chapel Hill prenatal diagnosis clinics (Chapel Hill, NC and Raleigh, NC) between July 2014 and July 2016. Approval from The University of North Carolina at Chapel Hill Institutional Review Board (13-4084) was obtained prior to patient consent and enrollment. Inclusion criteria include the following: 1) pattern of anomalies highly suggestive of an underlying genetic disorder; 2) unknown diagnosis based on karyotype, microarray, and in some cases, gene specific sequencing; 3) Fetal and parental DNA available. Trios were identified prospectively and retrospectively, enabling us to obtain fetal specimens at various gestational ages. Prospectively, women pregnant with a singleton fetus suspected to have a lethal anomaly consistent with a genetic disorder were approached for participation after they made the decision to continue the pregnancy. In the case of non-continuing pregnancies, the research study was not mentioned or offered until after the couple had made a decision to terminate the pregnancy. Retrospective identification of potential trios was accomplished by querying the UNC Perinatal Database to identify women with a history of fetal or neonatal death who had not received an explanatory diagnosis by standard prenatal testing. We contacted women who previously indicated a desire to be re-contacted if additional fetal testing options become available and who had fetal cells archived and available for DNA extraction for potential enrollment. Additional participants in the retrospective cohort were either self-referred or referred by a clinician aware of our current study recruitment. Once participants were enrolled, we collected parental blood and retrieved stored fetal samples for ES analysis. After the first 7 trios were enrolled, we expanded enrollment to individuals not receiving care at UNC; by using Skype to facilitate counseling, consent and results discussion in non-local cases. The sample size of 15 trios is a convenience sample for this pilot study.

Mothers and fathers from both retrospective and prospective groups had pretest counseling about ES and the possible results it can provide. Consent was obtained separately from mothers and fathers; both were informed about the possibility of ES revealing non-paternity. Participants were given the option to opt out at any time during the study. Because of the complexity of the genetic information that results from ES, consent and return of results were performed by a Certified Genetic Counselor who was not involved in the patient's

clinical care to avoid bias and undue pressure on the patient to participate. All participants agreed to learn of 1) any diagnostic findings with potential to explain the fetal phenotype, 2) any medically actionable incidental findings in a parent that would have medically actionable implications for that parent,[11] and 3) carrier status for significant autosomal recessive conditions in which both parents are carriers. Diagnostic results were classified into seven categories (Table 2). More than one result could be provided for a trio. After consent, we obtained parental blood and extracted DNA in the Biospecimen Processing Facility (BSP) or, for non-local cases, received DNA directly from an outside institution. If previously isolated DNA was not available, we extracted fetal DNA from stored products of conception, fetal amniocytes or villi (retrospective) or from umbilical cord blood, amniocytes, or chorionic villi (prospective). We split the DNA and sent a duplicate sample to the UNC Molecular Genetics Laboratory (MGL), a CLIA-certified and CAP-accredited facility, where it was stored and used for Sanger sequencing confirmation of genetic variants returned to participants. Duplicate samples streamline the process of variant confirmation and allow for quality checks between samples, as well as making results eligible for inclusion in the medical record. After confirmation with Sanger sequencing, parents were given the option to sign a separate consent form to have their own or their deceased child's variants placed in the electronic medical record.

ES and Variant Analysis

We created ES libraries and exome capture from maternal, paternal, and fetal DNA samples as previously described[12] and transferred them to the UNC High Throughput Sequencing Facility for sequencing using the Illumina Hi-Seq 2500. We processed, mapped, and aligned raw-read data, and identified variants using a standard bioinformatics pipeline developed for the NCGENES project in collaboration with colleagues in the Department of Genetics and the Renaissance Computing Institute.[13]

We captured quality metrics at all stages of processing to determine whether outputs could be used for analysis. Metrics include checks on input file correctness, distributions of nucleotide and quality scores, percent of reads aligned, read gap distributions, percent of reads with pairs, metrics on coverage across the genome and from targeted regions, and metrics from genome analysis toolkit (GATK) on called variants. Variants were annotated with information regarding predicted molecular effect (SnpEff)[14] and population allele frequencies (ExAC).[15] These additional annotations and trio data were used to filter and prioritize variants according to inheritance patterns (*de novo*, compound heterozygous variants, and homozygous recessive variants) within the trio using GEMINI.[16] Similar to whole exome sequencing used post-natally, all protein coding regions of the genome were interrogated. We also used a "gene list prioritization" approach to present all known pathogenic, rare truncating, and rare missense variants in genes known to have an association with the fetal phenotype (examples of such gene lists are shown in Supplementary Tables). Gene lists specific to the phenotype in question were curated using the primary literature and by reviewing previously developed panels currently in use. When no finding was identified using a gene list, all homozygous variants and compound heterozygous variants in autosomal recessive disorders, and de novo variants in autosomal dominant disorder were manually reviewed. Variants were manually reviewed by molecular

analysts using multiple sources (e.g., mutation databases, Online Mendelian Inheritance of Man (OMIM), PubMed, Exome Aggregation Consortium (ExAC)) for potential function in relation to the phenotype.

A committee of clinical and laboratory geneticists, obstetricians, genetic counselors, and pediatricians who were not involved in the patient's clinical care reviewed all findings of the molecular analysts to make a final determination about return to participants and result classification (e.g. positive-probable, uncertain VUS, etc.) using criteria developed by Richards et al. (Table 2).[17]. All variants thought to be potentially causative were reviewed by the committee within two weeks of the primary analyst identifying the variant. Results believed to clearly (or possibly) explain the fetal phenotype were reported to parents after confirmation in a CLIA-certified molecular genetics clinical laboratory. Also, all parental samples were analyzed for a small subset of "medically actionable" genes (e.g., *BRCA1/2*) per the American College of Medical Genetics and Genomics and any findings in the parents were reported.[11,17–21] Parents also consented to return of carrier status for significant autosomal recessive conditions in which both parents are carriers. All reported variants, whether diagnostic or incidental, were confirmed by Sanger sequencing in a CLIA-certified molecular genetics laboratory. The diagnostic results were categorized into seven categories (Table 2).[11,21,22]

Assessment of Maternal Perspectives and Understanding

We completed a mixed-methods assessment using questionnaires and semi-structured interviews with 15 mothers. We focused on the mother's perspectives and understanding in this pilot study. After informed consent, each mother completed a pre-sequencing questionnaire (8 questions related to demographics) and literacy genomic knowledge scale (25 true-false questions to assess recall and understanding of the structure and function of genes, how they are inherited, their relation to health, and strengths and limitations of ES). The literacy assessments were modified for prenatal use from previously used scales from the NCGENES project. A Wilcoxon rank sum test was performed to compare literacy genomic knowledge scores with income levels; a p-value less than or equal to 0.05 was defined as significant. This was followed by a semi-structured interview with the mother to identify expectations, understanding, and perceptions. To reduce bias, a trained research assistant rather than the genetic counselor or the PI, conducted the in-person interview with the mother (~45 minutes) adapted from a study of diagnostic genome sequencing in adult and pediatric patients (NCGENES; PI: Evans).

A trained research assistant conducted follow-up telephone post-quantitative and interview assessments with the mother 4 weeks after return of results to measure understanding and the impact of the information on future decisions.

RESULTS

Participant demographics of the cohort are shown in Table 1. Most (13/15) participants enrolled shortly after routine fetal genetic testing (CVS or amniocentesis for karyotype and microarray) was completed. All enrolled pregnancies had both normal karyotype and single nucleotide polymorphism (SNP) prenatal microarray. However, 2/15 were enrolled 5–10

years after the prior affected pregnancy (cases 1 and 2). Turn-around time to identify pathogenic variants once sequencing was performed ranged from 0 days to 28 days (mean 21 days). Gene lists were developed and used for cases with skeletal findings, non-immune hydrops, and for genitourinary abnormalities. Two of the three skeletal cases were diagnosed using the skeletal dysplasia gene list prioritization approach (*COL1A1* and *DYNC2H1*) and had the shortest turn around time (0 days to identify pathogenic variants once sequencing data was available).

Molecular Diagnoses

Genotype and phenotype data are listed in Table 4. In 7 (47%) of 15 trios, ES provided a diagnosis or possible diagnosis of the following disorders: osteogenesis imperfecta type 3 (*COL1A1*), fetal akinesia sequence (*MUSK*), scalp-ear nipple syndrome (*KCTD1*), primordial microcephaly-dwarfism syndrome (*RTTN*); Meckel-Gruber syndrome (*TMEM*67); lymphatic dysplasia *(PIEZO1)*; short rib polydactyly syndrome (*DYNC2H1*). Of the mutations found, there were two de novo mutations in the proband fetuses (*COL1A1 and KCTD1*) and five autosomal recessive disorders (*MUSK, RTTN, TMEM67, PIEZO1, DYNC2H1*) conferring a 25% risk of recurrence in a subsequent pregnancy. ES provided evidence for expanding the phenotype in one of these syndromes (scalp-ear nipple syndrome; *KCTD1*) to the fetal period. There was a significant family history in only one fetal case (case 5; fourth pregnancy affected with arthrogryposis phenotype). Two cases (case 7 short rib polydactyly and case 9 meckel-gruber syndrome) had sufficient ultrasound findings to enable the provider to send the correct gene-specific panel for the specific phenotype of interest. Although other variants in our positive diagnoses could be detected by a gene panel (case 1 osteogenesis imperfecta), the ultrasound phenotype was not detailed enough (shortened long bones with bowing) for the provider to reliably pick the correct panel by the ultrasound findings alone. In addition, autopsy and skeletal survey findings can suggest the wrong diagnosis (case 1: autopsy and skeletal survey suggested hypophosphatasia when OI, type 3 was the diagnosis) which would have led the provider astray.

Demonstrating the potential of ES in fetuses to reveal new candidate genes for developmental disorders, in one case with complex cardiac defect and abnormal kidney location, a *de novo* stop gain mutation was found in *MAP4K4*. This gene is known to be integrally involved in vascular development and cell migration and is embryonic lethal in knockout mouse models but no human phenotype has yet been described.[23] Because this gene has not been associated with human disease, the clinical significance of this variant is uncertain.

In two other cases, a single mutation in a gene associated with autosomal recessive inheritance of a phenotype consistent with the fetal presentation was identified. Incomplete sequencing coverage and the possibility of undetected deletions or duplications beyond what could be detected with microarray (all fetuses enrolled had normal microarrays) precluded exclusion of a second mutation.

We found only one medically actionable finding in a parent (familial hypercholesterolemia, *LDLR*); it was confirmed with Sanger sequencing. The parent in this case was already being

treated for high cholesterol and has a strong family history of hypercholesterolemia. The participant was encouraged to share the information with family members in the post-test counseling session. None of the couples had significant carrier results to report. Two couples chose to have fetalresults placed in the medical record. They plan to have prenatal diagnosis in a future pregnancy if the same anomalies are noted.

The mothers' self-report of knowledge and attitudes revealed a median perceived likelihood of 5.2 on a 10 point likert scale (range=2–7) that ES would provide a result for the abnormalities identified in a couple's fetus. Median genomic knowledge prior to sequencing was high (median 92; range 76–100). The study was not powered to detect a difference in genetics knowledge base assessment by socioeconomic background but there was a statistically significant finding that women in the highest socioeconomic group (>$90,000 annually) had higher pre-sequencing genomic knowledge (median 95 (95% CI: 91.6–98.4) than their lower income counterparts (<$90,000 annually) (median 88 (95% CI: 85–92.6) [$p<0.001$]. Seventy five percent of the women who scored above the mean were in the highest income bracket. In the post-assessments, all of the women expressed understanding of their ES results and felt having ES was a good decision in the post-results surveys and interviews. In a qualitative interview, the parent who received the incidental finding felt having ES was beneficial to his long-term health.

DISCUSSION

Our series of non-continuing anomalous pregnancies shows that the diagnostic utility of ES after normal standard genetic testing yields a definitive or possible explanation in up to (7/15) 47% of cases where a fetal genetic diagnosis was highly suspected. This is on the higher end of prenatal yields reported by other authors of similarly small series which range from 10–57% and confirms that exome sequencing increases the diagnostic yield prenatally in a select group of anomalous fetuses who fail to receive a diagnosis with standard genetic testing.[8–10,24] Criteria for study inclusion criteria, sample size, and diagnostic yield (DY) of other published studies are as follows: fetal demise or termination of pregnancy with multiple congenital anomalies with normal karyotype using trios (n=7) [DY=57%],[10] increased nuchal translucency (>3.5mm) and/or other abnormality with normal karyotype (n=24) [DY=21%],[25]diverse structural abnormalities on ultrasound using trios (n=30) [DY=10%].[24] It is important to note that diagnostic yield of any test depends on the prior probability of detectable conditions within that cohort, so it is likely that our apparently higher yield and that of Alamillo et al. reflects the inclusion of fetuses with a higher likelihood of a genetic etiology given that both studies only included fetuses in non-continuing pregnancies with multiple congenital anomalies. In addition, both our study and Alamillo et al. used trios consistently which improves diagnostic rates. The other studies with lower yields included fetuses with a single structural abnormality. The yield in a larger sample with broader inclusion criteria may be lower. In addition to selection of a cohort with a high likelihood of genetic etiologies, the interpretation of findings also influences diagnostic yield. Our approach was consistent with guidelines by Richards et al. and use of pre-established multidisciplinary variant analysis committees put in place for NCGENES (PI: Evans), thus, we do not feel our diagnostic yield was overinflated. Our study performed both karyotype and microarray on all included fetuses whereas other studies did not

consistently perform microarrays. Given that ES cannot detect larger copy number variants, we felt it important that chromosomal microarray be done prior to ES. Our findings suggest that ES will improve the accuracy of prenatal diagnosis in a select cohort of fetuses with multiple congenital abnormalities because ES has increased diagnostic capability when karyotype and microarray are normal.

Strengths of our study include the use of trios which enhances diagnostic yield and was not consistently used by other studies,[8,26] development of fetal specific gene lists to optimize turn-around time, development and use of trio-specific bioinformatics pipelines, and use of a multidisciplinary genetics team to evaluate classification of all results reported with respect to pathogenicity of the variants and (for diagnostic results) the likelihood that those variants explained the phenotype.[8,26] Our study also found that ES was useful in cases where a clinically available phenotype-driven panel did not provide an answer because we identified variants in genes that were not on the specific prenatal panel for the phenotype being tested, either because the gene had not been described at the time the panel was validated or because the phenotype was so heterogeneous that a complete panel could not be made (hydrops). Because we included only cases of non-continuing pregnancies, the postnatal exam of the fetus by a geneticist with autopsy was available in some cases to assist in refining the phenotype allowing us to specifically target genes associated with a particular phenotype and adding confidence when pathogenic variants were identified. Our study, along with previous studies, provides pilot data indicating that ES can improve prenatal diagnosis.

Given the important counseling issues inherent in ES, we also explored the important and critical issue regarding how mothers perceive and understand exome sequencing. Efforts to understand the psychosocial and behavioral impact of integrating genomic technologies into adult and pediatric practice are ongoing.[27–29] To date, little empirical work has been done to understand the unique challenges of applying exome or genome sequencing to the prenatal context. The experience of prenatal diagnosticians and patients regarding response to variants of uncertain significance and incidental identification of maternal pathology after prenatal chromosomal microarray (CMA) has been studied, and raise a range of similar issues.[30,31] These include complexities of trade-offs between better diagnostic ability than standard karyotype[32] but also greater risk of results with uncertain clinical significance. While prenatal diagnosticians have incorporated pre- and post-test counseling into their practice to explain nuanced results, the issues are magnified by the use of ES in this population given the higher incidence of uncertain variants in a sequencing context.

We found that women with lower income levels scored significantly lower on the genetics literacy assessment compared to women in higher income levels. We also found that women had high hopes and expectations (Likert scale 5.4) that ES would provide a result despite pre-test counseling by a genetic counselor that ES has previously been shown to yield a result approximately 30% of the time. However, when using a Likert scale participants may choose the neutral option because picking a neutral option allows people to avoid the cognitive effort needed to choose between their positive and negative feelings on an issue.[21] Attitudes towards prenatal screening and diagnosis are influenced by ethnicity, socioeconomic status, cultural and religious beliefs, acceptability of termination of

pregnancy, and experiences with disability and further research on this critical topic is needed to ensure that patient's needs are being met as new technologies inevitably become implemented in clinical practice.[33–35]

Our study also demonstrates how ES in this context can extend understanding of known and novel diseases that disrupt fetal development. The finding of a likely pathogenic variant in *KCTD1* expands the phenotype of a known Mendelian disorder (scalp ear nipple syndrome) to the fetal period. The discovery of a *de novo* truncating mutation in *MAP4K4* in a fetus with a complex heart defect makes this gene a novel candidate gene for a human developmental disorder given this gene's critical role in embryonic development of the heart in mouse models.[23,36,37] Further supporting the possibility of this gene as causative of the described phenotype is its *de novo* status. Further in vivo studies are planned using a zebrafish model to explore this intriguing finding.

Limitations of our study include relatively small sample size and selection of cases with a high *a priori* likelihood of having a genetic etiology. As cost decreases, ES may be more cost-effective than pursuing multi-gene panels, although analytic considerations, such as depth of coverage and coverage across exons may be optimal with molecular panels. Our study was not powered to identify statistical differences in outcomes related to maternal expectations and understanding; this is an area that needs further exploration in larger clinical studies of prenatal ES especially given that trends from this study show lower knowledge scores related to socio-economic status.

While ES is a promising diagnostic technology in the prenatal, childhood, and adult settings, there remain important limitations and ethical issues with the use of this technology, including provision of adequate counseling and informed consent. False negatives should be expected with ES given that most platforms cover only 85–90% of exons. Turn-around time has been cited as an issue when ES is applied prenatally but use of phenotype specific gene lists and trio analysis, as in the current study, has substantially decreased turn-around time.[38] Certainly, before ES is routinely implemented prenatally, turn-around time needs to be optimized so that reproductive decisions can be made in a timely manner. There are also ethical issues related to trio-sequencing including disclosure of identifying non-paternity, consanguinity, and medically actionable findings parents. In addition, if ES is applied in ongoing pregnancies, the additional ethical issue of being able to report a predisposition to adult onset disorders from fetal information arises. These issues will require ongoing ethical consideration as well as access to comprehensive genetic counseling by a certified genetic counselor with prenatal experience.

The results of the current study show that ES provides information to families, expands clinical phenotypes to the fetal period, and will likely enhance our knowledge of genes critical to fetal development. Neither the ACMGG nor the American College of Obstetrics and Gynecology recommends that ES be used routinely.[20,39] Questions about the most cost-effective and efficient way of identifying pathogenic variants in fetuses that do not receive a result with CMA should be addressed in larger clinical trials. Given the importance of responsibly applying new technologies to the broadest population possible, including traditionally underserved patients, decision aids in conjunction with a genetic counseling

session should be developed and studied to determine whether these interventions improve understanding of the types of results ES may provide. Further studies on both diagnostic utility and maternal expectations and understanding of prenatal ES are crucial before this technology becomes routinely incorporated into prenatal care.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Table 1

Demographics of the mothers

Characteristics	Study Cohort (n=15)
Age (years)	
Mean (SD [range])	32.0 ± 5.11(22–39)
Race	
Caucasian White	14 (93.3%)
African American	1 (6.6%)
Education Level	
High school graduate or equivalent	1 (6.6%)
College education	11 (73.3%)
Graduate or professional degree	3 (20.0%)
Total family income	
44,999 or less	4 (26.6)%
45,000–89,999	5 (33.3%)
90,000 or higher	6 (40%)
Prior genetic testing to look for causes of health problems	
Yes	8 (53.3%)
No	7 (46.6%)
Married	
Yes	13 (86.7%)
No	2 (13.3%)

Table 2

Classification scheme of case-level results[10,18,20,21]

Positive	
Positive-Definitive	Known pathogenic variant(s) in a known disease gene and consistent with inheritance pattern; fetal phenotype consistent with the reported disease spectrum
Positive-Probable	Likely pathogenic variant(s) in a known disease gene and consistent with the inheritance pattern; fetal phenotype consistent with the reported disease spectrum
Positive-Possible	A single rare or novel VUS known to be in trans with a pathogenic/known pathogenic variant in a gene that explains the phenotype
Uncertain	
Uncertain-VUS	Variant(s) of uncertain significance in a known disease gene and consistent with the inheritance pattern; fetal phenotype consistent with the reported disease spectrum. (e.g. uncertainty is limited to the pathogenicity of the variant).
Uncertain-AR Het	Single heterozygous variant (known pathogenic, likely pathogenic, or highly suspicious variant of uncertain significance) identified in a disease gene implicated in a recessive condition; fetal phenotype consistent with the reported disease spectrum
Uncertain-Contributory	Known pathogenic or likely pathogenic variant(s) in a known disease gene, but fetal phenotype is not completely consistent with the reported disease spectrum and thus the finding may contribute to but not completely explain the phenotype
Uncertain-Other	Category of other findings having uncertain case-level significance, including potential novel gene discoveries. For example, predicted deleterious variant(s) in a novel candidate gene that has not previously been implicated in human disease or for which the published data to support human disease association may not yet be definitive. Supporting data could be based on model organism data, CNV data, tolerance of the gene to sequence variation, data about tissue or developmental timing of expression, or knowledge of the gene function and pathway analysis. Further research is required to evaluate any of the suggested candidate genes.
Negative	
Negative	No variants in genes associated with the reported phenotype identified.

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Table 3

Phenotype and genotype information for the cohort

Case ID	Ultrasound Findings	Postnatal/Autopsy Results	ES result	Variant(s)	Classification
1	Skeletal dysplasia; shortened long bones and bowing	Skeletal survey post-mortem showed shorted long bones in upper and lower extremities, limited ossification of all bones with multiple fractures noted, broad metaphyses of humeri and tibia. Autopsy and skeletal surveys raises suspicion for hypophosphatasia.	De novo splice site mutation in ***COL1A1*** consistent with osteogenesis imperfecta.	c.1875+1G>A **Likely pathogenic**	Positive-probable
2	Postnatal exam showed skin sloughing	Not Applicable	De novo rare missense mutation in the BTB domain of the ***KCTD1*** gene, which is associated with scalp ear nipple syndrome.	c.86A>G p.Asn29Ser **Likely pathogenic**	Positive-probable
3	Severely malformed calvarium, microcephaly intracranial cyst, growth restriction, cerebellar hypoplasia, absent corpus callosum	Not Applicable	Compound heterozygous missense mutations in ***RTTN*** gene which causes brain malformations, microcephaly, growth retardation and ciliary dysfunction;	c.4480C>T p.Thr1398Met **Variant of uncertain clinical significance** c.5143 A>G p.Asn1715Asp **Variant of uncertain clinical significance**	Uncertain-VUS
4	Fetal akinesia sequence	Autopsy consistent with fetal akinesia sequence, no additional findings	Compound heterozygous missense mutations in the ***MUSK*** gene associated with fetal akinesia sequence;	c.1724T>C p. Ile575Thr **Likely pathogenic** c. 2408A>G p.Tyr803Cys **Variant of uncertain clinical significance**	Positive-possible
	4 affected pregnancies with an arthrogryposis phenotype	Not Applicable	One rare missense mutation in ***CHRND*** gene which causes lethal multiple pterygium syndrome; Copy number variant studies recommended to identify deletion not found by microarray or ES	c.817G>A p.Asp273Asn **Likely pathogenic**	Uncertain-AR Het
	Suspected autosomal recessive polycystic kidney disease; large echogenic kidneys; anhydramnios	Not Applicable	One rare missense mutation in the ***PKHD1*** gene; Copy number variant studies recommended to identify deletion not found by microarray or ES	c. 1342G>C p. Gly448Arg **Variant of uncertain significance**	Uncertain-AR Het
5	Suspected short rib polydactyly	Postnatal exam by a pediatric geneticist showed severe micromelia, bilateral postaxial hexadactyly, microcephaly and dysmorphic facial features; autopsy showed gut malrotation hepatomegaly, enlarged kidneys, abnormal position of heart vessels	Compound heterozygous mutations in the ***DYNC2H1*** gene which causes short rib polydactyly	c.10594C>T p.Arg3532Ter Novel nonsense mutation; **Likely pathogenic**. c. 8012T>C p.Met2671Thr Missense variant previously reported in an affected individual, considered a **variant of uncertain significance**	Positive-possible

Case ID	Ultrasound Findings	Postnatal/Autopsy Results	ES result	Variant(s)	Classification
6	Non-immune hydrops	Not Applicable	Compound heterozygous mutations in ***PIEZO1*** gene which has been recently associated with autosomal recessive generalized lymphatic dysplasia and non-immune hydrops fetalis	c. 307C>T p. Arg103Ter Novel nonsense mutation; **likely pathogenic**. c.7129+1G>C Novel splice site variant; **variant of uncertain significance**	Positive-possible
7	Multiple brain anomalies including Dandy Walker malformation, absent cerebellum, encephalocele. Large echogenic kidneys, oligohydramnios, hands not well visualized	Not Applicable	Compound heterozygous mutations in ***TMEM67*** gene which causes Meckel Gruber syndrome type 3 and Joubert syndrome type 6. Both previously described; **known pathogenic**	c.579_580delAG p.Gly195fs Previously described; **known pathogenic** c. 622A>T p.Arg208Ter Previously described; **known pathogenic**	Positive-Definitive
	Complex heart defect (hypoplastic left heart, atrial septal defect, aortic atresia); Right kidney fused to lower pole of left kidney)	Not Applicable	*De novo* nonsense mutation in the ***MAP4K4*** gene which has recently been shown to be important for endothelial cell migration and vascular angiogenesis; This gene has not previously been associated with human disease	c. 3568C>T p. Gln1190* De novo nonsense novel mutation; variant of uncertain clinical significance	Uncertain-Other (novel gene)
	Non-immune hydrops	Not Applicable	No identifiable etiology using ES		Negative
	Shortened long bones	Not Applicable	No identifiable etiology using ES		Negative
	Renal agenesis	Not Applicable	No identifiable etiology using ES		Negative
	Heterotaxy with complex heart defect and omphalocele	Not Applicable	No identifiable etiology using ES		Negative
	Multiple pregnancies with genitourinary abnormalities	Not Applicable	No identifiable etiology using ES		Negative

Table 4

Details of variants identified in positive and possible cases

Case ID	Gene	Diagnosis	Alteration	Inheritance	Classification	Origin	Previously reported	Notes
1	COL1A1	Osteogenesis imperfecta	c. 1875+1G>A splice site variant	AD	Likely pathogenic	De novo	Yes in a father and daughter with OI, type 1[40]	Rare variant at a cnonical RNA splice donor site.
2	KCTD1	Scalp ear nipple syndrome	c. 86 A>G p. Asn29Ser	AD	Likely pathogenic	De novo	No	Rare missense variant highly conserved in 'bric-a-brac, tram track, and broad complex" (BTB) domain. Several nearby misense changes with the same BTBdomain previously been associated with scalp ear nipple syndrome. Anomalies of the kidneys have been reported and the neonatal demise showed several areas of skin sloughing and hypoplastic nails consistent with previously described phenotype.[41]
3	RTTN	Microcephalic primordial dwarfism	c. 4480C>T Thr1398Met	AR	VUS	Inherited	No	Both rare, highly conserved missense variants. Other studies also report missense variants in this gene. RTTN is important in maintaining ciliary structure.[42–44]
			c. 5143 A>G p. Asn1715Asp				No	
4	MUSK	Fetal akinesia deformation sequence (FADS)	c. 1724T>C p. Ile575Thr	AR	Likely pathogenic	Inherited	Yes	Dutch founder mutation in 14 fetuses with FADS; Ile575Thr disrupts multiple functions of the encoded protein.[45–46]
			c. 2408A>G p. Tyr803Cys		VUS		No	Novel missense variant that alters a well-conserved amino acid from a tyrosine to a cysteine within a tyrosine kinase domain of the protein.
5	DYNC2H1	Short-rib polydactyly syndrome	c. 8012 T>C p. Met2671Thr	AR	VUS	Inherited	Yes, in an individual with short rib thoracic dysplasia, type 3 with or without polydactyly	Rare missense variant in the AAA kinase domaine previously reported in an individual with similar phenotype. However, without further clinical or functional information, it is a VUS.
			c. 10594C>T p. Arg3532Ter		Likely pathogenic			Rare nonsense mutation predicted to result in premature protein truncation. Other

Case ID	Gene	Diagnosis	Alteration	Inheritance	Classification	Origin	Previously reported	Notes
								truncating variant in this gene have been shown to cause disease and it is therefore likely pathogenic.
6	PIEZO1	Non-immune hydrops	c. 7129+1G>C splice site variant	AR	VUS	Inherited		
			c. 307C>T p. Arg103Ter		Likely pathogenic			
7	TMEM67	Meckel Gruber syndrome	c. 579_580delAG p.Gly195fs	AR	Known pathogenic	Inherited	Yes, previously reported as homozygous in a fetus with MKS and in two individuals with Joubert syndrome with limited clincial information.[47,48]	Predicted to result in premature truncation of the protein.
			c. 622A>T 0. Arg208Ter		Known pathogenic		Yes, previously reported in several fetuses with MKS.[49,50]	Nonsense mutation; Predicted to result in premature truncation of the protein.

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