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Fetal exome sequencing for isolated increased nuchal translucency: Should we be doing it?

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Introduction

An increased nuchal translucency (NT) greater than 3.5mm detected at first trimester ultrasound screening is associated with fetal chromosomal abnormalities, structural anomalies (such as congenital heart malformations), and a wide range of genetic disorders.[1,2] Investigation of fetuses with increased NT typically comprises rapid aneuploidy testing and chromosomal microarray (CMA) on a fetal DNA sample obtained through chorionic villus sampling (CVS) or amniocentesis. A chromosomal abnormality will be identified in approximately 30% of cases[3,4] but euploid fetuses with increased NT remain at increased risk of adverse outcomes, proportionally related to the degree of NT enlargement.[2–4]

In chromosomally normal fetuses with structural anomalies, prenatal exome sequencing (ES) has been shown to increase the diagnosis of monogenic conditions, with diagnostic rates varying widely across different phenotypes.[5–8] Two large, prospective studies of unselected fetuses with any structural abnormality showed that ES provided additional diagnosis in 8.5% and 10.3% of cases respectively.[5,6] However, in fetuses with multisystem or skeletal abnormalities the diagnostic rate was over 15% while those with isolated increased NT (≥3.5mm) saw the lowest diagnostic rate of 3.2% and 2.9% respectively.[5,6] Similar low diagnostic rates have also been reported recently for isolated increased NT,[9,10] questioning the clinical utility or cost effectiveness of prenatal ES in this situation.

With increasing availability of sequencing technology, decreasing costs and improved speed of bioinformatic analytical pipelines, rapid fetal ES for prenatal diagnosis is moving beyond the research arena and has recently been implemented in the UK National Health Service (NHS) and in many prenatal diagnosis centres across the US and Europe. Thus a clear evidence-base is required to enable most efficient use of this new technology. Here we review the final, extended datasets of the UK Prenatal Assessment of Genomes and Exomes (PAGE) and USA Columbia (CUIMC) studies to identify all cases presenting with increased NT, aiming to further delineate which pregnancies benefit most from prenatal ES and aid in prospective prognosis allocation. We review natural histories, outcomes and diagnostic variants and explore factors influencing diagnostic yields to inform development of guidelines for the use of prenatal ES in the presence of increased NT in clinical practice.

Methods

The study cohort comprised fetuses presenting with increased NT (≥3.5mm) recruited to the **Prenatal Assessment of Genomes and Exomes (PAGE)**[5] and the **Columbia (CUIMC) fetal WES**[6] **studies.**

PAGE study

Here we review 876 fetuses and 1727 matched parental samples (851 fetus-parent trios and 25 fetus-parent duos), of which 610 cases (596 trios and 14 duos) have been reported.[5] Study methodology and eligibility criteria were as previously published[5] but in brief, couples undergoing invasive testing for any ultrasound identified fetal abnormality, including isolated increased NT, were consented for trio ES where fetal karyotype/CMA were normal. Whole exome sequencing (WES) was performed with analysis targeted to a virtual panel of 1628 genes associated with developmental disorders.

CUIMC Study

CUIMC recruited a total of 494 fetuses with matched parental samples, of which 234 trios have been reported.[6] The study consented parents with pregnancies complicated by any fetal abnormality, including isolated increased NT, for invasive testing or collection of a cord sample after birth. Untargeted trio WES was performed when karyotype/CMA was non-causative of the anomaly. The bioinformatic analysis is described elsewhere.[6]

Variant interpretation

In both studies, candidate pathogenic variants were curated and discussed in consensus with relevant clinicians and scientists at a multidisciplinary clinical review panel (CRP). Variants classified as pathogenic (Class 5) or likely pathogenic (Class 4) according to ACMG guidelines[11] *and* judged likely to cause the observed structurally abnormal phenotype in the fetus were considered as positive diagnostic results, validated using Sanger sequencing and reported to parents after delivery in the PAGE study or at the time of diagnosis in the CUIMC study.[5,6]

Procedures

Interrogation of the study databases identified all fetal cases presenting at 11-14 weeks' gestation with any of the following terms recorded: "Increased nuchal translucency" (HP:0010880); "Fetal cystic hygroma" (HP:0010878); "Cystic hygroma" (HP:0000476); "Thickened nuchal skin fold" (HP:0000474),

whether in isolation or in combination with other phenotypes. No distinction was made between increased NT and cystic hygroma on the basis that practitioners documenting the fetal phenotype at the time of recruitment may have used the terms interchangeably and clinical management is the same in either case.

To ascertain cases with isolated increased NT at presentation, clinical information was manually reviewed, including the phenotypes (HPO terms and free text) recorded in the study databases and ultrasound scan reports at presentation. Following manual review of this information, any fetus without other structural anomalies at the point of presentation was classified as 'initially isolated increased NT'. Of note, both cohorts included some cases previously classified and published in other phenotypic groups as those classifications were originally based upon the predominant phenotype in the pregnancy as a whole, whereas here the classifications are based specifically upon the phenotype at initial presentation at 11-14 weeks' gestation.

For all cases with initially isolated increased NT at presentation, further ultrasound scan reports and clinical information from later in pregnancy were reviewed to ascertain whether the increased NT resolved, remained isolated, or if additional structural abnormalities were detected at a later gestation. Cases presenting with features consistent with established or evolving fetal hydrops (generalised oedema, pleural or pericardial effusions, ascites) were classed as non-isolated increased NT, since fetal hydrops is a distinct clinical entity with different prognostic implications from isolated increased NT. Pregnancy outcomes, and postnatal clinical information or post-mortem findings were ascertained from participating fetal medicine units.

Outcomes

The primary outcome assessed in both this and the previously published studies[5,6] was the detection of diagnostic genetic variants considered to have caused the observed fetal structural anomaly. We reviewed the exome sequence variants found by the PAGE and CUIMC studies in this increased NT cohort and calculated diagnostic rates for fetuses with: (1) non-isolated increased NT at presentation; (2) initially isolated increased NT with additional abnormalities detected later in pregnancy and; (3) isolated increased NT which remained isolated or resolved. We also calculated diagnostic rates according to the measured thickness of NT at presentation.

Statistical analysis

The two-tailed Fisher's exact test was used to compare rates of diagnostic genetic variants between sub-groups and Bonferroni correction for multiple testing was applied.

Funding

The PAGE study was funded by the Health Innovation Challenge Fund (HICF) from the UK Department of Health and Wellcome Trust (no. HICF-R7-396). The CUIMC study was supported by the Columbia Institute for Genomic Medicine and OB/GYN departments. External funders of these studies had no role in study design, data collection, data analysis, data interpretation, or writing of the report.

Ethical approval

All clinical information was accessed in pseudo-anonymised format, with participants' written informed consent, and the ethical approval of relevant Research Ethics Committees (South Birmingham – REC

reference number 14/WM/0150, Harrow – REC reference number 01/0095, and New York, NY – Columbia University College of Physicians and Surgeons protocol #AAAO8009).

Patient involvement

Design and conduct of the PAGE study was informed by input from patients and the public through collaboration with the charity Antenatal Results and Choices. The CUIMC study was designed and implemented by faculty of the Department of OBGYN and the Institute for Genomic Medicine. There was no additional patient involvement for the analysis presented here.

Results

In total, 213 fetuses with increased NT at 11-14 weeks of gestation were identified; 159 were classified as initially isolated, while 54 had additional structural abnormalities or fetal hydrops at presentation (in the first trimester). Following review and classification of candidate variants by the studies' multidisclipinary CRPs, 28 (13.1%) of 213 cases had a diagnostic variant identified (Tables 1-4).

An additional eight variants (Table 5) were designated as 'potentially clinically relevant' by the PAGE study CRP, because either there was insufficient evidence to classify the variant as (likely) pathogenic and/or the prenatal phenotype was not specific enough to be unequivocally attributed to the variant. Six of these were in fetuses with additional abnormalities and two in fetuses with isolated increased NT (Table 5). Variants previously published in the PAGE and CUIMC studies[5,6] are indicated in Tables 1-5.

Fetuses with increased NT and other anomalies

Diagnostic variants were detected in 12 (22.2%) of 54 fetuses presenting with non-isolated increased NT (Table 1). Of the 155 pregnancies presenting with initially isolated increased NT and with follow up to term (Fig. 1), additional abnormalities were detected in 37 cases (23.9%) later in pregnancy with diagnostic variants detected in 12 (32.4%). Noonan syndrome accounted for 4/12 (33.3%) of the diagnoses made (Table 2). A further six fetuses had variants designated 'potentially clinically relevant', of which 2/6 (33.3%) were also in RASopathy genes (Table 5).

Fetuses with isolated increased NT

In the 111 cases where no other fetal anomalies developed, and the increased NT either resolved or was not commented on later in pregnancy, a diagnostic variant was detected in two (1.8%) (Table 3). One was a diagnosis of chromosome 15 uniparental disomy (UPD), in a fetus presenting with an NT of 4.8mm at 13 weeks' gestation who was born at term with no apparent congenital abnormalities observed on clinical examination. The second was a fetus presenting with isolated NT of 3.5mm and found to have a *de novo* frameshift variant in the gene *RERE*. This fetus also had no apparent congenital abnormalities at birth, but at 8 months of age had clinical features consistent with *RERE*-related disease, at which point the prenatally detected variant was reclassified as pathogenic by the study MDT. Two further cases had 'potentially clinically relevant' variants. One, with a variant in *KMT2A,* had a sacral dimple at birth but no other problems were noted on follow-up to two years of age to allow a diagnosis of Wiedemann-Steiner syndrome to be made. The second had a *KMT2D* variant and whilst there were no problems detected on clinical examination at birth, examination at 18 months revealed fetal finger pads, arched eyebrows and a sacral dimple, which allowed confirmation of a diagnosis of Kabuki syndrome.

Fetuses with no follow-up

In seven cases the pregnancies ended soon after the initial presentation with no further scans performed. Diagnostic variants were detected in two (28.6%) of these cases (Table 4). Post-mortem examination confirmed findings compatible with Cornelia de Lange syndrome in the fetus with a *de novo* pathogenic *NIPBL* truncating variant. In the other, with a *de novo* likely pathogenic *PTPN11* variant, post-mortem confirmed the presence of a cystic hygroma. Four further cases, with no diagnostic variants identified, were lost to follow up and scan reports from later in the pregnancies were not available for review. These 11 cases are excluded from further analysis of diagnostic rates.

Sub-analysis according to the presence of additional structural abnormalities compared to pregnancies with 'truly' isolated increased NT, showed a significant increase in the diagnostic rate both where additional abnormalities were seen at presentation (1.8% vs 22.2% $P < 0.001$), and where additional abnormalities developed later (1.8% vs 32.4% $P < 0.001$). There was no statistically significant difference in the frequency of diagnostic variants between fetuses with additional abnormalities at presentation and those developing additional abnormalities at a later gestation (22.2% vs 32.4% $P = 0.336$).

Review of sequencing results in relation to the size of isolated increased NT at presentation (Table 4) showed that diagnostic rate increased with increasing size of NT, from 1.6% (1/63 cases) where NT was between 3.5-4.4mm, to 28.6% (4/14 cases) where NT was >7.5mm ($P <0.05$).

Discussion

Main Findings

In this cohort of pregnancies enrolled in the first trimester with an increased NT ≥3.5mm, we observed a low rate of diagnostic variants (1.8%) from prenatal ES for isolated increased NTs that remained isolated throughout the pregnancy. However, there was an increased diagnostic rate where fetuses had additional structural anomalies or hydrops, either at presentation (22.2%) or developing later in pregnancy (32.4%) We also observed significantly higher diagnostic rates where the size of the isolated increased NT was larger at presentation.

It is of note that in the studies we describe there were some likely pathogenic/pathogenic variants that did not explain the fetal phenotype. In the PAGE and CUIMC studies, the protocols dictated that pathogenic results were only reported to parents if they explained the phenotype in the fetus. In two cases - a fetus with an *RERE* variant and one with a *KMT2D* variant - it was only on longer term follow up that the recognised postnatal phenotype became clear and the pathogenicity was confirmed and reported to parents. This highlights how we are expanding our understanding of fetal phenotype-genotype relationships previously only recognised postnatally and that this growing knowledge is essential for accurate prenatal interpretation and complete reproductive genetic counselling in future cases.

It is also notable that this cohort includes three diagnoses of Noonan syndrome where causative variants were inherited from undiagnosed affected parents (PP2567, PP0503, fetal0222). In two cases there was a history of previous pregnancy loss with relevant phenotypes (large cystic hygroma and fetal hydrops, respectively), and in two cases the affected parent had unrecognised clinical features of Noonan

syndrome. These cases highlight the need for careful review of family history and previous obstetric history, as well as careful, expert parental examination when considering the underlying aetiology of increased NT in order to guide molecular testing, particularly where genes exhibit variable penetrance or expressivity.

Strengths and Limitations

A strength of this study is its relatively large sample size, drawn from the two largest published prenatal ES cohorts to date. Further, the prospectively collected, unselected nature of the cohort, and the detailed approach to examining the natural histories of the pregnancies presenting with isolated increased NT make this study relevant to clinical practice where rapid ES may be considered in an ongoing pregnancy.

However, varied interpretations of 'isolated' increased NT (e.g. isolated at presentation vs. isolated throughout the entire pregnancy, and whether or not 'soft markers' of genetic abnormality are classed as additional abnormalities) limit comparison of results between studies. A further limitation of prenatal ES for the investigation of isolated increased NT is the difficulty in interpreting genetic variants in the absence of specific fetal phenotypes, exacerbated by a dearth of publically available data regarding the complete spectrum of Mendelian disease in the fetal period.

Interpretation (in light of other evidence)

Other recent small studies of prenatal trio ES have also observed relatively low diagnostic rates of 0 – 3% for isolated increased NT,[9,10] particularly when specifically reporting cases without structural abnormalities developing later in pregnancy.[12] These low numbers of molecular diagnoses from prenatal ES are consistent with an existing body of evidence indicating that, once chromosomal abnormalities are excluded, if detailed follow-up scanning demonstrates resolution of the increased NT and the absence of any major abnormalities, then the chance of delivering a healthy infant with no major abnormalities is >95%.[1–3] Our observation that diagnoses from prenatal ES increased with enlarging size of NT at presentation is also in keeping with the known association between significant underlying pathogenicity and increasing NT thickness.[1]

In contrast to our findings, a recent smaller retrospectively collected cohort study reported by Choy and colleagues using prenatal whole genome sequencing (WGS) reports a diagnostic yield of 17.2% (5/29 cases) amongst fetuses with isolated increased NT and normal CMA, and found no significant difference between isolated and non-isolated increased NT groups.[13] The pathogenic variants reported comprised one case of mosaic Turner syndrome (45,X) not detected on CMA, and 4 variants in the genes *ARMC4*, *ANKRD11*, *GATA4* and *NSD1,* all of which would have been amenable to detection by WES in the PAGE and CUIMC studies. Differences in the approach to reporting variants may contribute to the difference in diagnostic rates between these studies. In the study by Choy *et al*. findings were not reported back to families, whereas diagnostic findings from the PAGE and CUIMC studies were confirmed in a clinical laboratory and reported to families after the end of the pregnancy. These studies' CRPs took a stringent approach to reporting only variants classified (likely) pathogenic *and* considered causative of the fetal phenotype. With a non-specific fetal phenotype such as isolated increased NT, it may be challenging to make a definitive genotype-phenotype correlation as well as there being some subjectivity in reporting decisions. This is especially true for novel variants as reported by Choy *et al.* This highlights an important point about the need for clear (international) consensus guidelines for reporting variants detected by prenatal ES or WGS in clinical practice, where results will be largely returned during an ongoing pregnancy and will have implications for counselling and management in that pregnancy. Other

retrospective studies have used a 'targeted' approach using chromosomal microarray testing and RD pathogenic variants testing (of nine known genes) and noted a high pathologic detetction rate in fetuses with first trimester increased NT (Sinajon P, Chitayat D, Roifman M, Wasim S, Carmona S, Ryan G, Noor A, Kolomietz E, Chong K. Microarray and RASopathy-disorder testing in fetuses with increased nuchal translucency. Ultrasound Obstet Gynecol. 2020 Mar;55(3):383-390).

Conclusion

These findings have clinical implications for offering prenatal ES in obstetric practice, where testing should aim to maximise benefit to patients without unduly increasing parental anxiety, and are particularly pertinent in view of the recent introduction of rapid fetal exome sequencing in the UK National Health Service[14]. Guidelines must take into account both clinical utility and cost-effectiveness in order to direct finite resources appropriately. Since diagnostic yield for completely isolated increased NT is low, a suggested strategy is to offer prenatal ES for increased NT only when additional fetal structural abnormalities are present. This not only increases the *a priori* likelihood of a monogenic disorder, but also facilitates variant interpretation and reporting. Therefore, timely and careful follow-up fetal ultrasound scanning to identify emerging anomalies will allow testing to focus on the pregnancies with the highest likelihood of a monogenic disorder. Such an approach would integrate well into existing care pathways as many providers already have established protocols for following up isolated increased NT detected at first trimester scanning with detailed anomaly scanning and/or fetal echocardiography at a later gestation. Since many of these pregnancies with an increased NT in the first trimester will have undergone CVS for detection of aneuploidy and CNVs, DNA can be saved at the time of the initial diagnostic testing which can subsequently be used for ES if second trimester ultrasound reveals an emerging phenotype.

In our combined cohort (from two countries), such a strategy would have avoided 116 negative ESs but missed three diagnoses (Noonan syndrome in a fetus with isolated increased NT of 9.9mm at 12 weeks' gestation where the pregnancy was terminated soon after on the basis of the ultrasound findings, chromosome 15 UPD in a fetus with isolated increased NT of 4.8mm at 13 weeks' gestation and normal scans thereafter, and RERE-related developmental disorder in a fetus with isolated increased NT of 3.5mm and normal scans thereafter). As reported by others,[1] the risk of underlying pathology increases with increasing NT size. In our cohort 4/14 (28.6%) of cases with an isolated NT ≥7.5mm in the first trimester had a diagnostic pathogenic variant. The numbers are small and further study is required, but a policy of offering ES for isolated NT of this size may be worth considering.

Where panel testing for RASopathies is available prenatally, this could provide an alternative option for investigating very large isolated increased NT.[15] The case of Kabuki syndrome described above (PP0722), together with other published evidence,[6,7,13] demonstrates that Kabuki syndrome can present prenatally with increased NT and so limited analysis for this condition as well as Noonan spectrum disease may be worth consideration in the future where significant and persistent isolated increased NT is identified. A potential alternate strategy here to limit costs may be to sequence the fetus alone and investigate parents only where a relevant variant is found in the fetus. Should a limited panel approach be offered, clinicians must provide clear counselling regarding the benefits and limitations of analysing a small gene set.

Our findings further highlight the significant challenges of variant interpretation in the prenatal setting when the fetal phenotype is incomplete or non-specific. In the PAGE study results were analysed and returned after the end of the pregnancy but in clinical practice, where ES results will be returned rapidly in an ongoing pregnancy we need guidelines on reporting when the prenatal phenotype is incomplete and the phenotype-genotype correlation is uncertain. As experience with prenatal ES increases and the variations in prenatal phenotypes are further recognised, interpretation and reporting will become clearer.

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