DETECTION OF PATHOGENIC VARIANTS RELATED TO SEVERE DOMINANT MONOGENIC DISEASES BY NON-INVASIVE PRENATAL TESTING (NIPT-SGD)

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Non-invasive prenatal testing (NIPT) as a screening method for common chromosomal abnormalities such as trisomy 21, 18, and 13 has been widely adopted. In the last five years, the possibility of NIPT to detect common single-gene disorders (SGD) due to de novo mutations or paternal inherited had been reported worldwide. Our report describes the first cases in Vietnam that identified pathogenic/likely pathogenic variants by NIPT-SGD in fetuses. These findings were compared with the diagnostic testing (whole exome sequencing/WES) on amniotic fluid/placenta tissue/umbilical cord blood samples. Single-gene NIPT detected pathogenic variants in the fetuses on TSC2, FGFR3, FGFR2 (two cases), and PTPN11 genes. All results coincided with the subsequent diagnosis. Preliminary research showed the potential of cell-free fetal DNA analysis for prenatal screening of dominant single-gene mutations.

Keywords: Single-gene disorders, NIPT-SGD, TSC2, FGFR3, FGFR2, PTPN11.

I. INTRODUCTION

The recognition of cell-free fetal DNA (cffDNA) in maternal peripheral blood has altered the traditional model of prenatal screening tests, enabling safer, earlier, more effective detection of genetic disorders in the fetus. Global adoption of noninvasive prenatal testing (NIPT) for fetal aneuploidies has accelerated. The commercial sector has expanded the scope of cell-free DNA (cfDNA) screening to include sex chromosome aneuploidies, rare autosomal trisomies, copy-number variants (CNVs), indels, and single nucleotide variants based on the genome-wide nature of next-generation sequencing (NGS).[1]

Approximately sixty percent of monogenic diseases that develop severe conditions after birth are dominant, mostly resulting from newly arising mutations (*de novo*). Despite the rare prevalence of each dominant monogenic disease, the accumulative incidence of these disorders is significant. However, screening for these conditions has not been widely applied, mainly due to the necessity of more supporting evidence, clinical validity, and cost-effectiveness assessment. Zhang J. et al. (2019) demonstrated that NIPT could provide valuable genetic information, allowing for

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Received: 19/04/2023
Accepted: 28/04/2023

the identification of a wide range of dominant conditions with a high *de novo* rate or paternally inherited dominant gene variants.[2] In 2022, Mohan et al. evaluated the clinical experience of NIPT in screening for 25 single-gene diseases (NIPT-SGD) with a combined population incidence of 1 in 600, providing a screen-positive rate of 5.7%. NIPT-SGD can aid in the early detection of various SGDs, particularly when abnormal ultrasound results or positive family history. Confirmatory prenatal or postnatal diagnostic testing and follow-up were recommended for all screen-positive patients.[3] In Vietnam, a study was conducted on the launch of the NIPT-SGD protocol using the NGS technique with ultra-deep sequencing > 10,000x, targeting 30 genes, to search for pathogenic or likely pathogenic variants implicated in 25 dominant conditions (listed in Methods) in 2022.[4] We present in this study the application of NIPT-SGD for the discovery of pathogenic variants in fetuses (with abnormal ultrasound findings) associated with four severe dominant monogenic diseases, including Tuberous sclerosis 2, Thanatophoric dysplasia, Apert syndrome, Crouzon syndrome, and Noonan syndrome all leading to poor prognosis of the fetus or serious effect to postnatal life quality.

II. SUBJECTS AND METHODS

1. Subjects

Five singleton pregnant women (and their husbands) were counseled at Hanoi Medical University Hospital and Hanoi Obstetrics and Gynecology Hospital between January 1, 2022, and December 31, 2022, due to abnormal fetal ultrasound findings from the second trimester (Pregnant women without bone marrow/organ transplantation or known cancer or blood transfusion within the two prior weeks). They accepted to participate in the study and obtained both results: a positive NIPT-SGD (with a pathogenic variant detected in 30 investigated genes) and fetal diagnostic gene testing.

2. Methods

Research design

Descriptive study of case series.

Clinical information, including parents' age, family history, gestational age (when they entered the study and when abnormalities were first detected via ultrasound), fetal ultrasound findings, and outcomes were collected.

NIPT-SGD was implemented in expectant women's peripheral blood specimens. Cell-free fetal DNA was extracted from maternal plasma samples and analyzed by next-generation sequencing targeting 30 genes at a depth of >10,000X. List of 30 genes with almost complete penetrance investigated in NIPT-SGD including *BRAF, MAP2K1, MAP2K2, HRAS, PTPN11, SOS1, RAF1, NRAS, RIT1, SOS2, KRAS, SHOC2, CBL, COL1A1, COL1A2, FGFR3, FGFR2, JAG1, CHD7, NIPBL, SMC1A, SMC3, RAD21, HDAC8, CDKL5, SYNGAP1, MECP2, NSD1, TSC2, TSC1.* These genes have been linked to a variety of conditions, including Noonan syndrome 1,3,4,5,6,7,8,9, Noonan-like syndrome with hair follicle loss, Noonan-like syndrome with/without acute myeloid leukemia, heart-face-skin syndrome 1,3; Costello syndrome, Ehlers-Danlos syndrome type VIIA/B, osteogenesis imperfecta type I, II, III, IV, achondroplasia, CATSHL syndrome, Crouzon syndrome, cartilage hypoplasia, Muenke syndrome, chondrodysplasia lethal type I, II, Antley-Bixler syndrome, Apert syndrome, Jackson Weiss syndrome, Pfeiffer syndrome types 1, 2, 3, Alagille syndrome, CHARGE syndrome, Cornelia de Lange syndrome 1,2, 3,4,5, *CDKL5*-related epileptic encephalopathy, *SYNGAP1*-related intellectual

disability, Rett syndrome, Sotos syndrome 1, tuberous sclerosis type 1, type 2. NIPT-SGD concurrently identified chromosomal numerical aberrations.

NIPT-SGD was positive when a pathogenic gene variant with a variant allele fraction (VAF) exceeding 2% and minimal coverage of 200X was identified. Sanger sequencing/ amplicon sequencing was performed from the genomic DNA extracted from the peripheral blood of the parents to ascertain if the fetus carried a *de novo* mutation or a paternally inherited variant.

Amniotic fluid/umbilical cord blood/ placenta tissue (product of conception, POC) were collected for Whole exome sequencing (detection of clinically related pathogenic/likely pathogenic variants) and CNV-seq (detection of chromosomal numerical and structural alterations (deletion/duplication size is greater than 400,000 base pairs)). The NIPT-SGD results were compared to the fetal genetic diagnosis.

III. RESULTS

The mean ages of expectant women and their husbands were 31.6 and 39.2 years, respectively, as shown in **Table 1**. The average gestational week for the study's subjects was 24 (as early as 17 weeks and as late as 32 weeks). Most ultrasound features of fetuses suggest genetic diseases/syndromes; however, they are not apparent until the late second and third trimesters.

Table 1. Age characteristics and fetal ultrasound

Case	Code	Age		GA (w)	Abnormal Ultrasound Findings
		Mat	Pat		
1	NSG031*	28	28	32	32 weeks: Small for gestational age, right ventricle dilation, myocardium tumors.
2	NSG039	42	50	17	17 weeks: short limbs and rib hypoplasia. (12w: Short limbs)
3	NSG042	24	33	26	26 weeks: Craniosynostosis, ventricular enlargement, corpus callosum agenesis, and thickening of the nuchal fold. Fetal MRI and ultrasound: Crouzon syndrome was likely. *(23 weeks gestation: left ventricle dilation, nuchal fold thickening, and abnormal cranial ossification. Amniocentesis with normal Microarray result)*
4	NSG044	38	47	24	22w, the skin thickness at the corner of the nose is 3.3mm, nuchal fold thickness of 8.9mm. Thicken placenta. *(IVF pregnancy, 12 weeks of increased NT, 17 weeks of nuchal fold thickening, amniocentesis with normal Microarray result).*

Case	Code	Age		GA (w)	Abnormal Ultrasound Findings
		Mat	Pat		
5	NSG054	26	38	20	20w: Lemon-shaped skull, the skull joints were obscured. Wide-set eyes (hypertelorism: 20mm). Syndactyly of the fingers and toes. Apert syndrome was likely. *(17w: syndactyly (finger); amniocentesis with normal CNV-seq)*
	Average	31.6	39.2	24	

GA (w): gestational age (weeks). *Positive family history: the first child had epilepsy.

Table 2. NIPT-SGD results, Sanger sequencing for gDNA of parental blood, and fetal genetic diagnostic results#

Code	Gene	NIPT-SGD				Sanger	WES	
		Genotype (Het)	Class (ClinVar)	Phenotype	FF/ VAF (%)	Origin	Sample	Genotype
NSG031	*TSC2*	NM_000548.5: c.4375C>T	P	Tuberous sclerosis 2	23 14,2	Paternal	Fresh amniotic fluid (AF)	NM_000548.5: c.4375C>T
NSG039	*FGFR3*	NM_000142.4: c.1111A>T	P	Thanato-phoric dysplasia	6 5	De novo	Fresh AF	NM_000142.4: c.1111A>T
NSG042	*FGFR2*	NM_000141.4: c.1124A>G	P/LP	Crouzon syndrome	11 12,8	De novo	Umbilical cord blood (TOP)	NM_000141.4: c.1124A>G
NSG044	*PTPN11*	NM_002834.4: c.598A>T	P/LP	Noonan syndrome	6,2 6	De novo	AF & placenta tissue (TOP)	NM_002834.4: c.598A>T
NSG054	*FGFR2*	NM_000141.5: c.755C>G	P	Apert syndrome	15,1 5,2	De novo	Fresh AF	NM_000141.5: c.755C>G

#: Neither the NIPT test nor the CNV-seq test detected aneuploidies in any of the cases in this study.

No fetus was identified with abnormal deletion/duplication by CNV-seq.

* ClinVar: is the National Institutes of Health's disease statistics database for clinical variant data.

P/LP = Pathogenic/Likely Pathogenic: FF: fetal fractions: fetal DNA in total cell-free DNA in maternal blood; VAF: Variant allele fraction: frequency of DNA molecules carrying the variant; TOP: Termination of pregnancy.

The rate of cffDNA in NIPT-SGD test is >4%. The percentage of DNA molecules carrying the variant is >2%. The results of NIPT-SGD detected P/LP variants on the genes *TSC2, FGFR2* (two cases*), FGFR3, and PTPN11* and were similar to the diagnostic test results on amniotic fluid/placenta tissue. /umbilical cord blood. Four out of five cases involved de novo variants (Sanger/amplicon sequencing did not detect the variant in the parents, except NSG031, which was inherited from the father).

IV. DISCUSSION

The mean age of the five pregnant women and spouses in the study was 31.6 and 39.2, respectively. In four cases (cases 2, 3, 4, and 5), the pathogenic variants were confirmed to be *de novo* mutations, the average age of the mother was 32.50 years old, and the average age of the father was 42 years old. In spermatogenesis, the probability of de novo mutations increased for males older than 40.[5] One case was found to have a positive family history. Factually, up to 80% of genetic diseases and congenital abnormalities have no aberrant family history. 4/5 (excluding case 4) cases with relatively typical ultrasound findings of disease/syndrome; however, these suggestive features usually become apparent at the end of the second or third trimester of pregnancy, which makes prenatal counseling for families challenging. The pathogenic variants (according to the ClinVar database) detected by NIPT-SGD matched the results of coding-DNA region sequencing on 22,000 genes (WES) in POC samples. Additionally, these gene variants were associated with fetal ultrasound phenotype.

Case 1: (NSG031) recorded with a first child diagnosed with epilepsy (other clinical/ subclinical data could not be employed). When Sanger sequencing for c.4375C>T in *TSC2* (detected by NIPT-SGD) was performed on the parents' peripheral blood sample, it was determined that the father also possessed this variant. The clinical examination of the father revealed numerous painless subcutaneous nodules on his face and a few hypopigmented macules on the body. Other organs have detected no abnormalities. . At 32 weeks of gestation, an ultrasound detected abnormalities in the fetus. The family continued ultrasonographic monitoring of the fetus and was referred for postnatal examination and monitoring for anomalies in the nervous and cardiovascular systems. Currently, the infant is five months old, weighs 5.5 kg, and has not yet displayed any clinical manifestations of abnormality (without examination by clinicians). NIPT-SGD can detect the dominant monogenic variant inherited from the father.[2] Tuberous sclerosis (TSC) is inherited in an autosomal dominant pattern. Two-thirds of affected individuals have TSC as the result of a de novo pathogenic variant. The offspring of an affected individual are at a 50% risk of inheriting the pathogenic variant. Prenatal testing and preimplantation genetic testing are possible. Tuberous sclerosis type 2 (prevalence 1-2:10,000) caused by a pathogenic variant in *TSC2*, often has a more severe phenotype than TSC1, which can affect multiple organs such as the brain, heart, skin, kidneys, and lungs... causing seizures, mental retardation, and behavioral disorders. The penetrance of TSC is now thought to be 100%. TSC exhibits both inter- and intrafamilial variability in clinical features. Therefore, this child should received long-term follow-up,

Case 2: (NSG039) PARA 3003, natural fourth

pregnancy. The 12-week ultrasound detected short limbs - surveillance for achondroplasia - and the family was informed of the fetus's poor prognosis. They decided to continue monitoring the pregnancy and made a prenatal diagnosis at 17 weeks. NIPT-SGD and WES both identified the *FGFR3* mutation c.1111A>T. On ClinVar, eight submissions of this pathogenic variant were associated with Thanatophoric dysplasia type 1 (incidence 1-2:10,000), frequently resulting in perinatal mortality or stillbirth. This condition Type 1 is represented by short limbs, curved femurs, short ribs, a narrow chest, and a large cranium. After obtaining the amniocentesis results and consulting on the fetus's prognosis, the family decided to terminate the pregnancy at 23 weeks.

Cases 3 and 5: (NSG042 and NSG054) Pathogenic variants in *FGFR2* were detected using NIPT-SGD on maternal plasma and WES on umbilical cord blood samples or amniotic fluid. These two cases previously had normal microarray/CNV-seq results. In cases with craniosynostosis signs (with or without syndactyly), gene sequencing for early prenatal diagnosis should be implemented, along with the combination of ultrasound and fetal MRI to monitor lately observed symptoms. The *FGFR2* pathogenic variants c.1124A>G and c.755C>G were found to be associated with Crouzon syndrome and Apert syndrome, accordingly. The specific identification of disease-causing mutations in the gene also provides additional insight into the fetus' prognosis. In these two instances, the family chose to terminate the pregnancy. NIPT-SGD research detecting pathogenic mutations in *FGFR3* and *FGFR2* also demonstrated high accuracy, particularly when using next-generation sequencing (NGS) methods.[6,7]

Case 4: (NSG044) IVF pregnancy (PARA: 4014) exhibited an increase in nuchal translucency beginning at 12 weeks. A webbed neck was observed at 17 weeks, and the amniocentesis at a hospital of Obstetrics and Gynecology yielded a normal microarray result. The family continued keeping track of the pregnancy until 22 weeks when a fetal ultrasound identified an enlarged neck skin fold measuring 8.9mm. A physician informed the family of the possible presence of single-gene mutations causing Noonan syndrome with some common symptoms in pregnancy, namely lymphatic dysplasia, nuchal translucency, cystic hygroma, pleural effusion, and ascites. After birth, children may have different degrees of growth retardation, excess skin folds in the neck, lymphatic dysplasia, chest wall abnormalities, short stature, etc.). The expectant mother registered for additional amniocentesis at the other Obstetrics and Gynecology Hospital and received WES on the amniotic fluid (gene testing was carried out on the placenta sample after the family elected to terminate the pregnancy). The pathogenic variant in *PTPN11* detected by NIPT-SGD resembled the diagnostic results obtained from two types of POC. This mutation was confirmed *de novo* via Sanger sequencing on the gDNA of the parents. Benn et al.'s 2020 study also employed NIPT-SGD to detect pathogenic mutations in a gene panel associated with Noonan spectrum disorders/Noonan syndrome. 14/25 cases are caused by *PTPN11* mutations, and 13/25 cases had confirmed diagnoses. According to this study, the positive screening rate for Noonan syndrome was 8% (18/225) in fetuses with increased nuchal translucency, cervical lymphatic cysts, or fetal edema. Early detection of high-risk pregnancies with Noonan syndrome can provide valuable fetal prognostic information and enhance pregnancy management and postpartum care.[8]

Initial study results suggested the potential benefits of cell-free fetal DNA analysis for prenatal screening of dominant single-gene mutations. However, a larger sample size would provide a more comprehensive understanding of the clinical utility of NIPT-SGD in identifying pathogenic variants associated with dominant monogenic disorders.

V. CONCLUSIONS

The presence of *de novo* pathogenic variants or paternally inherited dominant gene variants in *TSC2, FGFR2* (2 cases), *FGFR3*, and *PTPN11* that cause four common and severe dominant single-gene disorders, including Tuberous sclerosis 2, Thanatophoric dysplasia, Apert syndrome, Crouzon syndrome, and Noonan syndrome can be determined by NIPT-SGD. NIPT-SGD results are precisely compatible with those of fetal diagnostic tests. These pathogenic variants' associated phenotypes correspond to abnormal ultrasound findings of the fetus. Early detection of pathogenic variants on genes associated with severe dominant monogenic diseases is beneficial to monitor and manage pregnancy.

Ethics approval

The study was approved by the Institutional Review Board for Ethics in Biomedical Research - Hanoi Medical University. The study conformed with the Declaration of Helsinki regarding the use of human samples and identifiable information. Informed consent was obtained from the subjects regarding the use of the samples for research purposes.

Competing interests

The authors declare no conflict of interest.

Author Contributions

Dao Thi Trang, Luong Thi Lan Anh, and Nguyen Duy Anh conceived and designed the study and analysis. Nguyen Huu Duc Anh, Nguyen Thi Hao, and Nguyen Thi Sim contributed to the subject's recruitment. Tran Vu Uyen, Nguyen Hoai Nghia, Tang Hung Sang, Giang Hoa, and Dao Thi Trang participate in data collection and carry out the experiments, performed analysis, and finalized the results. Dao Thi Trang and Luong Thi Lan Anh contributed to the drafting of the manuscript. All authors have read and approved the final version for publication.

ACKNOWLEDGMENTS

We sincerely thank the patients for giving us consent and allowing us to publish the data. We thank our colleagues at Hanoi Medical University Hospital, Hanoi Obstetric and Gynecology Hospital who helped us in patient recruitment and clinical data collection. This work is funded by Gene Solutions JSC, Vietnam.

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