

## Research Article

# Contribution of Pathogenic CNVs and Noonan Syndrome in Fetuses with Increased Nuchal Translucency and Persistently Increased Nuchal Fold

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## Introduction

In prenatal screening it is not uncommon to observe an altered venous-lymphatic differentiation of the endothelial cells in the jugular lymphatic sacs to occur in the first trimester, causing nuchal edema beginning in the second trimester [1,2]. Fetal increased Nuchal Translucency (NT) is recognized as a sensitive marker for chromosomal disorders, and has been applied in routine first trimester Down syndrome screening programs in many countries [3,4], first used to screen for DS by Professor Nicolaides [5]. In the second trimester, about one-third of DS fetuses have a Nuchal Fold (NF) thickness >6mm [6]. NF is the most frequently investigated marker for fetal chromosomal abnormalities [7,8]. Increased NT and increased NF are also associated with many genetic disorders, structural abnormalities and syndromes [9,10].

Chromosomal Microarray Analysis (CMA) has significant advantages over karyotyping in both the prenatal and postnatal genetic diagnosis. CMA is rapidly becoming the preferred diagnostic test for fetuses with structural malformations [11-13]. Pathogenic chromosomal Copy Number Variants (CNVs) can be diagnosed by CMA accurately in fetuses with NT >99<sup>th</sup> percentile ( $\geq 3.5$ mm) [14,15]. A meta-analysis by M. Grande et al included 17 studies showed the incremental yield of CMA in detecting pathogenic CNVs after a

## Abstract

Prenatal genetic diagnosis in euploid fetuses with increased Nuchal Translucency (NT) and persistently increased Nuchal Fold (NF) is challenging. The aims of this study is to evaluate the prevalence of pathogenic copy number variants and Noonan Syndrome (NS) in fetuses with increased NT and persistently increased Nuchal Fold (NF) to provide recommendations for pre-natal diagnostic strategies. This is a prospective study from 118 prenatal samples from fetuses with increased NT ( $\geq 3.5$ mm) in first trimester. Multiplex Ligation Dependent Probe Amplification (MLPA), Chromosomal Microarray Analysis (CMA) and karyotyping were conducted. For fetuses with increased NF ( $\geq 6$ mm) in the second trimester with normal karyotype and CMA, targeted Next-Generation Sequencing (NGS) tests for NS were carried out. A total of 118 fetuses had an NT measurement of  $\geq 3.5$ mm performed MLPA and karyotyping, 89 euploid fetuses were further investigated with CMA, which yielded eight pathogenic CNVs (size ranged from 0.85Mb to 14.5Mb). Twenty fetuses had persistently increased NF at the second trimester. NS testing revealed 3/20 (15%) fetuses had pathogenic variants, and one (5%) with a novel variant of uncertain clinical significance inherited from the father. Our study suggested that NS targeted sequencing facilitates additional genetic diagnosis in fetuses with high NT and persistently increased NF.

**Keywords:** Nuchal translucency; Nuchal fold; Noonan syndrome; Next generation sequencing; Prenatal diagnosis

normal karyotype was 5%, although CMA cannot detect balanced rearrangements and single gene mutations [15].

Noonan Syndrome (NS:MIM163950) is regarded as the single gene disorder which is the most frequently associated with increased NT, with an incidence of up to 7% in the first trimester, and 10% in the second trimester pregnancy [16-19]. NS is an autosomal dominant disorder, with a prevalence of 1 in 1000-2500 [20]. This disease is characterized by distinct craniofacial dysmorphisms, postnatal growth retardation, and congenital cardiac defects such as pulmonary valve stenosis, atrial septal defects, and hypertrophic cardiomyopathy. Craniofacial features include broad forehead, hypertelorism, downslanting palpebral fissures, ptosis, and low-set posteriorly rotated ears [21]. NS is a genetically heterogeneous disease, more than 10 genes are associated with NS. Three major genes, including PTPN11 (~50%; MIM 176876) [22], SOS1 (10-15%; MIM 182530) [23] and RAF1 (5-10%; MIM 164760) [24,25] are known to be causative genes with higher detection rates for NS. The other disease causing genes are KRAS (MIM: 190070), LZTR1 (OMIM 600574), SOS2 (MIM 601247), CBL (OMIM 165360), RIT1 (OMIM 609591), and RRAS (MIM 165090). Molecular diagnostic techniques have developed rapidly, such as targeted Next Generation Sequencing (NGS) tests to enable accurate, faster and cost-effective diagnosis of

NS [26]. Bakker M et al. proposed a cost-effective selection of clinical options [27]. They showed that the diagnosis of NS can be suspected prenatally in fetuses with increased nuchal translucency and one or more of the following characteristics: persistently increased nuchal fold or cystic hygroma, hydrops fetalis, pleural effusion, cardiac anomalies, polyhydramnios. At present, managing pregnancy with increased NT involves continuing assessment to establish a proper diagnostic strategy, and to provide parents with realistic information about fetal outcomes. The aims of this study is to evaluate the prevalence of copy number variants and NS in unique cohort of fetuses with increased Nuchal Translucency (NT) and persistently increased Nuchal Fold (NF) to provide data for prenatal diagnostic strategies.

## Materials and Methods

### Patients and samples

From January 2015 to October 2017, study subjects were referred to two prenatal diagnosis centers for increased NT investigation. The doctors who obtained NT measurement were certified by The Fetal Medicine Foundation, London, UK. NT measurements were performed between gestational ages of 11-13+6 weeks. The NF was measured on a frontooccipital transverse view, including the cavum septum pellucidum, cerebellum and the posterior fossa, as the distance between the median point of the outer curve of the occipital bone and the outer skin edge in 15-20 weeks, the cut-off value is 6mm [28]. 118 pregnancies with  $NT \geq 3.5$ mm were recommended to receive prenatal diagnostic testing in Dongguan Maternal and Child Health Care Hospital and Shenzhen Bao'an Maternal and child health hospital.

### Multiplex ligation dependent probe amplification

Multiplex Ligation Dependent Probe Amplification (MLPA) analysis were performed using the SALSA, RMLPA Rprobemix P095 aneuploidy assay (MRC-Holland, Amsterdam, the Netherlands). MLPA assays were used to assess copy numbers of chromosome 13, 18, 21, X and Y. MLPA results were verified by karyotype analysis and CMA test.

### Chromosomal Microarray Analysis (CMA)

Genomic Copy-Number Variants (CNVs) were detected using the Fetal DNA Chip (Agilent Technologies, Inc. Santa Clara, CA, USA), a targeted high-resolution 8X60k oligonucleotide array (Fetal DNA Chip), specifically constructed for prenatal diagnosis with the intention of targeting common trisomic aneuploidies and most well-known micro-deletion/microduplication syndromes, or the Affymetrix CytoScan 750k array.

### The inclusion criteria for Noonan testing

The inclusion criteria to carry out NGS for NS were: 1. NT measurement  $\geq 3.5$ mm in the first trimester and  $NF \geq 6$ mm in the second trimester; 2. Normal karyotype and CMA results in the prenatal chorionic villus sampling or amniocentesis.

### Noonan testing

DNA extracted from the invasive samples were sent to a diagnostic laboratory to detect NS genes by target capture and sequencing including BRAF, HRAS, KRAS, MAP2K1, MAP2K2, PTPN11, RAF1, SHOC2, SOS1, CBL, NRAS, RIT1. Results with known pathogenic variants in any of the tested genes were considered positive, while

those with variants of uncertain clinical significance were considered VUS. No clinically significant variants detected are considered negative. The positive results were confirmed by sanger sequencing.

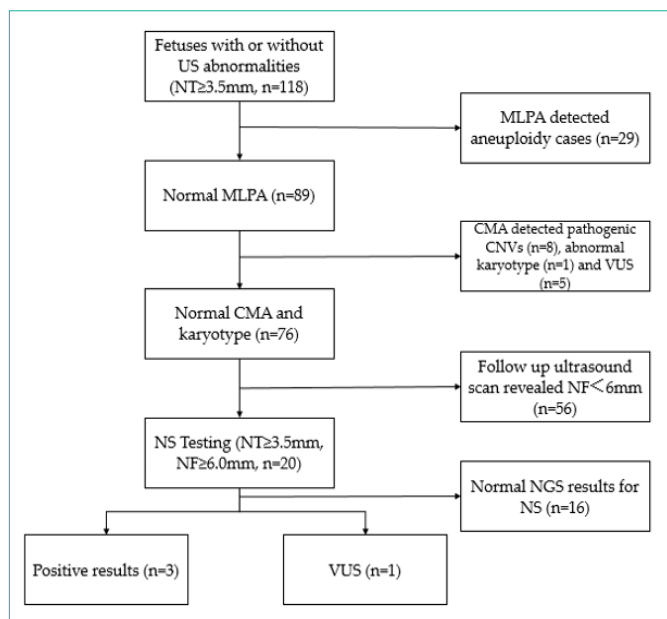
### Target capture and sequencing

**Target capture and sequencing:** The genomic DNA of the samples was fragmented by a Q800R Sonicator (Qsonica) to generate 300-500bp insert fragments. The paired end libraries were prepared following the Illumina library preparation protocol. Custom designed NimbleGen SeqCap probes (Roche NimbleGen, Madison, Wis) were used for in-solution hybridization to enrich target sequences. Enriched DNA samples were indexed and sequenced on a NextSeq500 sequencer (Illumina, San Diego, Calif) with 100-150 cycles of single end reads, according to the manufacturer's protocols.

**Variant annotation and interpretation:** Primary data were received in fastq format after image analysis and base calling conducted using the Illumina Pipeline. The data were filtered to generate 'clean reads' by removing adapters and low quality reads (Q20). Sequencing reads were mapped to the reference human genome version hg19 (2009-02 release, <http://genome.ucsc.edu/>). Nucleotide changes observed in aligned reads were called and reviewed by using NextGENe software (SoftGenetics, State College, Pa). Beside detection of deleterious mutations and novel single nucleotide variants, coverage-based algorithm developed in-house, eCNVscan, was used to detect large exonic deletions and duplications. The normalized coverage depth of each exon of a test sample was compared with the mean coverage of the same exon in the reference file to detect Copy Number Variants (CNVs). Sequence variants were annotated using population and literature databases including the 1000 Genomes Project, dbSNP, GnomAD, Clinvar, HGMD and OMIM. Some online software were used to analyze the structure of the protein, predict the conservation domain, function domain and perform the multiple sequence alignment. Variant interpretation was performed according to the American College of Medical Genetics and Genomics (ACMG) guidelines. The study was approved by the research ethics committee of the institutions.

## Results

A total of 118 fetuses had NT measurements of  $NT \geq 3.5$ mm had MLPA and karyotyping with either CVS or amniocentesis samples detected 29 aneuploidies (Figure 1). The remaining 89 euploid fetuses performed CMA and found eight pathogenic CNVs and five VUS (Table 1 and Table 2). Twenty euploid fetuses had persistently increased NF ( $\geq 6$ mm) in the 15-20 gestational week ultrasound scan with normal CMA results and were offered Noonan Syndrome (NS) gene panel testing. The NS gene panel yielded positive findings in three fetuses (15%, 3/20), a variant of uncertain clinical significance in one (5%, 1/20), and negative results in 16 (80%, 16/20) fetuses (Table 3). The NT/NF thickness measurements of fetuses with NS positive gene panel results were 8mm/11.5mm (case 1), 5mm/10mm (case 2), 5.2mm/8mm (case 3) (Table 3). Fetuses with positive NS variants had a thicker median NT and NF than those with negative results (Table 4). There were no other ultrasonographic structural abnormalities detected in any of the fetuses tested positive for NS. Two fetuses carried known pathogenic mutations in the PTPN11 gene, the other with a known pathogenic mutation in the BRAF gene. Case 1 was found with polyhydramnios. All positive NS results and VUS were



**Figure 1:** Summary of prenatal testing of fetuses with increased nuchal translucency.

NS: Noonan Syndrome Testing; CMA: Chromosomal Microarray Analysis; CNV: Copy-Number Variant; MLPA: Multiplex Ligation-Dependent Probe Amplification; NT: Nuchal Translucency; NGS: Next-Generation Sequencing; VUS: Variants of Uncertain Clinical Significance; NF: Nuchal Fold.

confirmed by Sanger sequencing. The fetuses tested positive for NS were terminated, and case 4 with a VUS in BRAF (Table 4) resulted in a live birth. Follow up at the age of ~2 years old revealed mild autistic features. In the remaining 16 fetuses with negative NS results, 3 fetuses had prenatal ultrasound abnormalities. There was one fetus with hydrocephalus, one with hypoplastic left heart, and one with tetralogy of fallot, cerebellar dysplasia and micrognathia. In these three cases, the parents opted for termination of the pregnancy in the second trimester. Follow up of remaining cases reported live birth and healthy babies in all 13 cases (Supplementary Table 1).

## Discussion

In this prospective study, 15% (3/20) of euploid fetuses with increased NT and persistently increased NF and normal CMA were diagnosed genetically with NS using targeted next-generation sequencing. This information will be helpful for clinicians and parents

**Table 1:** Molecular and clinical characterization of 8 fetuses with pathogenic CNVs.

Case	NT (mm)	NF (mm)	Findings on US (second trimester)	CNVs results <sup>a</sup>	CNV type	Size (Mb)	Inheritance	Karyotype results	Outcome
1	7	NA	NA	arr 4p16.3 (68345_3449602) x 1	Del	3.3	de novo	Normal	TOP
2	3.5	6.9	Micrognathia, microtia	arr 22q11.21 (18909032_21307800) x 3	Dup	2.4	de novo	Normal	TOP
3	4.1	5.3	No structural abnormality	arr 13q14.2q14.3 (49301719_53625881) x 1	Del	4.3	NA	Normal	TOP
4	3.7	5.6	Tetralogy of fallot	arr 22q11.21 (18909032_21357982) x 1	Del	2.4	de novo	Normal	TOP
5	3.8	5.3	Defect endocardial cushion	arr 16p13.11p12.3 (14,892,975-16,858,332) x 1	Del	1.97	de novo	Normal	TOP
6	3.8	6.5	No structural abnormality	arr 16p11.2 (29340592_30190029) x 1	Del	0.85	de novo	Normal	TOP
7	6.3	8.8	Cystic hygroma	arr 5p13.2q11.1 (34985928_49441945) x 3	Dup	14.5	de novo	Abnormal	TOP
8	3.5	7.5	No structural abnormality	arrXp22.31 (6455151_8143509) x 0	Del	1.7	de novo	Normal	TOP

NT: Nuchal Translucency; NF: Nuchal Fold; US: Ultrasound Scan; CNVs: Copy Number Variations; a: UCSC Genome Browser hg19 coordinates; NA: Not Available; TOP: Termination of Pregnancy.

to assess the risks for pregnancy management and future pregnancies. In this study, we describe an increased incidence of pathogenic CNVs (8.98%, 8/89) among the euploid fetuses with increased NT which is similar to the literature [14,29].

We chose the cut-off value of increased NT as 3.5mm according to the 99<sup>th</sup> percentile of the normal range [9]. Meanwhile, we chose persistently increased NF as a necessary indicator for NS testing based on the literature that this marker is associated with aneuploidies and genetic syndromes [8,10] but it is challenging in prenatal diagnosis. There are no data about the relevance of NS in increased NT and persistently increased NF with normal CMA in literature. In euploid fetuses, increased NT (≥3.0mm) is associated with a high risk 10% (4/39) of NS [30], our study also indicated that NT ≥3.5mm and NF ≥6mm showed a similar risk (15%, 3/20) of NS. Taken together, NS using targeted next-generation sequencing should be recommended for this group of fetus with normal CMA results.

Croonen EA et al. [31] recommend genes including PTPN11, RAF1 and KRAS to be tested when increased NT is combined with one or more of polyhydramnios, hydrops fetal, cystic hygroma, cardiac anomalies, hydrothorax, the detection rate of NS was 17%. Targeted NGS could allow a rapid, less costly and an accurate diagnosis for Noonan syndrome [26]. A targeted NGS for Noonan syndrome could provide further information about this monogenic disease to parents for genetic counseling and decision-making. In our study, 12 targeted genes which were known to be associated with NS were tested, including BRAF, HRAS, KRAS, MAP2K1, MAP2K2, PTPN11, RAF1, SHOC2, SOS1, CBL, NRAS, RIT1 [26,32].

In the NS positive cases, case 1 had an increased NT (8mm) and NF (11mm), but no obviously ultrasound structural abnormalities were found except polyhydramnios until 23 gestation weeks. The positive case 2 (NT: 5.0mm/NF: 10.0mm) and case 3 (NT: 5.2mm/NF: 8mm) also had no ultrasound structural abnormalities (Table 4). The features of NS include craniofacial features, common heart malformations such as pulmonary valve stenosis, atrial septal defects and hypertrophic cardiomyopathy which are not easily detectable in first trimester or early second trimester ultrasound scans. In our study, all pathogenic NS cases had increased NT and persistently increased NF without prenatal severe structural abnormalities. Consequently, if increased NT is detected by prenatal ultrasound, especially when NF is increased persistently, targeted capture and sequencing for NS

**Table 2:** Molecular and clinical characterization of 5 fetuses with VUS CNVs.

Case	NT (mm)	NF (mm)	Findings on US (second trimester)	CMA results <sup>a</sup>	CNV type	Size (Mb)	Inheritance	Karyotype results	Outcome
1	6.4	NA	Cystic hygroma	arr [GRCh37] 2q14.3 (126,978,133-127,743,822) x1	Del	0.766	Mat	Normal	TOP
				arr [GRCh37] 7p22.2 (2,955,638-3,704,743) x 3	Dup	0.749			
2	3.6	5.4	No structural abnormality	arr [GRCh37] 22q11.21 (18,970,561-21,800,471) x 3	Dup	2.83	Mat	Normal	LB
3	5.9	7.1	Ventricular septal defect	arr [GRCh37] 7q33 (136,578,081-137,248,769) x 1	Del	0.671	<i>de novo</i>	Normal	TOP
				arr [GRCh37] 13q31.1 (80,760,920-82,446,069) x 1	Del	1.68			
4	3.7	7	No structural abnormality	arr [GRCh37] 13q12.12 (23,532,645-24,870,530) x 3	Dup	1.3	Pat	Normal	LB
5	3.5	7.5	No structural abnormality	arr [GRCh37] 18p11.32 (179,030-1,900,396) x 1	Del	1.7	Mat	Normal	LB

NT: Nuchal Translucency; NF: Nuchal Fold; US: Ultrasound Scan; Conc: Conclusion; VOUS: Variants of Unknown Significance; CNVs: Copy Number Variations; a: UCSC Genome Browser hg19 coordinates; NA: Not Available; TOP: Termination of Pregnancy; LB: Live Birth.

**Table 3:** NT/NF thickness measurements of the fetuses with NS positive gene panel results.

	No. of fetuses	Maternal age at EDC (Yr)	Median NT thickness (mm)	Median NF thickness (mm)
Positive NS	3	32	6.1	9.8
Negative NS	16	30	4.2	7.1
VOUS	1	33	4.1	6.7

NT: Nuchal Translucency; NF: Nuchal Fold; NS: Noonan Syndrome; VOUS: Variants of Unknown Significance.

**Table 4:** Molecular and clinical characterization of 3 fetuses with positive NS gene panel results and 1 VUS.

Case	NT	NF	Findings on US	NS Results	Conc.	Outcome
1	8	11.5	Polyhydramnios	<i>PTPN11</i> c.184T>G (p.Y62D)	Likely Path.	TOP
2	5	10	No structural abnormality	<i>PTPN11</i> c.1528C>G (p.Q510E)	Path.	TOP
3	5.2	8	No structural abnormality	<i>BRAF</i> c.1411G>T (p.V471F)	Path.	TOP
4	4.1	6.7	No structural abnormality	<i>BRAF</i> c.1120A>G (p.I374V)	Vus.	LB

NS: Noonan Syndrome; VUS: Variants of Unknown Significance; TOP: Termination of Pregnancy; LB: Live Birth.

or even whole exome sequencing is suggested to rule out NS or other single gene disorders.

One fetus carried a VUS in *BRAF* c.1120A>G (p.I374V), which was inherited from her father. The father showed no abnormalities from detailed physical and ultrasound examination by a geneticist. However, follow up of this child at the age of ~2 years old revealed mild autistic features, including poor verbal skills and attention. Although the penetrance of NS is complete, NS is also known to show variable expressivity. Further follow up assessments of both father and child including cognitive, physical examination and cardiovascular examinations are warranted to investigate whether there is causative association.

In the 16 fetuses with negative NS panel results, all achieved a healthy live birth. Follow up available in 14 cases showed healthy children. The remaining two were lost to follow up. This indicates when NS is excluded, fetuses with increased NT and persistently increased NF have good outcomes. At present, managing pregnancies with increased NT involves continuous assessment to establish a proper diagnostic strategy. Pregnant woman can undergo the CMA as a first-tier test in fetuses with increased NT or, alternative, after common aneuploidies have been ruled out by normal MLPA or QF-PCR results. These rapid molecular diagnostic techniques can be performed immediately after screening in first trimester or in the early second trimester. Fetuses with a normal CMA and karyotype are recommended to perform screening 2-3 weeks later to assess the

structural anomalies such as cardiac defects, skeletal deformity and so on, that are known to be associated with increased NT [10], and the measurement of NF is also important for risk assessment. Detailed scan at 20 weeks of gestation is essential to assess the fetal anatomy. When the NF remains increased, the risk of an unidentified anomaly or genetic syndrome remains. In these cases, Noonan testing results could provide parents with realistic information about fetal outcome and help them make informed decisions. With further research and development of whole exome sequencing tests [33], studies with large sample sizes to detect more single gene disease among increased NT fetuses with normal karyotype and chromosomal microarray are needed [34].

The limitation of this study is the small sample size of the increased NT and persistently increased NF, with the reduced cost of WES. A larger sample size study using WES should be conducted in the future.

## Conclusion

In summary, we describe an increased rate of pathogenic CNVs (8.98%, 8/89) among euploid fetuses with increased NT. Target capture sequencing for NS yielded an additional 15% (3/20) diagnostic rate among fetuses with normal CMA, increased NT and persistently increased NF. Our study suggested that CMA and targeted sequencing of NS genes facilitates genetic diagnosis and enables more accurate prediction of fetal prognosis and genetic risk of recurrence in future pregnancies.

## Author Contributions

Yangyang Lin, Huilin Wang and Zhu Yuanfang designed the research, Yangyang Lin, Huilin Wang, Matthew Hoi Kin Chau, Jiwei Lou, Xiumei Zeng, Yuanhao Liang, Zhuo Meng, Rui Zhang, Rungui Xie, Wenbin Zhong, Yanhui Liu, Fubing Yu, Yuanfan Zhu performed research; Huilin Wang, Wei Zhang analyzed data; Yangyang Lin and Huilin Wang, wrote the paper, Yangyang Lin, Huilin Wang, Matthew Hoi Kin Chau and Kwong Wai Choy revised the paper.

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## Institutional Review Board Statement

The study was conducted according to the guidelines of the Declaration of Helsinki, and approved by the Ethics Committee of Dongguan Maternal and child healthcare hospital.

## Informed Consent Statement

Informed consent was obtained from all subjects involved in the study.

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