



Clinical application of chromosomal microarray for pathogenic genomic imbalance in fetuses with increased nuchal translucency but normal karyotype

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Purpose: To evaluate the additive value of prenatal chromosomal microarray analysis (CMA) in assessing increased nuchal translucency (NT) (≥ 3.5 mm) with normal karyotype and the possibility of detecting clinically significant genomic imbalance, based on specific indications.

Materials and Methods: Invasive samples from 494 pregnancies with NT ≥ 3.5 mm, obtained from the Research Center of Fertility & Genetics of Hamchoon Women's Clinic between January 2019 and February 2020, were included in this study and CMA was performed in addition to a standard karyotype.

Results: In total, 494 cases were subjected to both karyotype and CMA analyses. Among these, 199 cases of aneuploidy were excluded. CMA was performed on the remaining 295 cases (59.7%), which showed normal (231/295, 78.3%) or non-significant copy number variation (CNV), such as benign CNV or variants of uncertain clinical significance likely benign (53/295, 18.0%). Clinically significant CNVs were detected in 11 cases (11/295, 3.7%).

Conclusion: Prenatal CMA resulted in a 3% to 4% higher CNV diagnosis rate in fetuses exhibiting increased NT (≥ 3.5 mm) without other ultrasound detected anomalies and normal karyotype. Therefore, we suggest using high resolution, non-targeting CMA to provide valuable additional information for prenatal diagnosis. Further, we recommend that a genetics specialist should be consulted to interpret the information appropriately and provide counseling and follow-up services after prenatal CMA.

Key words: Nuchal translucency measurement, Increased nuchal translucency (≥ 3.5 mm), Prenatal chromosomal microarray, Prenatal chromosomal microarray analysis.

Introduction

Nuchal translucency (NT) is the thickness of fluid collection in the fetal neck. This may be observed by an ultrasound scan, that is performed between 10 and 13 weeks 6 days of gestation. An increased NT (≥ 3.5 mm, or >99 th percentile) is correlated not

only with chromosomal aneuploidies, but also with important defects of the heart and arteries, skeletal dysplasia to a considerable extent, as well as some genetic syndromes. In addition, when these defects occur, there is an increased risk of miscarriage, intrauterine fetal death, or delayed development [1,2].

Cytogenetic analysis of amniotic fluid (AF) or chorionic villi

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Conflict of interest: The authors declare that they do not have any conflicts of interest.

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samples (CVS) has been the gold standard prenatal diagnostic method for the detection of abnormal karyotypes. This method reliably analyzes chromosomal aneuploidies, as well as structural abnormalities. Chromosomal microarray analysis (CMA) is a novel method that detects not only the chromosomal aneuploidies, but also the copy number variations (CNVs). These CNVs appear in genomic imbalances, such as microdeletions and microduplications, which may be correlated with known genetic syndromes, or abnormal clinical phenotypes [3,4].

The latest meta-analysis, regarding the need for CMA in cases with increased NT and normal karyotype, showed progressive diagnosis rates between 4% (increased NT) and 7% (complex ultrasound malformation), including aberrations that involved 22q11.2 [5,6].

Most researchers have proposed CMA for prenatal diagnosis, in cases with NT ≥ 3.5 mm. Also, the increased resolution achieved by CMA provides a broader scope to diagnose chromosomal aberrations, as well as an increase in the number of uncertain findings, secondary findings, or adult-onset disease indicators. Uncertain findings or secondary results are the major dilemmas in prenatal genetic counseling. In our study, we aimed to evaluate the clinical interpretations of prenatal CMA, for the inspection of genomic imbalances in specimens from fetuses with an increased NT (≥ 3.5 mm) and a normal karyotype [7-9].

Materials and Methods

The invasive samples from 494 pregnant women with fetuses having a NT ≥ 3.5 mm included in this study were obtained from the Research Center of Fertility & Genetics of Hamchoon Women's Clinic between January 2019 and February 2020. These were subjected to prenatal diagnosis using rapid aneuploidy detection (RAD), G-band karyotyping and CMA. Details regarding the analysis flow are shown in Fig. 1.

All the samples underwent either quantitative fluorescence polymerase chain reaction (QF-PCR) or the direct method for RAD. Conventional cytogenetic analysis was carried out on CVS. This was in accordance with the standard protocols used for examining the numerical and structural aberrations of chromosomes, in direct cytotrophoblastic cell-preparations and long-term cultures of mesenchymal tissue (GTG-banding, 550 band level). Cytogenetic analysis using AF was also carried out according to the standard protocols.

Genomic DNA for prenatal CMA was extracted from T25-flask-cultured fetal cells using a Qiagen DNA mini kit (Qiagen, Valencia, CA, USA), according to the manufacturer's protocol. For single nucleotide polymorphism (SNP) array analysis, the genomic DNA was screened using CytoScan 750K (Affymetrix Inc., Santa Clara, CA, USA), and analyzed using Affymetrix gene chip kit software ChAS 3.2. Our CNV results were compared with those in public CNV databases (Database of Genomic Variants [<http://projects.tcag.ca/variation/>]; Decipher [[```

graph TD
 A\["Pregnancies with NT ≥ 3.5 mm n = 494"\] --> B\["RAD \(QF-PCR or direct CVS\)
CVS=482 AF=12"\]
 B --> C\["Abnormal Karyotype n=199"\]
 B --> D\["Normal Karyotype n=295"\]
 D --> E\["CMA"\]
 E --> F\["CNVs n=53
Loss=29 Gain=34"\]
 F --> G\["Pathogenic or VOUS n=11"\]

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\*\*Abnormal Karyotype n=199\*\*

|            |      |
|------------|------|
| Trisomy 13 | n=7  |
| Trisomy 18 | n=66 |
| Trisomy 21 | n=94 |
| Monosomy X | n=27 |
| 47,XXY     | n=3  |
| 47,XXX     | n=2  |

\*\*CNVs n=53\*\*

|      |    |      |    |
|------|----|------|----|
| Loss | 29 | Gain | 34 |
|------|----|------|----|

\*\*Pathogenic or VOUS n=11\*\*](http://decipher.</a></p>
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**Fig. 1.** Characteristics of pregnant women and aberrant CMA findings in fetuses with increased NTs ( $\geq 3.5$  mm). CMA, chromosomal microarray; NT, nuchal translucency; RAD, rapid aneuploidy detection; QF-PCR, quantitative fluorescence polymerase chain reaction; CNVs, copy number variations; AF, amniotic fluid; VOUS, variants of uncertain clinical significance.

**Table 1.** Significant genomic imbalanced CNVs detected in 11 fetuses with increased NT ( $\geq 3.5$  mm) and normal chromosome

| Case | GA (wk) | Indication   | Karyotype | Sample types | CMA result (ISCN 2016)                                                                          | Size & type of CNV                     | Parantal test  | Related syndrome/gene                                                          |
|------|---------|--------------|-----------|--------------|-------------------------------------------------------------------------------------------------|----------------------------------------|----------------|--------------------------------------------------------------------------------|
| 1    | 12+5    | INT (3.5 mm) | 46,XY     | Cultured CVS | arr[GRCh37]1q41q42.11(222512810_224178383)×3, 8q11.23(53366164_53931053)×1                      | 1,666 kb dup, 565 kb del               | ND             | 17 genes (9 morbid OMIM genes) and 3 genes (2 morbid OMIM genes)               |
| 2    | 13+6    | INT (3.8 mm) | 46,XY     | Cultured CVS | arr[GRCh37]16p11.2(29428531_30190029)×1 dn                                                      | 761 kb del                             | <i>De novo</i> | 24 genes (24 morbid OMIM genes)                                                |
| 3    | 12+5    | INT (3.9 mm) | 46,XY     | Cultured AF  | arr[GRCh37]16p11.2(32022076_34061205)×3, 16p13.3(6568310_6963896)×1, Xp22.31(6537109_8167604)×2 | 2,039 kb dup, 396 kb del, 1,630 kb dup | ND             | 11 genes (1 morbid OMIM gene), RFX1 (605104) and 6 genes (4 morbid OMIM genes) |
| 4    | 14+3    | INT (4.4 mm) | 46,XY     | Cultured CVS | arr[GRCh37]15q24.1q24.2(74409679_76035913)×3 dn                                                 | 1,626kb dup                            | <i>De novo</i> | 28 genes (28 morbid OMIM genes)                                                |
| 5    | 11      | INT (4.7 mm) | 46,XX     | Cultured AF  | arr[GRCh37]9p23.1(11525018_11808598)×1 dn                                                       | 284 kb del                             | <i>De novo</i> | 6 genes (4 morbid OMIM genes)                                                  |
| 6    | 13      | INT (4.9 mm) | 46,XY     | Cultured CVS | arr[GRCh37]3p26.3p26.2(2534659_2852016)×1 mat                                                   | 317 kb del                             | Maternal       | 1 gene                                                                         |
| 7    | 13+2    | INT (5.2 mm) | 46,XY     | Cultured CVS | arr[GRCh37]1q21.1q21.2(145895746_147830830)×1 dn                                                | 1,935kb del                            | <i>De novo</i> | 13 genes (13 morbid OMIM genes)                                                |
| 8    | 12+6    | INT (5.8 mm) | 46,XY     | Cultured CVS | arr[GRCh37]15q25.2(82533402_83625545)×1 dn                                                      | 1,092 kb del                           | <i>De novo</i> | 19 genes (12 morbid OMIM genes)                                                |
| 9    | 18+2    | INT (6.5 mm) | 46,XX     | Cultured AF  | arr[GRCh37]8q22.2(99217277_99642055)×1, 18p11.32p11.31(136226_3812306)×1                        | 425 kb del, 3676 kb del                | ND             | 3 genes (1 morbid OMIM gene) and 30 genes (17 morbid OMIM genes)               |
| 10   | 13+6    | INT (7.7 mm) | 46,XY     | Cultured CVS | arr[GRCh37]2q21.1(130704336_131171569)×1 dn                                                     | 467 kb del                             | <i>De novo</i> | 5 genes (1 morbid OMIM gene)                                                   |
| 11   | 12+4    | INT (8.9 mm) | 46,XY     | Cultured CVS | arr[GRCh37]22q11.21(18648855_21800471)×1                                                        | 3,152 kb del                           | ND             | DGeorge syndrome (OMIM # 188400) /47 genes (47 morbid OMIM genes)              |

CVS, chorionic villi sample; NT, nuchal translucency; GA, gestational age; CMA, chromosomal microarray analysis; CNV, copy number variation; INT, increased nuchal translucency; dn, *de novo*; dup, duplication; ND, not done; del, deletion; AF, amniotic fluid. Based on hg19.

sanger.ac.uk]; ISCA[<https://www.iscaconsortium.org>]; UCSC [<http://genome.ucsc.edu/>]; OMIM [<http://www.omim.org/>], by trained investigators. Further, they were classified as pathogenic, likely pathogenic, uncertain clinical significance, likely benign, or benign according to the guidelines of the American College of Medical Genetics [10]. CNVs were reported to the physician according to the guidelines of the Society of Obstetricians and Gynaecologists of Canada (SOGC)-Canadian College of Medical Geneticists (CCMG) [5]. If a pathogenic sample was detected, parental CMA testing was recommended in order to confirm the aberration. Also, eventually, it was reconfirmed via secondary genetic methods such as fluorescence *in situ* hybridization or multiplex ligation-dependent probe amplification (MLPA).

## Results

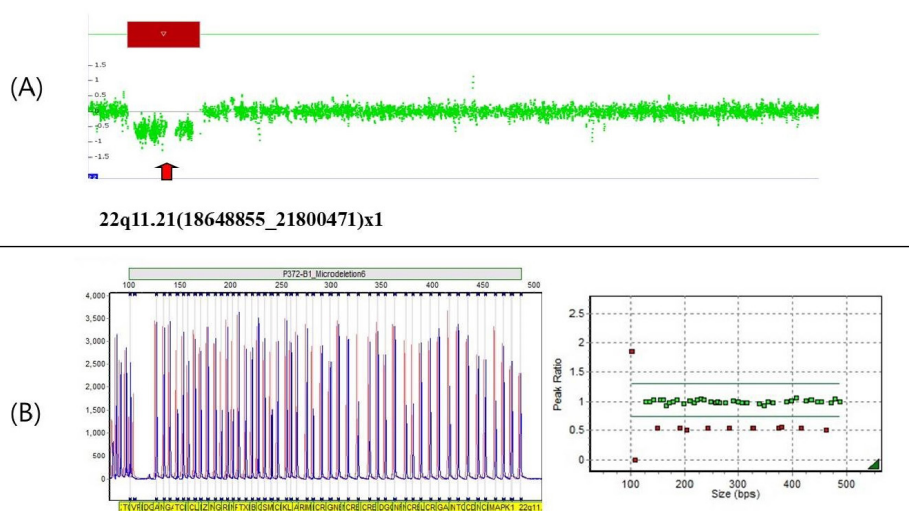
Fig. 1 summarizes the fetuses examined in this study. Out of the 494 pregnancies with increased fetal NT at 10 to 13 weeks 6 days of gestation that were analyzed in the study, the invasive procedures performed were CVS (482/494, 97.6%) or amniocentesis (12/494, 2.4%). Rapid aneuploidy testing (QF-PCR, direct CVS) showed 199 (40.3%) cases having an aneuploidy involving chromosomes 13, 18, 21 or X, consistent with the karyotype analyzed in the cultured fetal tissue. In total, 494 cases were subjected to both karyotype and CMA analyses, among which 199 cases of aneuploidy were excluded. The remaining 295 (59.7%) cases underwent CMA, and had a normal CMA result (231/295, 78.3%), or showed a non-significant CNV, such as benign CNV or VOUS likely benign (53/295, 18.0%). Clinically

significant CNVs were detected in 11 cases (11/295, 3.7%) (Table 1). The size of the CNV, its genomic position, and gene content were re-evaluated for clinical significance by referencing the latest published studies and public databases. Table 1 shows the list of cases with clinically significant genomic imbalances. Out of seven cases (Cases 2, 4–8, 10), six were *de novo* and one was inherited from the mother; in four cases (Cases 1, 3, 9, 11), the parents were not tested; however, out of three of these cases (Cases 1, 3, 11), one had an abnormal ultrasound finding in addition to the NT. Case 11 (DiGeorge syndrome) was reconfirmed with MLPA P372-B1 (Fig. 2) and matched.

## Discussion

In our study, 3.7% of the fetuses had a significantly increased NT ( $\geq 3.5$  mm; 99th percentile); however, through CMA, even the normal karyotypes were found to have pathogenic genomic imbalances. This incidence was very similar to the guidelines of the 2011 SOGC-CCMG [5]. Therefore, our results corroborate that prenatal CMA identifies clinically significant CNVs, that are not detectable by conventional cytogenetic methods. The most frequent pathogenic CNVs that were related to increased NT were 22q11.2 microdeletions/microduplications. We previously reported the detection of 22q11.2 genomic imbalance by the MLPA method, which was used even though the karyotype was normal, despite the increased NT [11].

To date, karyotype analysis remains a reliable method in prenatal diagnosis. However, because of the limitations of chromosomal analysis, most researchers discuss the benefits



**Fig. 2.** Case 11 results in deleted 22q11.21. (A) CMA image showing a 3.152 kbp deletion. (B) MLPA results in the MRC-Holland P372-B1.

and limitations of using CMA and conventional cytogenetic methods for prenatal diagnostic testing of pregnant women, and propose additional options for CMA. CMA method has a higher resolution than conventional karyotyping, and is capable of diagnosing smaller submicroscopic imbalances, including the uniparental disomy and loss of heterozygosity, by SNP array [8,9,12]. For fetuses having an increased risk of submicroscopic chromosomal imbalances, such as those with increased NT, the utility of the supplemental data provided by CMA in prenatal diagnosis is evident, and helps in reducing the rate of undiagnosed diseases, or the likelihood of their occurrence. CMA could provide more accurate predictive perceptions as compared to karyotype analysis, thereby affecting pregnancy management and the concomitant outcomes.

Nevertheless the interpretation of CMAs and the policy for classifying CNVs is challenging. In our study, we noticed certain pathogenic variants or likely pathogenic variants, as they do not always correspond to severe defects, and may be inherited from a parent having very little or no clinical features. Therefore, it is suggested that the CMA must be interpreted with caution in prenatal diagnosis, by using proper guidelines. Our team interpreted the results based on the guidelines of the SOGC-CCMG, and informed the pregnant women and their families.

For clinically significant imbalances (pathogenic), it is necessary to carry out parental testing. Due to incomplete penetrance and a variable phenotype, these CNVs also raised concerns and frustration among parents, which lead to confusion regarding the future health and development of the offspring. In the present study, among seven cases (Cases 2, 4-8, 10) [13-19], six were de novo, while one was maternally inherited; in four cases (Cases 1, 3, 9, 11) [20-23], the parents were not tested; however, from three of the (Cases 1, 3, 11) cases, one was observed with an abnormality in the ultrasound, in addition to the NT. Nevertheless, the clinical significance of these variants cannot be predicted before parental confirmation. CMA was inadequate for accurately indicating the clinical significance of a formerly unreported CNV. Instead, parental confirmation is necessary in order to rule out some CNVs [24,25], which tend to be benign. Fortunately, with the application of CMA and sustained improvements in the data from the database, the incidence of VOUSs can be reduced. It is advisable to reconfirm the findings using other genetic diagnostic methods, if possible.

We encountered both counseling challenges and ethical dilemmas with the initiation of prenatal CMA. These challenges included cases that were classified into: VOUS, CNVs with incomplete penetrance, and conditions with the onset of adult-

hood. Clearly, such cases require additional extensive counseling, as opposed to informing a patient about a test result being normal or abnormal, which is the case with karyotype results. A key aspect in such cases is comprehensive genetic counseling by an expert, who is a specialist in interpreting prenatal CMA information.

In our study, CMA showed a 3% to 4% increase in the number of CNV diagnosed fetuses, with increased NT ( $\geq 3.5$  mm), and an absence of other ultrasound anomalies and normal karyotype. Therefore, we suggest using a high resolution, non-targeting CMA to provide useful additional information for prenatal diagnosis. A key aspect in such cases is comprehensive genetic counseling by an experienced provider, having a specialization in interpreting the prenatal CMA. Also, we recommend involving of a genetics specialist service in prenatal CMA interpretation, counseling, and follow-up.

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