ARTICLE

Isochromosome 21q is overrepresented among false-negative cell-free DNA prenatal screening results involving Down syndrome

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Abstract
False-negative cell-free DNA (cfDNA) screening results involving Down syndrome are rare, but have high clinical impact on patients and their healthcare providers. Understanding the biology behind these results may allow for improved diagnostic follow-up and counseling. In 5 different centers offering cfDNA prenatal screening, 9 false-negative results were documented in 646 confirmed cases of trisomy 21; a false-negative rate of 1.4% (95% CI, 0.7–2.6). False-negative results included 4 cases of classical trisomy 21 and 5 cases with a de novo 21q;21q rearrangement. Two out of five rearrangements had molecular studies and were confirmed as isochromosomes. When combined with reports from the cfDNA screening literature, 8 out of 29 (28%) Down syndrome cases with a false-negative "non-invasive prenatal test" (NIPT) were associated with a 21q;21q rearrangement, compared with 2% reported in live born children with Down syndrome. In our laboratory series, evidence for placental or fetal mosaicism was present in 3 out of 3 true-positive cases involving a 21q;21q rearrangement and was confirmed in one false-negative case where placental material was available for study. Isochromosome 21q rearrangements are thus overrepresented among false-negative cfDNA screening results involving Down syndrome. Postzygotic isochromosome formation leading to placental mosaicism provides a biological cause for the increased prevalence of these rearrangements among false-negative cases. For clinical practice, a low trisomic fraction (z-score or equivalent measure) relative to the fetal fraction suggests placental mosaicism. Care should be taken as these cases may not reflect confined placental mosaicism, but rather full trisomy in the presence of a placenta containing normal cells.

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Introduction

Cell-free DNA (cfDNA)-based prenatal screening, also known as non-invasive prenatal testing (NIPT), has unparalleled sensitivity and specificity for trisomy 21, when compared to other screening modalities [1, 2]. Detection rates typically exceed 99%, with a false-positive (FP) rate of <0.1% [3]. Rare false-negative (FN) cases have been documented in larger clinical series [4, 5]. Low fetal fraction (FF) and mosaicism involving a euploid cell line have been implicated in some FN results, while other cases remain unexplained [6]. Here, we describe an overrepresentation of chromosome 21q;21q rearrangements among FN cfDNA screening reports and provide evidence that placental mosaicism arising from postzygotic isochromosome 21q formation is the likely biological cause. A low trisomic fraction [7] relative to the FF may suggest confined placental mosaicism (CPM), or, as described in this report, a full trisomy present in the fetus with a placenta containing normal cells.

Materials and methods

Five clinical laboratories (Academic Medical Center Amsterdam, the Netherlands; Victorian Clinical Genetics Services (VCGS), Melbourne, Australia; Genea, Sydney, Australia; Aarhus University Hospital, Denmark and Fleury Medicina & Saude, Sao Paulo, Brazil), reviewed their cfDNA screening outcome records for trisomy 21 true-positive (TP) and FN cases. All prevailing cfDNA screening technologies were utilized: massive parallel shotgun sequencing (MPSS), targeted MPS (tMPS), targeted microarray (tMA), and targeted single nucleotide polymorphism (tSNP)-based methodologies, using a number of bioinformatics algorithms. Clinical samples obtained from consented patients with average or elevated risk and received between 29 July 2013 and 29 September 2017 were included. Laboratory databases were interrogated for cytogenetic summary reports for all trisomy 21 cases, and for chromosome 21 z-scores [8] (or equivalent measure such as NCV_score [7]) and FF estimates [2, 9–11] in FN cases. One laboratory also reviewed its cytogenetic records for all trisomy 21 cases where cfDNA screening was done by external commercial providers. Genetic tests were performed on chorionic villi, amniotic fluid, newborn blood and/or placental material when available. The false-negative rate (FNR) was calculated from confirmed FN and TP cases involving trisomy 21 [(FN)/(TP+FN)]. CfDNA screening technologies and methods used to estimate FF for cases with 21q;21q rearrangements are listed in Table 1. CfDNA screening technologies associated with FN results for all known Down syndrome cases with karyotype data, including those described in literature, are listed in Table 2. Placental mosaicism for trisomy 21 was suspected in positive cfDNA samples that demonstrated a low trisomic fraction [7], relative to the FF, as described previously [11]. The trisomic fraction can be calculated directly from sequence tag-count data [7] and is the relative increase in sequence counts above the expected euploid state seen with massive parallel shotgun sequencing (MPSS) based NIPT methods [11].

For the identification of cytogenetically documented FN cfDNA screening cases, we relied on a systematic review published in July 2017 [3]. We included all publications used in this meta-analysis and listed in supplementary files, except for proof-of-concept studies, which we excluded. Additional records were found by cross-referencing.

Results

Five participating laboratories tested 58,504 cfDNA clinical samples and recorded 6 FN results from 500 confirmed cases of trisomy 21. Another 3 FN cases were documented in 146 diagnostic samples with trisomy 21 karyotype where cfDNA screening was done by external commercial providers. In total, 9 FN cases were documented from 646 confirmed cases of trisomy 21. The FNR for the combined series was 1.4% (95% CI, 0.7–2.6). FN results were recorded using targeted sequencing, whole genome sequencing, array-based and SNP-based techniques.

Five out of 9 FN samples involved a chromosome 21q;21q rearrangement; the other 4 cases had classical trisomy 21 karyotypes. All 5 cases with rearrangements resulted in Down syndrome live births (Table 1). In cases 1 and 3 the rearrangement was an isochromosome 21q based on the results of quantitative fluorescence PCR (QF-PCR) analysis. In cases 2, 4, and 5 no molecular studies were done. All 5 rearrangements were de novo based on normal parental karyotypes. The average fetal fraction at first analysis in FN cases with 21q;21q rearrangements was 10.1% (range 3–17.2%).

In case 1, ultrasound findings after low risk cfDNA screening led to amniocentesis and a non-mosaic isochromosome 21q was found. After birth, mosaicism involving a disomic cell-line was seen in chorionic villi from the placenta [12]. In cases 2–5, the newborn had a non-mosaic karyotype. The placenta was not available for analysis to confirm or exclude placental mosaicism in these cases (Table 1).

Three TP cases of trisomy 21 involving 21q;21q rearrangements (cases 6–8) were recorded in the laboratory series (Table 1). In case 6, true fetal mosaicism for trisomy 21 was reported after amniocentesis and a low-grade mosaicism involving an isochromosome 21q was confirmed in newborn blood. The placenta was not available. The two remaining TP cases with 21q;21q rearrangements demonstrated placental mosaicism based on cytogenetic analysis of chorionic villi (case 7) and/or bioinformatics analysis of cfDNA (cases 7 and 8) (Table 1 and Fig. 1).

In total 77 papers were reviewed for FN reports; 70 from the published systematic review [3] and another 7 via cross-referencing [13–19]. In total 16 papers mentioned FN cfDNA screening results for Down syndrome and 7 specified the karyotype. After combining these data with the results from the laboratory series, 8 out of 29 FN results for Down syndrome (28%) involved a 21q;21q rearrangement (Table 2).

Discussion

Down syndrome caused by 21q;21q rearrangements occurs in ~2% of cases [20]. Some 21q;21q rearrangements are Robertsonian translocations between two different chromosome arms, but most represent true isochromosomes [21]. Isochromosomes have two identical chromosome

Table 1 Cases of Down syndrome due to 21q;21q rearrangement reported in the 5 participating centers

Case No. and cfDNA screening technology	Indication for NIPT	GA (wk +d)	z-score or NCV score for c'some 21	FF (method)	Trisomic fraction	PND findings (wk+d)	Postnatal results	Conclusion
Chromosome 21q;21q rearrangements not identified by cell-free DNA prenatal screening (False negatives)								
1. MPSS [12]	cFTS 1/140 for T21	13+5	0.68	10.5%	NA	Abnormal US (20+2)	Live birth, Down syndrome	Confirmed iso21q; placental trisomy mosaicism confirmed
		20+6	2.5	10.9%	NA	Amniocentesis (20+2)	Placental Villi ST nuc ish 0% T21	
		32+2	6.7	14.3% (SANE-FALCON) [10]	NA	uAF QF-PCR T21[a] AF Karyotype 46,XX,i(21)(q10)dn[7]	Placental Villi LT nuc ish 66% T21 Blood karyotype 46,XX,i(21)(q10)dn[7]	
2. MPSS	Not recorded	10+6	0.63 1.06 (repeat)	7% (platform specific)[e]	NA	Normal US (19+2)	Live birth, Down syndrome Blood karyotype 46,XX,+21,der(21;21)(q10;q10)dn[15]	21q rearrangement (presumed iso21q)[b]; placenta not available for study
3. MPSS	cFTS 1/300-1/700 for T21	12+3	0.83	3%[d] (platform specific)[e]	NA	Normal US (19+4)	Live birth, Down syndrome QF-PCR T21[a] Blood karyotype 46,XX,i(21)(q10)dn[10] Blood CMA arr(21)x3	Confirmed iso21q; placenta not available for study
4. tMPS	US Markers	23	NA	17.2% (platform specific)[f]	NA	Short nasal bone, short femur, borderline umbilical cord Doppler values	Live birth, Down syndrome Blood karyotype 46,XX,+21,der(21;21)(q10;q10)dn[20]	21q rearrangement (presumed iso21q)[b]; placenta not available for study
5. tMPS	US Markers	13	NA	12.7% (platform specific)[f]	NA	Increased nuchal translucency, transitory tricuspid regurgitation	Live birth, Down syndrome Blood karyotype 46,XX,+21,der(21;21)(q10;q10)dn[20]	21q rearrangement (presumed iso21q)[b]; placenta not available for study
Chromosome 21q;21q rearrangements identified by cell-free DNA prenatal screening (True positives)								
6. MPSS	Primary screen	12	7.44	5.8% (relative Y count)[g]	4.8%	Normal US Amniocentesis (17) uAF nuc ish 24% T21 uAF SNP CMA 20% T21[c]	Live birth, mos T21 (clinically normal) Blood smear nuc ish 7% T21 Blood karyotype 46,XY,i(21)(q10)dn[3]/46,XY [82]	Confirmed iso21q; mosaicism in newborn; placenta not available for study
7. MPSS	Primary screen	10+3	4.64	7.8% (SeqFF) [9]	3%		TOP	

Table 1 (continued)

Case No. and cfDNA screening technology	Indication for NIPT	GA (wk+d)	z-score or NCV score for c'some 21	FF (method)	Trisomic fraction	PND findings (wk+d)	Postnatal results	Conclusion
						Normal US CVS (11) uCVS nuc ish 44% T21 LT CVS Karyotype 46,XX,+21,der(21;21) (q10;q10) [60]		21q rearrangement (presumed iso21q)[b]; placental mosaicism confirmed and reflected in low trisomic fraction as compared to FF
8. MPSS	cFTS 1/65 for T21	14+2	8.25	9.9% (relative Y count)[g]	5.30%	Declined PND	Live birth, Down syndrome Blood karyotype 46,XY, +21,der(21;21)(q10;q10) dn[60]	21q rearrangement (presumed iso21q)[b]; placenta not available for study, low trisomic fraction as compared to FF reflects placental mosaicism

AF Amniotic Fluid, cFTS combined First Trimester Screening, CVS Chorionic Villus Sampling, dn de novo, FF Fetal Fraction, GA gestational age, iso21q isochromosome 21q; LT Long Term Culture (mesenchyme); mos mosaic, MPSS Massive Parallel Shotgun Sequencing, NA not available, nuc ish interphase in situ hybridization, PND Prenatal Diagnosis, SNP CMA Single Nucleotide Polymorphism Chromosome Microarray, ST Short Term Culture (cytotrophoblast chorionic villi), T21 Trisomy 21, TOP Termination of Pregnancy, tMPS targeted Massive Parallel Sequencing, uAF uncultured Amniotic Fluid, uCVS uncultured whole Chorionic Villi, US ultrasound, wk+d weeks+days

[a] biallelic trisomy pattern for all informative markers consistent with isochromosome 21q

[b] molecular studies not performed; 21q;21q rearrangement could be an isochromosome or a Robertsonian translocation

[c] SNP CMA genotyping profile consistent with mosaicism for isochromosome 21q

[d] FF is low but within acceptable limits for platform

[e] FF estimation performed from sequence read counts according to the elastic net approach [9, 11]

[f] FF estimation as described according to DANSR/FORTE methodology [2]

[g] FF estimation based on linear regression relationship between NCV_X and NCV_Y in male pregnancies [7]

Table 2 Reports of false-negative cfDNA screening results for Down syndrome where karyotype is recorded

First author and Year of Publication	Cases (n)[a]	cfDNA screening technology	False negative T21 (n=29)			True positive	
			Standard T21 (n)	iso21q T21 (n)	Mosaic T21 (n)	T21[b] (n=2092)	FNR : FN/ (TP+FN)
Futch et al. [31]	5974	MPSS	2	0	0	52	3.7%
Wang et al. [18]	NA	MPSS	0	1	1	NA	NA
Stumm et al. [32]	472	MPSS	1	0	1	40	4.8%
Zhang et al. [5]	146,958	MPSS	5	1	0	720	0.8%
Willems et al. [15]	9928	tMPS	1	1	0	121	1.6%
Taneja et al. [4]	85,298	MPSS	5	0	1	522	1.1%
This study							
TRIDENT study [12]	1386	MPSS	0	1	0	29	3.3%
Aarhus University Hospital, Aarhus	810	MPSS	0	1	0	5	16.6%
Genea, Sydney	23,550	MPSS	1	1	0	185	1.1%
Fleury Medicina & Saude, Sao Paulo	951	tMPS	0	2	0	30	6.3%
Fleury Medicina & Saude, Sao Paulo	1551	MPSS	0	0	0	34	0.0%
VCGS, Melbourne	30,256	MPSS	0	0	0	211	0.0%
VCGS, Melbourne cytogenetic referrals	NA	tMA/tSNP	3[c]	0	0	143	2.1%
Total No.			18	8	3	2092	

iso21q isochromosome 21q, *FN* False Negative, *FNR* False negative rate, (*n*) No. cases, *NA* not available, *MPSS* massive parallel shotgun sequencing, *tMPS* targeted massive parallel sequencing, *tMA* targeted microarray, *tSNP* targeted SNP-based, *T21* trisomy 21, *TP* True Positive, *VCGS* Victorian Clinical Genetics Services

[a]outcome data may be incomplete in some study cohorts

[b]includes all chromosomal forms of trisomy 21

[c]cfDNA testing done by external commercial providers (1x tMA, 2x tSNP false negative)

arms, and arise de novo from a post-fertilization event involving centromere misdivision or U-type exchange between sister chromatids [21, 22]. Depending on the timing of this event, mosaicism for the initial euploid cell line may persist in the placenta. Chorionic villus sampling (CVS) literature describes FN cases of trisomy 21 associated with de novo isochromosome 21q. The chromosome aberration is present in the fetus and usually the mesenchymal core of the chorionic villi (long-term CVS culture), but is absent from the villi's outer cytotrophoblast layer (short-term CVS culture) [23–26]. Placental cytotrophoblast cells are more distantly related to the embryo than are cells from the mesenchymal core, which derive from the inner cell mass, along with the embryo [27]. If de novo isochromosome 21q formation occurs in inner cell mass precursors, then the mesenchymal core and fetus would have trisomy 21, while the cytotrophoblast cells would remain predominantly euploid. As the cytotrophoblast is the primary source of the "fetal" cfDNA obtained from maternal plasma [28], isochromosome 21q might also be seen among FN cfDNA screening results, regardless of the cfDNA technology used.

Our data show that 8 of 29 (28%) FN results with karyotype information involved 21q;21q rearrangements; a 14-fold increase over the 2% of cases reported in live born children with Down syndrome. In 21q;21q rearrangement cases, placental mosaicism can lead to a cfDNA "trisomic" fraction that is reduced or absent relative to the FF (Fig. 1). The likelihood of a FN result will be influenced by variables such as the proportion of abnormal cells in the placental cytotrophoblast, the FF, and the assay's limit of detection. Furthermore, a low trisomic fraction relative to FF may be seen in cases of true fetal mosaicism for trisomy 21 and in cases of CPM with normal fetal karyotype (Fig. 1). Only an invasive diagnostic test can distinguish these mosaic outcomes, with amniocentesis providing the most definitive prenatal result. It should be noted that FFs estimated by different methods are not directly comparable [29] and each cfDNA-based screening assay will need to establish its own method for assessing possible mosaicism.

In case 1, repeated cfDNA testing showed an increase in chromosome 21 z-scores as the FF increased with gestational age and placental mass. The pregnancy was eventually called at high risk at 32 weeks of gestation; the FF
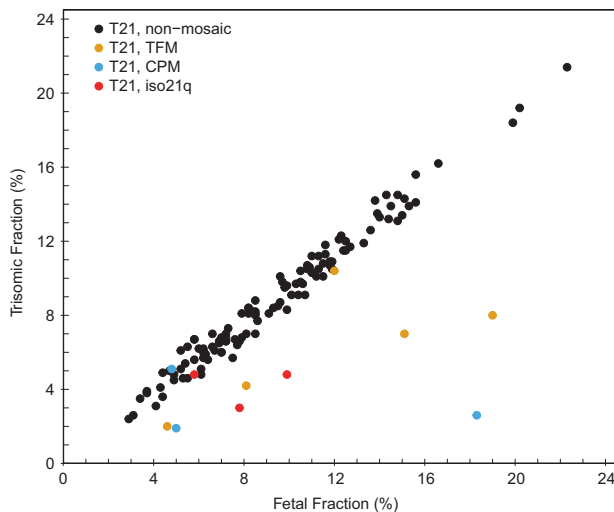
Fig. 1 Singleton pregnancies ($n = 132$) at increased risk for trisomy 21 after cfDNA screening and their cytogenetic outcomes. For each sample, the *x*-axis indicates the fetal fraction (FF) and the *y*-axis shows the trisomic fraction. Non-mosaic true positive samples with trisomy 21 (black circles) lie close to the unity line ($y = x$). Samples that plot below the unity line have a trisomic fraction that is substantially lower than the FF. These samples were associated with true fetal mosaicism (TFM) for trisomy 21 (yellow circles) or mosaicism confined to the placenta (CPM) (blue circles). Trisomy 21 samples with TFM and CPM may occasionally plot on the unity line. Three true positive trisomy 21 samples with 21q;21q rearrangements are represented by red circles. Two plot well below the unity line, indicating placental mosaicism, but were associated with non-mosaic trisomy 21 in the fetus (cases 7 and 8 in main text). The third case plots on the unity line and was associated with TFM for trisomy 21 after amniocentesis, with a low proportion of trisomic cells (3/85) confirmed in the newborn (case 6 in main text)

was 14.3% (Table 1). Placental mosaicism was proven after birth with disomic cells being confined to the cytotrophoblast. For TP cases 7 and 8, the trisomic fraction was calculated [7, 11] and was substantially lower than the estimated FF, consistent with placental mosaicism involving cytotrophoblast (Table 1 and Fig. 1). The mosaicism was confirmed in case 7 but placental tissue was not available in case 8.

In case 1, ultrasound anomalies prompted amniocentesis and the diagnosis was made prenatally. In the 4 other cases either ultrasound was normal or "soft markers" or an enlarged nuchal translucency were observed and no invasive testing was done, consistent with current guidelines [30], and parental choice.

A limitation of our study design is the selection bias in favor of centers confronted with FN cases of Down syndrome involving 21q;21q rearrangements. We therefore sought to confirm the data by reviewing published cases of FN cfDNA screening results. Although only about half of the studies reporting FN results specified the trisomy 21 karyotype, our findings were confirmed, with 3 out of 20 (15%) FN results recording 21q;21q rearrangements. We

acknowledge that reports of FN cases in the literature might also be influenced by publication bias. Thus, our total calculated prevalence of 28% for FN 21q;21q rearrangements is likely to be an upper estimate.

We conclude that Down syndrome due to de novo isochromosome 21q is more likely to record a FN result than standard trisomy 21 during cfDNA screening. This can be explained by placental mosaicism. For centers reporting cfDNA screening outcome data we recommend recording the karyotype of all discrepant results. For clinical practice, a low trisomic fraction relative to the FF may suggest placental mosaicism. Identification and interpretation of potential mosaicism helps guide further patient counseling and management, such as recommending amniocentesis rather than CVS for follow-up studies. Care should be taken as these cases may not reflect confined placental mosaicism, but rather full trisomy in the presence of a placenta containing normal cells. Finally, the results of our study will help clinicians and patients understand that the biology of cfDNA screening is complex. False-negative results can occur through mechanisms other than poor quality, technical error or negligence.

Compliance with ethical standards

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