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Fetoplacental mosaicism: potential implications for false-positive and false-negative noninvasive prenatal screening results

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Purpose: Noninvasive prenatal screening for fetal aneuploidy analyzes cell-free fetal DNA circulating in the maternal plasma. Because cell-free fetal DNA is mainly of placental trophoblast origin, false-positive and false-negative findings may result from placental mosaicism. The aim of this study was to calculate the potential contribution of placental mosaicism in discordant results of noninvasive prenatal screening.

Methods: We performed a retrospective audit of 52,673 chorionic villus samples in which cytogenetic analysis of the cytotrophoblast (direct) and villus mesenchyme (culture) was performed, which was followed by confirmatory amniocentesis in chorionic villi mosaic cases. Using cases in which cytogenetic discordance between cytotrophoblast and amniotic fluid samples was identified, we calculated the potential contribution of cell line–specific mosaicism to false-positive and false-negative results of noninvasive prenatal screening.

Results: The false-positive rate, secondary to the presence of abnormal cell line with common trisomies in cytotrophoblast and normal amniotic fluid, ranged from 1/1,065 to 1/3,931 at 10% and 100% mosaicism, respectively; the false-negative rate was calculated from cases of true fetal mosaicism, in which a mosaic cell line was absent in cytotrophoblast and present in the fetus; this occurred in 1/107 cases.

Conclusion: Despite exciting advances, underlying biologic mechanisms will never allow 100% sensitivity or specificity.

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Key Words: cell-free placental DNA; false-negative rate; false-positive rate; fetoplacental mosaicism; noninvasive prenatal screening

INTRODUCTION

Although the utility of analyzing cell-free DNA (cfDNA) in maternal circulation as a means of ascertaining important information about a pregnancy was recognized almost 2 decades ago,[1] it is only recently that noninvasive prenatal screening (NIPS) for fetal aneuploidy using cffDNA has been available commercially.[2,3] Although NIPS is described as a "fetal" screening test and authors refer to "cell-free fetal DNA" (cffDNA) in the context of the test,[4,5] this is a misnomer because the DNA actually originates from apoptosis of placental cytotrophoblast and syncytiotrophoblast cells.[6–8]

Studies to date in which NIPS was performed demonstrate high sensitivity and specificity for detection of the common aneuploidies (trisomies 13, 18, and 21) with a rapid rise in the uptake of NIPS worldwide. However, concerns regarding the paucity of scientific data, performance characteristics, and consequent clinical utility are now being raised[9] and, as with all screening tests, false-positive (FP) and false-negative (FN) results occur.[10–13] There are various explanations for FP and FN results, with fetoplacental mosaicism, in which the cytotrophoblast but not the fetus (FP)—and vice versa (FN)—contains the aneuploid cell line, being a primary potential mechanism.

Determining the frequency of confined placental mosaicism (CPM) and true fetal mosaicism (TFM) requires that, in the analysis of villus samples, karyotyping of both the cytotrophoblast and the mesenchyme is performed together with the confirmatory analysis on amniotic fluid (AF) in case of mosaicism detected in chorionic villi. Although cytogenetic analysis of the cytotrophoblast layer from which NIPS DNA is derived is no longer conducted in many laboratories, we continue to assess both placental tissues (cytotrophoblast and mesenchyme) on all chorionic villus sampling (CVS) studies. Using our extensive database, our aim was to calculate the potential contribution of fetoplacental mosaicism to NIPS FP and FN results.

MATERIALS AND METHODS

Study conduct and laboratory procedures

After approval of the TOMA Laboratory Institutional Review Board, a retrospective cytogenetic audit of 52,673 consecutive prenatal diagnoses of CVS analyzed by the TOMA laboratory in a 13-year period (May 2000–May 2013) was performed.

Cases underwent similar procedures using consistent evaluation criteria and procedures in agreement with the Italian and

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European guidelines, as previously reported.[14,15] In the direct preparation technique, metaphases were obtained "directly" from spontaneously dividing in vivo cytotrophoblasts using the protocol originally developed by Simoni et al. in 1983.[16] Mesenchymal core analysis was performed on cultured cells, as previously described.[17] A total of at least 16 chorionic villi metaphases were routinely counted and paired. When an abnormal direct preparation was identified along with a normal female mesenchyme culture, a total of 40 metaphases from two cultures were counted to minimize the risk of maternal cell contamination.

Mosaicism was defined as a normal cell line plus either (i) the presence of at least two cells with the same trisomy or structural rearrangement or (ii) three cells showing the same monosomy in at least one placental tissue (cytotrophoblast and/or mesenchyme).[18] In mosaic CVS cases, a confirmatory amniocentesis was always recommended. Karyotyping from AF was performed using the in situ technique, and we analyzed 20 metaphases from a minimum of 10 colonies taken from at least two in situ cultures. TFM was defined as the presence of at least two colonies derived from two different cultures showing the same abnormality in AF as observed in CVS. A nonmosaic fetoplacental discrepancy was the homogeneous presence of an abnormal cell line in only one of the analyzed tissues.[12] Mosaicism was classified according to the distribution of the abnormal cell line and is described in **Table 1**.[18]

Data analysis

To calculate the potential contribution of mosaicism to FP and FN NIPS, we evaluated mosaics in which the cytotrophoblast was cytogenetically discrepant from the fetus. Specifically, we evaluated CPM type 1, in which the chromosomal abnormality is only in the cytotrophoblast and not in the mesenchyme and AF; CPM type 3, in which the chromosomal abnormality is found both in the cytotrophoblast and the mesenchymal core and not in AF; and TFM type 5, in which aneuploid cells are absent in the cytotrophoblast but present in the villus mesenchyme and the fetus. CPM types 1 and 3 would contribute to FP NIPS results and TFM type 5 to FN results.

We selected only mosaics involving chromosome imbalances targeted or potentially identifiable by NIPS: trisomies 13, 18, and 21, monosomy X (MX), 47,+i(13q), 47,+i(21q), 47,XXX/XXY/XYY, 46,X,del(Xq), 46,X,i(Xq), mosaic 45,X/47,XXX and 47,+i(18p). In this calculation, 47,+i(13q) and 47,+i(21q) were counted as T13 and T21, respectively (**Table 2**).

The potential contribution to the FP rate for each chromosome abnormality was calculated by dividing the number of CPM 1 and 3 cases by the number of normal cases: number of FP(CPM1 + CPM3)/true negative cases + FP(CPM1 + CPM3). The contribution to the FN rate was calculated by dividing the number of TFM type 5 by the number of all abnormal cases: number of FN(TFM5)/true positive cases + FN(TFM5). Data are further presented stratified by the percentage of abnormal cells in the cytotrophoblast to illustrate the effect and the role of percentage mosaicism on FP rates. The total FN rate is calculated as 1 minus sensitivity and the FP rate as 1 minus specificity.

RESULTS

Of the 52,673 cases, 308 had CPM type 1, 90 had CPM type 3, and 51 had TFM type 5; among these, 45 of CPM1 (14.6%), 13 of CPM3 (14.4%), and 25 of TFM5 (29.4%) cases involved the common trisomies 13, 18, and 21.

FP rate

The potential contribution to the FP rate from CPM type 1 or 3 with trisomic cell lines quantified between ≥10% through 100% is reported in **Table 2**. Even using a conservative assumption that a high percentage of mosaicism ≥70% abnormal cells would be required to produce sufficient fetal fraction to generate a "positive" by NIPS, the FP rate for the common trisomies (13, 18, and 21) would be 0.033% or 1 in 3,006 cases reported as normal (95% confidence interval (CI): 1/1,877–1/4,813); for common trisomies and monosomy X, the FP rate would be 0.08%, which is equivalent to 1 in 1,243 cases reported as normal (95% CI: 1/917–1/1,687); and for all targeted or potentially identifiable chromosome abnormalities, the FP rate would be 0.091%, which is equivalent to 1 in 1,105 cases reported as normal (95% CI: 1/829–1/1,474).

FN rate

FN cases involving T13, 18, and 21 are projected to occur in 1/107 karyotypes reported as abnormal (95% CI: 1/65–1/176); for T13, 18, and 21 and MX, it will occur in 1/68 abnormal karyotypes (95% CI: 1/46–1/99); for all targeted or potentially identifiable chromosome abnormalities (including structural anomalies

Table 1 Schematic representation of the types of mosaicism and the expected NIPS results

Type of mosaic	Cytotrophoblast (direct preparation or short-term culture)	Mesenchyme (long-term culture)	Amniocytes	Expected NIPS result
CPM1	Abnormal	Normal	Normal	FP
CPM2	Normal	Abnormal	Normal	TN
CPM3	Abnormal	Abnormal	Normal	FP
TFM4	Abnormal	Normal	Abnormal	TP*
TFM5	Normal	Abnormal	Abnormal	FN
TFM6	Abnormal	Abnormal	Abnormal	TP*

CPM, confined placental mosaicism; FN, false negative; FP, false positive; NIPS, noninvasive prenatal screening; TFM, true fetal mosaicism; TN, true negative; TP*, true positive assuming a consistent percentage of the mosaic abnormal cell line in cytotrophoblast.

Table 2 Prediction of contribution of FP and FN rates to imbalances of chromosomes 13, 18, 21, X, and Y due to CPM1, CPM3, or TFM 5

		Type of imbalance												
Mosaic type	**Abnormal cell (%)**	**T13[a]**	**T18**	**T21[a]**	**MX**	**47,XXY/ XYY/XXX**	**Other[b]**	**T13[a]**	**T18**	**T21[a]**	**MX[c]**	**T13[a], 18, 21[a]**	**T13[a], 18, 21[a], MX**	**T13[a], 18, 21[a], MX, etc.[d]**
		Potential for FN (*n*)						FN rate[e] 1/X (95% CI)						
TFM5	0	1	6	8	11	4	1	136 (25–770)	64 (30–139)	135 (69–266)	14 (8–26)	107 (65–176)	68 (46–99)	61 (43–87)
		Potential for FP (*n*)						FP rate[f] 1/X (95% CI)						
CPM1	≥10	19	9	8	38	12	2	2,022	4,359	5,161	1,073	1,065	526	443
CPM3		7	3	2	11	1	0	(1,380–2,962)	(2,494–7,620)	(2,804–9,501)	(812–1,418)	(804–1,412)	(431–642)	(369–531)
CPM1	≥20	12	7	6	34	8	3	2,920	5,231	6,451	1,223	1,420	646	547
CPM3		6	3	2	9	1	0	(1,847–4,615)	(2,842–9,629)	(3,269–12,730)	(908–1,646)	(1,026–1,966)	(518–805)	(447–670)
CPM1	≥30	9	4	5	29	6	3	3,754	7,472	7,372	1,421	1,826	785	678
CPM3		5	3	2	8	1	0	(2,236–6,301)	(3,620–15,425)	(3,572–15,219)	(1,031–1,958)	(1,263–2,638)	(616–1,000)	(541–850)
CPM1	≥40	8	3	4	25	4	3	4,042	8,717	8,601	1,642	2,045	895	795
CPM3		5	3	2	7	0	0	(2,363–6,917)	(3,996–19,020)	(3,942–18,766)	(1,164–2,318)	(1,385–3,018)	(691–1,159)	(623–1,015)
CPM1	≥50	8	2	4	22	4	3	4,042	13,075	8,601	1,812	2,222	981	862
CPM3		5	2	2	7	0	0	(2,363–6,917)	(5,085–33,622)	(3,942–18,766)	(1,262–2,602)	(1,481–3,335)	(748–1,286)	(669–1,112)
CPM1	≥60	5	2	2	21	4	3	5,255	13,075	12,901	2,102	2,839	1,186	1,017
CPM3		5	2	2	4	0	0	(2,855–9,674)	(5,085–33,622)	(5,017–33,174)	(1,424–3,103)	(1,796–4,488)	(880–1,597)	(772–1,341)
CPM1	≥70	6	1	2	20	2	3	5,255	17,433	12,901	2,190	3,006	1,243	1,105
CPM3		4	2	2	4	0	0	(2,855–9,674)	(5,929–51,260)	(5,017–33,174)	(1,472–3,258)	(1,877–4,815)	(917–1,687)	(829–1,474)
CPM1	≥80	5	1	2	17	1	3	5,839	17,433	12,901	2,502	3,194	1,378	1,240
CPM3		4	2	2	4	0	0	(3,072–11,097)	(5,929–51,260)	(5,017–33,174)	(1,637–3,825)	(1,966–5,189)	(1,000–1,899)	(914–1,682)
CPM1	≥90	4	1	2	13	1	3	6,568	17,433	12,901	3,503	3,407	1,699	1,495
CPM3		4	2	2	2	0	0	(3,329–12,962)	(5,929–51,260)	(5,017–33,174)	(2,123–5,779)	(2,065–5,621)	(1,190–2,425)	(1,070–2,089)
CPM1	=100	3	0	2	11	1	3	7,506	26,150	12,901	4,041	3,931	1,960	1,695
CPM3		4	2	2	2	0	0	(3,637–15,496)	(7,172–95,353)	(5,017–33,174)	(2,362–6,915)	(2,298–6,726)	(1,338–2,872)	(1,187–2,419)
TN		52,538	52,297	51,600	52,525	52,540	52,670					51,089	50,941	50,805
TP		135	376	1,073	148	133	3					1,584	1,732	1,868
Total CVS		52,673												

False-positive rate prediction is stratified by frequency of abnormal cell lines: from ≥10% to 100% of the cells assessed. Results are provided for individual trisomies and in combination for the aneuploidies currently available for detection using NIPS.

CI, confidence interval; CPM1 and 3, confined placental mosaicism types 1 and 3; CVS, chorionic villus sampling; FN, false negative; FP, false positive; NIPS, noninvasive prenatal screening; TFM 5, true fetal mosaicism type 5; TN, true negative (total number of CVS true positives; TP, true positive).

[a]Including +i(13q) or +i(21q). [b]Including 46,delXq, 45,X/47,XXX, 46,X,i(Xq), 47,+i(18p). [c]Monosomy X. [d]Including all imbalances of chr13, 18, 21, X, and Y targeted or potentially detectable by NIPS. [e]Number of FN/number of abnormal cases (FN + TP). [f]FP/number of normal cases (FP + TN).

involving these chromosomes), it will occur in 1/61 abnormal karyotypes (95% CI: 1/43–1/87). The rate of FN, stratified by type of aneuploidy, is as follows: for T13, 1/136 (95% CI: 1/25–1/770); for T18, 1/64 (95% CI: 1/30–1/139); for T21, 1/135 (95% CI: 1/69–1/266); and for MX, 1/14 (95% CI: 1/8–1/26; **Table 2**).

Conclusion

NIPS has been a long-awaited addition to the tools available for the care of pregnant women. Although the sensitivity and the specificity of NIPS have been reported to approach 99%, several issues, such as performance characteristics of the different assays, remain, which are detailed in the study by Morain et al.[9] Even the lay press[19] has raised flags that FP and FN are expected in "screening" tests, but with marketing to both patients and doctors that stresses high sensitivity and specificity, there is confusion, and pitfalls abound with respect to informed consent. Because this screening test is being used on a more frequent basis in a larger population, providers must be able to inform patients regarding its limitations, including the risk of a FP or FN screen result.

CPM is a well-known biologic phenomenon that is likely to result from mitotic or meiotic nondisjunction errors and trisomy rescue. Recently published case reports have suggested this to be a mechanism underlying some false/discordant NIPS results.[12,13] Our analysis, based on a large cytogenetic data set that incorporates both the cytotrophoblast and the mesenchymal core, allows the prediction of the potential contribution of CPM and TFM to the rates of FP and FN NIPS results, respectively. However, it is important to note that verification of these predicted results would require prospective data to be obtained.

Patients undergoing CVS for cytogenetic analysis should be counseled that the tissue being sampled is not fetal and that mosaic conditions can occur. We propose that the term cell-free fetal DNA, "cffDNA," is more appropriate for sequences derived from AF.[20] Because NIPS is primarily a "placental" assay, this term should be changed to cell-free placental DNA, "cfpDNA." As a result, patients will be made aware that there is a small chance of a discrepancy that may require further confirmation through amniocentesis. Of note, the test is indeed accurate for the targeted abnormalities, because, in the vast majority of cases, the cytotrophoblast accurately reflects the fetal chromosomal status. However, comparable with CVS, a "positive" NIPS test may reflect only the cytotrophoblast and not the fetal karyotype and therefore must always be confirmed by invasive testing. Similarly, physicians must include in NIPS counseling that the outer placental cellular layer may be normal, but the fetus may still be affected and, therefore, a negative NIPS may be relatively reassuring due to a generally high negative predictive value; however, NIPS cannot completely rule out an abnormal fetal karyotype, even for the limited disorders being tested.

Discrepancies between NIPS and fetal results may fail to be explained by extensive cytogenetic analysis of term placenta. Syncytial sprouts, present from the early stage of pregnancy, are considered to be a morphological manifestation of villus growth and represent the first step in the development of lateral villi.[21] The number and size of these sprouts increase steadily during the first 3 months of pregnancy, paralleling the increase in serum level of gonadotropins.[22] At the end of 20 weeks of gestation, the cytotrophoblast completely disappears,[23] and this might explain such unexplained discrepancies.

The cytotrophoblast is the mitotically active cell of the villi, and cytogenetic results are obtained without in vitro culture, analyzing only spontaneous metaphases. Thus, the cytogenetic results used in this study reflect the actual constitution of the outer placental cellular layer. Although FP results are dependent on the percentage of mosaicism, the fetal fraction of the maternal plasma, and the type of NIPS (single-nucleotide-poymorphism-based or counting-based) technology, the estimated FN rate due to TFM type 5 can be considered a reliable estimation because it is independent of the technical and physiological variants. In the future, deeper sequencing might enable the identification of lower-level mosaics, and our data provide a projection of the increase in the FP rate attributed to CPM as the sensitivity of NIPS technologies increases.

There are other reported reasons for discrepancies between fetal karyotype and NIPS results, including a vanishing twin or a co-twin's demise,[24] nonmosaic maternal chromosome abnormality,[25] and maternal metastatic disease.[26] Particularly in the case of FN, low fetal fraction[27] may play a key role. This study quantifies the role of CPM/TFM and does not address these other potential etiologies.

Published studies report variable FP rates, which are summarized as 1/384 for T21 and 1/500 for T18.[28] FN rates (one minus the detection rate) were summarized as 1/200 for T21 and 1/63 for T18.[28] Our results support the hypothesis that CPM types 1 and 3 do contribute to the FP rate of NIPS and that TFM type 5 may be a major contributor to the suboptimal sensitivity.

Although our study indicates that FP and FN results may very well be the result of placental biology and not a failure in the actual test platform, it also demonstrates that although we can expect further technological and bioinformatic breakthroughs in the future, underlying placental–fetal genetic mechanisms will never allow 100% sensitivity.

DISCLOSURE

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