DOI 10.1515/cclm-2014-0034 — Clin Chem Lab Med 2014; 52(5): 585–588

**Editorial**

Stephen Siu-Chung Chim

# Potential application of fetal epigenetic markers on the non-invasive prenatal detection of chromosomal abnormality

Identification of fetal chromosomal aneuploidy is a predominant reason for pregnant women to undergo prenatal testing, most of which are invasive and carry a risk for fetal loss. The presence of fetal DNA in maternal circulation has offered an opportunity for non-invasive prenatal detection [1]. However, this fetal DNA exists only as a minor fraction among the co-existing background of maternal DNA [2, 3]. Hence, the use of fetal DNA in maternal plasma to detect fetal aneuploidy is technically challenging. With the advent of massively parallel genomic sequencing (MPGS) to shotgun (non-specifically) sequence all the fetal and maternal DNA molecules, non-invasive prenatal detection of fetal trisomy 21 could now be achieved at high sensitivity and specificity [4–6].

However, using MPGS for non-invasive prenatal detection of fetal aneuploidy requires a turn-around-time of 7–10 days [7], the use of expensive equipment and reagents and the use of relatively complex bioinformatics methods. Thus, investigators have been seeking alternatives for the non-invasive prenatal detection of aneuploidy. Most of these alternatives rely on the fetal-specific nucleic acid species or polymorphisms for analyzing the fetal DNA in maternal plasma. For instance, the existence of epigenetic (DNA methylation) signatures that are specific to the fetus, but not its mother, has facilitated the development of fetal epigenetic markers in maternal plasma for the non-invasive analysis of the fetal chromosome of interest. In this issue of *Clinical Chemistry and Laboratory Medicine*, Lim and colleagues have demonstrated that a fetal-specific DNA methylation pattern (fetal epigenetic marker) on chromosome 21 could potentially be used for such purpose [8].

The use of epigenetic marker to specifically identify the fetal DNA in maternal plasma was first demonstrated in 1999 [9]. However, that fetal epigenetic marker was polymorphism-dependent and could only be applied in certain fetal-maternal pairs. The first polymorphism-independent fetal epigenetic marker was the unmethylated form of the *serpin peptidase inhibitor, clade B (ovalbumin), member 5* (also known as *maspin*) gene (U-*SERPINB5* or U-*maspin*), as discovered by a candidate gene approach [10]. Since U-*SERPINB5* is located on chromosome 18, it has been further demonstrated for the first time that non-invasive detection of fetal trisomy 18 could be achieved using fetal epigenetic marker [11].

Seeing this promising demonstration, various investigators have launched screening efforts at high resolution and wide genome coverage to systematically identify more fetal epigenetic markers [12–17]. CpG islands (CGIs), which harbor a high density of CpG sites, often undergo DNA methylation. The first study to systematically investigate CGIs on chromosome 21 for fetal epigenetic markers at single-nucleotide resolution has covered 114 (76% of all the 149 CGIs defined by bioinformatics criteria) and involved the use of the Epityper platform, cloning and conventional Sanger sequencing techniques [12]. This study has provided the first empirical evidence that the fetal (placental) and the maternal (blood cell) genomes harbor a lot of DNA methylation differences. Since the placenta and maternal blood cells are the respective sources of fetal and maternal DNA in maternal plasma, these DNA methylation differences could be developed into fetal epigenetic markers. In that study, a panel of 22 (19% of 114 analyzed CGIs) fetal epigenetic markers have been identified, including the unmethylated form of the *phosphodiesterase 9A* gene (U-*PDE9A*), which has been developed by Lim and colleagues as a potential non-invasive prenatal test for trisomy 21 in 2011 [18].

Later, using more sophisticated screening techniques, namely combined bisulfite and restriction analysis (COBRA), investigators have screened beyond the CpG islands for fetal epigenetic markers on 51 regions on gene promoters located on chromosome 21 [14]. This study has discovered the methylated form of the *holocarboxylase synthetase* gene (M-*HLCS*) as a fetal epigenetic marker. To compare the relative chromosome dosage, the concentrations of this fetal **e**pi**g**enetic marker M-*HLCS* were normalized against those of a fetal **g**enetic marker on chromosome Y, *zinc finger protein, Y-linked* (*ZFY*). Hence,

this chromosome dosage approach was dubbed as the epigenetic-genetic (EGG) approach, and has been demonstrated to be useful for the non-invasive prenatal detection of trisomy 21 (Tables 1 and 2) [14].

In the current study by Lim and colleagues, the use of the fetal epigenetic markers for chromosome 21, M-*HLCS*, has already minimized the interference of the co-existing maternal chromosome 21 DNA sequences [8]. To compare the relative chromosome dosage, the authors normalized the concentrations of the fetal epigenetic marker M-*HLCS* by another fetal epigenetic marker on chromosome 3, namely the methylated form of the *Ras association (RalGDS/AF-6) domain family member 1* gene (M-*RASSF1A*). Using conventional quantitative polymerase chain reaction (qPCR) to measure M-*HLCS* and M-*RASSF1A*, the authors have already achieved a sensitivity of 90% and a specificity of 92.5% in the non-invasive prenatal detection of fetal trisomy 21. Despite this, there is room for improvement for Lim's approach.

To explore ways to improve the performance of using fetal epigenetic markers for non-invasive prenatal detection of trisomy 21, we have tabulated the salient features of other studies similar to this current one in Table 1. While each study has its own unique features and could not be directly compared with each other, it is noted that study using digital PCR seems to give better sensitivity and specificity, compared with the conventional quantitative polymerase chain reaction (qPCR). To perform a digital PCR experiment, one will dilute the template DNA from each sample to average concentrations below 1 molecule per well and analyze by PCR in literally hundreds or thousands of replicate PCR wells [19]. For each sample, some PCR wells will be positive, while others will be negative for the targeted marker. Since most positive wells contain just one template molecule, counting the positive wells will enable the absolute quantification of the original template DNA. Translating the exponential but analog nature of qPCR into a ‚1' or '0' signal in digital PCR [20], digital counting platforms should facilitate more precise and accurate quantification [21].

Besides the use of a digital PCR platform, we also note that those similar studies involved the use of fetal genetic markers, rather than fetal epigenetic markers, to quantify a reference chromosome for relative chromosome dosage analysis [14, 16, 22]. Normalizing against a fetal epigenetic marker, namely M-*RASSF1A* located on chromosome 3, Tong and colleagues have found that the relative dosage of chromosome 21 of more than half of the trisomy 21 placentas overlapped with the euploid (normal) reference interval, which was defined as the mean ratio of M-*HLCS* to M-*RASSF1A*±1.96 standard deviation (SD) of the euploid placentas as 0.86–1.63 (Table 2). In contrast, normalizing against a fetal genetic marker, namely *ZFY* located on chromosome Y, they have found that the relative dosage

**Table 1** Studies on non-invasive prenatal detection of trisomy 21 using fetal epigenetic markers.

| Publication | Pre-treatment | Quantification method | Fetal chr21 marker | Fetal reference chromosome marker (nature of marker) | No. of trisomy 21 fetuses tested positive/No. of trisomy 21 fetuses | No. of euploid fetuses tested negative/No. of euploid fetuses | Sensitivity | Specificity |
|---|---|---|---|---|---|---|---|---|
| Tong et al. [14] | MSRE | Digital PCR | M-*HLCS* | *ZFY* (genetic) | 5/5 | 23/24 | 100.0% | 95.8% |
| Lim et al. [8] | MBD | qPCR | M-*HLCS* | M-*RASSF1A* (epigenetic) | 9/10 | 37/40 | 90.0% | 92.5% |
| Lim et al. [18] | MBD | qPCR | U-*PDE9A* | M-*PDE9A*+U-*PDE9A* (epigenetic) | 15/18 | 85/90 | 83.3% | 94.4% |

chr21, chromosome 21; MBD, methyl-CpG binding domain-based enrichment; MSRE, methylation-sensitive restriction enzyme digestion; qPCR, quantitative polymerase chain reaction.

**Table 2** Data from Tong et al. [14] on the prenatal detection of trisomy 21 using fetal epigenetic or genetic markers as reference.

| Sample | Pre-treatment | Quantification method | Fetal chr21 marker | Fetal reference chromosome marker (nature of marker) | No. of trisomy 21 fetuses tested positive/No. of trisomy 21 fetuses | No. of euploid fetuses tested negative/No. of euploid fetuses | Sensitivity | Specificity |
|---|---|---|---|---|---|---|---|---|
| Placenta | MSRE | Digital PCR | M-*HLCS* | M-*RASSF1A* (epigenetic) | 3/12 | 10/10 | 25.0% | 100.0% |
| Placenta | MSRE | Digital PCR | M-*HLCS* | *ZFY* (genetic) | 12/12 | 10/10 | 100.0% | 100.0% |
| Maternal plasma | MSRE | Digital PCR | M-*HLCS* | *ZFY* (genetic) | 5/5 | 23/24 | 100.0% | 95.8% |

chr21, chromosome 21; MSRE, methylation-sensitive restriction enzyme digestion; qPCR, quantitative polymerase chain reaction.

of chromosome 21 of all trisomy 21 placentas were greater than the upper limit of the reference interval, which was calculated from the euploid placentas as 1.08–1.62 (Table 2). Therefore, by normalizing against a fetal genetic marker instead of fetal epigenetic marker, Tong and colleagues were able to improve the performance of the test. They reasoned that the M-*HLCS*/*ZFY* ratio has a smaller inter-individual variation that the M-*HLCS*/M-*RASSF1A* ratio because the genetic *ZFY* marker has less heterogeneity compared with the epigenetic M-*RASSF1A* marker.

The limitation of normalizing against a fetal genetic marker is that not all pregnancies could be covered. For instance, the M-*HLCS*/*ZFY* ratio is inapplicable to pregnancies with female fetuses, which contain no chromosome Y and hence no *ZFY*. To overcome this, one may increase the coverage by using a panel of paternally-inherited polymorphism markers located on autosomes as demonstrated in another publication [22]. Since these so-called autosomal genetic reference markers are commonplace in the genome, population coverage could be achieved readily.

In the current study, Lim and colleagues have demonstrated the potential use of fetal epigenetic marker for the non-invasive prenatal detection of trisomy 21 [8]. The use of digital PCR platform and normalization with a fetal genetic marker will further improve the performance of the test. Taken together with the panels of forthcoming fetal epigenetic markers systematically discovered from the increasingly comprehensive screening efforts [12–17], there is a good chance that they may serve as a supplement or an alternative to MPGS-based non-invasive prenatal detection of fetal chromosomal aneuploidy in the future.

### Conflict of interest statement

**Author's conflict of interest disclosure:** The author has held and filed patent applications on aspects of the use of nucleic acids in maternal blood for noninvasive prenatal testing, a proportion of which has been licensed to Sequenom, Inc.
**Research funding:** The author has published related work supported by a grant from the Research Grants Council of the Government of the Hong Kong Special Administrative Region, China (Project No. CUHK462909) and by the University Grants Committee of the Government of the Hong Kong Special Administrative Region, China, under the Areas of Excellence Scheme (AoE/M-04/06). The funders had no role in preparation of the manuscript.
**Employment or leadership:** None declared.
**Honorarium:** None declared.

## References

1. Lo YM, Corbetta N, Chamberlain PF, Rai V, Sargent IL, Redman CW, et al. Presence of fetal DNA in maternal plasma and serum. Lancet 1997;350:485–7.
2. Lun FM, Chiu RW, Chan KC, Leung TY, Lau TK, Lo YM. Microfluidics digital PCR reveals a higher than expected fraction of fetal DNA in maternal plasma. Clin Chem 2008;54:1664–72.
3. Lo YM, Tein MS, Lau TK, Haines CJ, Leung TN, Poon PM, et al. Quantitative analysis of fetal DNA in maternal plasma and serum: implications for noninvasive prenatal diagnosis. Am J Hum Genet 1998;62:768–75.
4. Chiu RW, Chan KC, Gao Y, Lau VY, Zheng W, Leung TY, et al. Noninvasive prenatal diagnosis of fetal chromosomal aneuploidy by massively parallel genomic sequencing of DNA in maternal plasma. Proc Natl Acad Sci USA 2008;105:20458–63.
5. Fan HC, Blumenfeld YJ, Chitkara U, Hudgins L, Quake SR. Noninvasive diagnosis of fetal aneuploidy by shotgun sequencing DNA from maternal blood. Proc Natl Acad Sci USA 2008;105:16266–71.
6. Chiu RW, Akolekar R, Zheng YW, Leung TY, Sun H, Chan KC, et al. Non-invasive prenatal assessment of trisomy 21 by multiplexed maternal plasma DNA sequencing: large scale validity study. Br Med J 2011;342:c7401.
7. Shea JL, Diamandis EP, Hoffman B, Lo YM, Canick J, van den Boom D. A new era in prenatal diagnosis: the use of cell-free fetal DNA in maternal circulation for detection of chromosomal aneuploidies. Clin Chem 2013;59:1151–9.
8. Lim JH, Lee DE, Kim KS, Kim HJ, Lee BY, Park SY, et al. Non-invasive detection of fetal trisomy 21 using fetal epigenetic biomarkers with a high CpG density. Clin Chem Lab Med 2014;52:641–7.
9. Poon LL, Leung TN, Lau TK, Chow KC, Lo YM. Differential DNA methylation between fetus and mother as a strategy for detecting fetal DNA in maternal plasma. Clin Chem 2002; 48:35–41.
10. Chim SS, Tong YK, Chiu RW, Lau TK, Leung TN, Chan LY, et al. Detection of the placental epigenetic signature of the maspin gene in maternal plasma. Proc Natl Acad Sci USA 2005;102:14753–8.
11. Tong YK, Ding C, Chiu RW, Gerovassili A, Chim SS, Leung TY, et al. Noninvasive prenatal detection of fetal trisomy 18 by epigenetic allelic ratio analysis in maternal plasma: theoretical and empirical considerations. Clin Chem 2006;52:2194–202.
12. Chim SS, Jin S, Lee TY, Lun FM, Lee WS, Chan LY, et al. Systematic search for placental DNA-methylation markers on chromosome 21: toward a maternal plasma-based epigenetic test for fetal trisomy 21. Clin Chem 2008;54:500–11.
13. Old RW, Crea F, Puszyk W, Hultén MA. Candidate epigenetic biomarkers for non-invasive prenatal diagnosis of Down syndrome. Reprod Biomed Online 2007;15:227–35.

14. Tong YK, Jin S, Chiu RW, Ding C, Chan KC, Leung TY, et al. Noninvasive prenatal detection of trisomy 21 by an epigenetic-genetic chromosome-dosage approach. Clin Chem 2009;56:90–8.
15. Papageorgiou EA, Fiegler H, Rakyan V, Beck S, Hulten M, Lamnissou K, et al. Sites of differential DNA methylation between placenta and peripheral blood: molecular markers for noninvasive prenatal diagnosis of aneuploidies. Am J Pathol 2009;174:1609–18.
16. Tsui DW, Lam YM, Lee WS, Leung TY, Lau TK, Lau ET, et al. Systematic identification of placental epigenetic signatures for the noninvasive prenatal detection of Edwards syndrome. PLoS One 2010;5:e15069.
17. Lun FM, Chiu RW, Sun K, Leung TY, Jiang P, Chan KC, et al. Noninvasive prenatal methylomic analysis by genomewide bisulfite sequencing of maternal plasma DNA. Clin Chem 2013;59:1583–94.
18. Lim JH, Kim SY, Park SY, Lee SY, Kim MJ, Han YJ, et al. Non-invasive epigenetic detection of fetal trisomy 21 in first trimester maternal plasma. PLoS One 2011;6:e27709.
19. Vogelstein B, Kinzler KW. Digital PCR. Proc Natl Acad Sci USA 1999;96:9236–41.
20. Pohl G, Shih Ie M. Principle and applications of digital PCR. Expert Rev Mol Diagn 2004;4:41–7.
21. Wang TL, Maierhofer C, Speicher MR, Lengauer C, Vogelstein B, Kinzler KW, et al. Digital karyotyping. Proc Natl Acad Sci USA 2002;99:16156–61.
22. Tong YK, Chiu RW, Akolekar R, Leung TY, Lau TK, Nicolaides KH, et al. Epigenetic-genetic chromosome dosage approach for fetal trisomy 21 detection using an autosomal genetic reference marker. PLoS One 2010;5:e15244.

***Corresponding author: Stephen Siu-Chung Chim,** PhD, Associate Professor, Department of Obstetrics and Gynaecology, Faculty of Medicine, The Chinese University of Hong Kong, 1/F Special Block EF, Prince of Wales Hospital, 30–32 Ngan Shing Street, Shatin, N.T., Hong Kong, P.R. China, Phone: +85 22 6321324, Fax: +85 22 6360008, E-mail: sschim@cuhk.edu.hk