Clinical Chemistry 56:1
90–98 (2010)

Noninvasive Prenatal Detection of Trisomy 21 by an Epigenetic–Genetic Chromosome-Dosage Approach

Yu K. Tong,[1,2] Shengnan Jin,[1,2†] Rossa W.K. Chiu,[1,2] Chunming Ding,[1,3†] K.C. Allen Chan,[1,2]
Tak Y. Leung,[4] Ling Yu,[3] Tze K. Lau,[4] and Y.M. Dennis Lo[1,2*]

BACKGROUND: The use of fetal DNA in maternal plasma for noninvasive prenatal diagnosis of trisomy 21 (T21) is an actively researched area. We propose a novel method of T21 detection that combines fetal-specific epigenetic and genetic markers.

METHODS: We used combined bisulfite restriction analysis to search for fetal DNA markers on chromosome 21 that were differentially methylated in the placenta and maternal blood cells and confirmed any target locus with bisulfite sequencing. We then used methylation-sensitive restriction endonuclease digestion followed by microfluidics digital PCR analysis to investigate the identified marker. Chromosome-dosage analysis was performed by comparing the dosage of this epigenetic marker with that of the *ZFY* (zinc finger protein, Y-linked) gene on chromosome Y.

RESULTS: The putative promoter of the *HLCS* (holocarboxylase synthetase) gene was hypermethylated in the placenta and hypomethylated in maternal blood cells. A chromosome-dosage comparison of the hypermethylated *HLCS* and *ZFY* loci could distinguish samples of T21 and euploid placental DNA. Twenty-four maternal plasma samples from euploid pregnancies and 5 maternal plasma samples from T21 pregnancies were analyzed. All but 1 of the euploid samples were correctly classified.

CONCLUSIONS: The epigenetic–genetic chromosome-dosage approach is a new method for noninvasive prenatal detection of T21. The epigenetic part of the analysis can be applied to all pregnancies. Because the genetic part of the analysis uses paternally inherited, fetal-specific genetic markers that are abundant in the genome, broad population coverage should be readily achievable. This approach has the potential to become a generally usable technique for noninvasive prenatal diagnosis.

© 2009 American Association for Clinical Chemistry

Screening for trisomy 21 (T21)[5] constitutes an important component of modern obstetrics care in many countries. Common screening methods routinely available in prenatal clinics target the epiphenomena associated with the chromosomal aneuploidy rather than directly targeting the actual chromosome dosage in the fetus *(1, 2)*. For a definitive test, conventional screening programs require invasive procedures such as chorionic villus sampling and amniocentesis; however, such methods can lead to procedure-related fetal loss *(3–5)*.

The discovery of cell-free fetal DNA in maternal plasma in 1997 has offered new possibilities for noninvasive prenatal diagnosis *(6, 7)*. Detection of the fetal-derived, paternally inherited genetic materials in maternal plasma samples has allowed the prenatal determination of fetal Rhesus D blood group *(8, 9)* and determination of fetal sex for sex-linked disorders *(10)*. The development of noninvasive prenatal diagnostic tests for fetal chromosomal aneuploidies, however, has been more challenging.

Two main groups of approaches have been developed for the direct detection of T21 from the analysis of nucleic acids in maternal plasma. The first group involves the analysis of allelic ratios of single nucleotide polymorphisms (SNPs) present in a fetal-specific nucleic acid marker *(7)*. Examples of this approach include the analysis of circulating placental mRNA (the RNA-SNP approach) *(11)* and DNA-methylation markers (the epigenetic allelic-ratio approach) *(12)*. The main disadvantage of the allelic ratio–based approach is that these methods are applicable only to fe-

[1] Centre for Research into Circulating Fetal Nucleic Acids, Li Ka Shing Institute of Health Sciences, [2] Department of Chemical Pathology, [3] Stanley Ho Centre for Emerging Infectious Diseases, and [4] Department of Obstetrics and Gynaecology, The Chinese University of Hong Kong, Prince of Wales Hospital, Shatin, New Territories, Hong Kong SAR, China.
[†] Current affiliation: Singapore Institute for Clinical Sciences, Agency for Science, Technology and Research, Singapore.
[*] Address correspondence to this author at: Department of Chemical Pathology, The Chinese University of Hong Kong, Rm. 38023, 1/F, Clinical Sciences Bldg., Prince of Wales Hospital, 30-32 Ngan Shing St., Shatin, Hong Kong SAR, China. Fax 852-2636-5090; e-mail loym@cuhk.edu.hk.
Received July 16, 2009; accepted September 22, 2009.
Previously published online at DOI: 10.1373/clinchem.2009.134114
[5] Nonstandard abbreviations: T21, trisomy 21; SNP, single-nucleotide polymorphism; EGG, epigenetic–genetic; MSRE, methylation-sensitive restriction endonuclease; COBRA, combined bisulfite restriction analysis; CGI, CpG island; IQR, interquartile range.

Downloaded from https://academic.oup.com/clinchem/article/56/1/90/5622561 by U.S. Department of Justice user on 16 August 2022

tuses that are heterozygous for the analyzed SNP. The second group of approaches uses single molecule–counting methods *(13)* such as digital PCR *(14)* and massively parallel genomic sequencing *(15, 16)*. In these approaches, individual plasma DNA molecules are counted. The precision of such methods allows the detection of the slight increase in chromosome 21–derived DNA molecules in the plasma of a pregnant woman carrying a T21 fetus. One disadvantage of the latter approaches is that counting of an extremely large number of molecules is required for markers with no fetal specificity (e.g., random sequences from chromosome 21), as is exemplified in recent experiments that used massively parallel genomic sequencing to analyze millions of molecules per case *(15, 16)*. This requirement is achievable with the use of expensive equipment and reagents and the use of relatively complex bioinformatics methods.

We propose a new approach for the noninvasive prenatal detection of T21 from maternal plasma that is based on measuring the ratio of the concentrations of a fetal-specific DNA-methylation marker on chromosome 21 and a fetal-specific DNA marker on a reference chromosome. We call this the epigenetic–genetic (EGG) chromosome-dosage approach. In contrast with the epigenetic allelic-ratio approach described above, the EGG approach does not require the fetal-specific DNA-methylation marker and the SNP to be present together within a short stretch of DNA; thus, population coverage is much easier to achieve. We also demonstrate that with a target locus on chromosome 21 and another on a reference chromosome, the EGG approach is more precise than an approach based purely on epigenetic markers.

For the EGG approach to work, however, it is crucial to have a good fetal-specific DNA-methylation marker on chromosome 21. The ideal marker would be hypermethylated in the fetus and hypomethylated in maternal blood cells so that a methylation-sensitive restriction endonuclease (MSRE) could be used to digest away the maternal sequences. Indeed, previous work has shown that the promoter of the *RASSF1A*[6] gene [Ras association (RalGDS/AF-6) domain family member 1A; GenBank accession no. NM_007182] is one such marker, except that it is on chromosome 3 *(17, 18)*. Despite reports of a number of fetal DNA-methylation markers on chromosome 21 *(19–21)*, a chromosome 21 marker that has characteristics comparable to *RASSF1A* has yet to be found. We thus began our study by looking for such a marker with combined bisulfite restriction analysis (COBRA) *(22)* targeting 35 gene promoter regions on chromosome 21. This effort led to the discovery of differential methylation of the putative promoter region of the *HLCS* gene [holocarboxylase synthetase (biotin-(proprionyl-Coenzyme A-carboxylase (ATP-hydrolysing)) ligase); GenBank accession no. NM_000411] as a new hypermethylated fetal-DNA marker. We then used the *HLCS* gene to implement the EGG concept.

[6] Human genes: *RASSF1A*, Ras association (RalGDS/AF-6) domain family member 1A; *HLCS*, holocarboxylase synthetase (biotin-(proprionyl-Coenzyme A-carboxylase (ATP-hydrolysing)) ligase); *ZFY*, zinc finger protein, Y-linked; *ZFX*, zinc finger protein, X-linked; *C21orf81*, chromosome 21 open reading frame 81.

Materials and Methods

STUDY PARTICIPANTS

Women with euploid and T21 pregnancies who visited the Department of Obstetrics and Gynaecology, Prince of Wales Hospital, Hong Kong, were recruited. Informed consent was obtained from the individuals who joined the study, and ethics approval was obtained from the Joint Chinese University of Hong Kong–New Territories East Cluster Clinical Research Ethical Committee. Details of sample collection and processing are described in the Data Supplement that accompanies the online version of this article at http://www.clinchem.org/content/vol56/issue1.

SCREENING OF DIFFERENTIALLY METHYLATED REGIONS ON CHROMOSOME 21

COBRA *(22)* was used to assess the methylation status of genomic sequences on chromosome 21 in DNA samples from placentas and maternal blood cells. The COBRA procedure is described in the online Data Supplement.

The PCR products of the *HLCS* locus produced in the COBRA analysis were used for cloning and bisulfite sequencing. The detailed protocol is presented in the online Data Supplement. The frequency of methylated sites was calculated by dividing the total number of methylated CpG sites by the total number of CpG sites in the cloned region *(18)*.

CONVENTIONAL REAL-TIME QUANTITATIVE PCR ANALYSIS OF THE *HLCS* LOCUS

We used the COBRA-screening and bisulfite sequencing results to develop an MSRE-digestion assay. We then used real-time quantitative PCR analysis to target *HLCS* locus region B2, which is hypermethylated in the placenta and unmethylated in maternal blood cells. The procedures are detailed in the online Data Supplement. The sequences for the primers and probe are listed in Table 1. Results for genomic DNA samples were expressed as a methylation index, which was calculated by dividing the *HLCS* copy number obtained after enzyme digestion by that obtained with mock digestion. We analyzed maternal plasma DNA to show

Downloaded from https://academic.oup.com/clinchem/article/56/1/90/5622561 by U.S. Department of Justice user on 16 August 2022

Table 1. Oligonucleotide sequences and specific PCR conditions for the real-time quantitative PCR and digital PCR assays.

Assay	Oligonucleotide	Sequences (5′ to 3′)	Specific thermal cycling conditions
HLCS (real-time quantitative PCR and digital PCR)	Forward primer	CCGTGTGGCCAGAGGTG	50 Cycles of 95 °C for 30 s and 60 °C for 1 min
	Reverse primer	AAAGGGCCAGGTCGGGA	
	Hydrolysis probe	FAM-AGGATTTGGGGCTGCGC (MGB)[a]	
RASSF1A (digital PCR)	Forward primer	AGCTGGCACCCGCTGG	50 Cycles of 95 °C for 30 s and 60 °C for 1 min
	Reverse primer	GTGTGGGGTTGCACGCG	
	Hydrolysis probe	VIC-ACCCGGCTGGAGCGT (MGB)[b]	
ZFX/ZFY (digital PCR)	Forward primer	CAAGTGCTGGACTCAGATGTAACTG	50 Cycles of 95 °C for 15 s and 57 °C for 1 min
	Reverse primer	TGAAGTAATGTCAGAAGCTAAAACATCA	
	Hydrolysis probe_*ZFY*	FAM-TCTTTACCACACTGCAC (MGB)	
	Hydrolysis probe_*ZFX*	VIC-TCTTTAGCACATTGCA (MGB)	

[a] FAM, 6-carboxyfluorescein; MGB, minor-groove binding.
[b] VIC, Applied Biosystems proprietary dye.

the postpartum clearance of the hypermethylated *HLCS* molecules.

CHROMOSOME-DOSAGE ANALYSIS BY MICROFLUIDICS DIGITAL PCR

We used PCR analysis on a microfluidics digital PCR platform to compare chromosome dosages for chromosome 21 and the reference markers *(23, 24)*. The loci for an epigenetic–epigenetic comparison were *HLCS* on chromosome 21 and *RASSF1A* on chromosome 3 *(18)*. Both are hypermethylated fetal DNA markers. The loci for an EGG comparison were *HLCS* on chromosome 21 and *ZFY* (zinc finger protein, Y-linked) on chromosome Y from pregnancies with a male fetus.

MSRE digestion. Restriction enzyme *Bst*UI (New England Biolabs) was used to digest hypomethylated DNA. Extracted DNA was digested with *Bst*UI at 60 °C for 16 h. For placental tissues and maternal blood cells, we used 40 U *Bst*UI to digest 200 ng DNA. A mock-digested aliquot without added enzyme was included. For plasma samples, we used 20–60 U *Bst*UI to digest the DNA from 3.4–9.1 mL of plasma.

Microfluidics digital PCR analysis. Microfluidics digital PCR assays were designed for the *HLCS*, *RASSF1A*, and *ZFY* loci (see Fig. S1 in the online Data Supplement), which represent the dosages of chromosome 21, chromosome 3, and chromosome Y, respectively. The *ZFX* (zinc finger protein, X-linked) and *ZFY* assays and the basis of the digital PCR analysis have been described previously *(25)*. The sequences of primers and probes are listed in Table 1. The digital PCR experiments were carried out on the BioMark System (Fluidigm) with the 12.765 Digital Arrays (Fluidigm). Each array consisted of 12 panels, each of which was further partitioned into 765 reaction wells. With each panel, the reaction was set up as a 10-μL mixture at the following final concentrations: 1× TaqMan Universal PCR Master Mix (Applied Biosystems), 125 nmol/L hydrolysis probe (Applied Biosystems) (Table 1), and 900 nmol/L of the forward and reverse primers (Integrated DNA Technologies) (Table 1). The reaction mixture was diluted with the assay loading buffer and sample loading buffer according to the manufacturer's protocol. The input DNA volume was 3.5 μL. Table 1 shows the specific thermal cycling conditions for each assay. The *HLCS* and *RASSF1A* assays were performed as monoplex assays. The *ZFX/ZFY* assays were performed as a duplex reaction.

The fetal specificity of the assays was determined by detecting the number of copies of *HLCS*, *RASSF1A*, *ZFX*, and *ZFY* in *Bst*UI-digested samples of genomic DNA and plasma DNA.

Chromosome-dosage results obtained with the epigenetic–epigenetic and EGG approaches. Digital PCR analyses of the *HLCS*, *RASSF1A*, *ZFX*, and *ZFY* loci were performed on euploid and T21 samples of placental DNA after *Bst*UI restriction enzyme digestion. For the analysis of maternal plasma, we compared the ratios of *HLCS* to *ZFY* in *Bst*UI-digested DNA samples obtained

Downloaded from https://academic.oup.com/clinchem/article/56/1/90/5622561 by U.S. Department of Justice user on 16 August 2022

from euploid and T21 pregnancies. Samples that used 1 digestion-reaction tube were diluted and distributed into 6 panels. Samples that used 2 digestion-reaction tubes (with a larger volume) were diluted and distributed into 12 panels, and so on.

STATISTICAL ANALYSIS

SigmaStat 3.5 software (Systat Software) was used for statistical analyses.

Results

COBRA SCREENING OF DIFFERENTIALLY METHYLATED REGIONS ON CHROMOSOME 21

Of the 35 promoter regions on chromosome 21 selected for COBRA screening of differential methylation, 30 were not associated with CpG islands (CGIs); the other 5 were associated with CGIs. The criteria for defining a CGI were as described previously *(26)*. We obtained DNA sequences proximal to transcription start sites (−1 kb to +500 bp, the transcription start site being 0) for the 35 promoters from the March 2006 human reference sequence of the UCSC Genome Bioinformatics Database (http://www.genome.ucsc.edu/; NCBI Build 36.1) and designed 51 COBRA assays to compare the methylation patterns of placental tissues and maternal blood cells (see Table S1 in the online Data Supplement).

With the 51 assays, we compared the methylation profiles of the promoter regions for placental tissues collected from the first and third trimesters with those for maternal blood cells (see Table S1 in the online Data Supplement). Of the screened regions, the putative promoter regions of *HLCS* and *C21orf81* (chromosome 21 open reading frame 81; GenBank accession no. AF326257) were identified by COBRA to be differentially methylated in placental tissues and maternal blood cells. Representative COBRA results are illustrated in Fig. S2A in the online Data Supplement for *HLCS* region B2, which was hypermethylated in placental tissues compared with maternal blood cells, and in Fig. S2B in the online Data Supplement for *C21orf81*, which was hypermethylated in maternal blood cells compared with placental tissues.

CLONING AND BISULFITE SEQUENCING ANALYSIS OF THE *HLCS* LOCUS

We used cloning and bisulfite sequencing experiments to further analyze the methylation of the *HLCS* region at the resolution of a single molecule; the results are shown in Fig. 1. The *HLCS* sequencing results confirmed that *HLCS* is hypermethylated in placental cells at a methylated site frequency of 0.435–0.699. Although we detected a low methylation level in maternal blood cell samples at the 3′ end (i.e., position −57 to +111) of the target sequence (methylated site frequency <0.100), we observed almost no methylation of CpG sites from −232 to −67. We did not further investigate the potential hypomethylated fetal DNA marker, *C21orf81*, because our aim in the current study was to develop hypermethylated fetal DNA markers.

REAL-TIME QUANTITATIVE PCR FOR MSRE-DIGESTED PLACENTAL, MATERNAL BLOOD CELL, AND PLASMA DNA SAMPLES

We compared the methylation profiles of the putative promoter region of *HLCS* from 8 maternal blood cell samples with the profiles from 2 first-trimester and 2 third-trimester placental tissue samples collected from euploid pregnancies. The results of restriction enzyme digestion followed by real-time quantitative PCR analysis are shown in Fig. S3 in the online Data Supplement. All DNA samples from maternal blood cells were mostly digested by restriction enzymes, yielding methylation indices approaching 0 [median, 0.027; interquartile range (IQR), 0.023–0.029]. Placental DNA samples were partially digested, yielding methylation indices ranging from 0.76 to 0.94.

Previous data suggested that the placenta was the predominant source of fetal DNA *(27)* and that maternal blood cells were the main contributor of the background maternal DNA *(28)* detectable in maternal plasma. We therefore hypothesized that the placenta-specific fraction of the *HLCS* DNA molecules—namely the methylated or nondigestible fraction—could be detected in maternal plasma. We collected 10 pairs of pre- and postdelivery samples of third-trimester maternal plasma for analysis. Positive *HLCS* signals were obtained for all of the enzyme-digested predelivery plasma samples. The clearance pattern for the enzyme-treated pre- and postdelivery plasma samples suggested that the digestion-resistant *HLCS* molecules were pregnancy specific ($P = 0.002$, Wilcoxon signed rank test; see Fig. S4A in the online Data Supplement). Median *HLCS* concentrations were 730 copies/mL (IQR, 650–790 copies/mL) and 35 copies/mL (IQR, 26–46 copies/mL) for enzyme-treated pre- and postdelivery plasma samples, respectively. With mock digestion, we expected unmethylated maternal *HLCS* molecules to be detected in addition to digestion-resistant placental *HLCS* molecules, and no postpartum clearance was observed ($P = 0.16$, Wilcoxon signed rank test; see Fig. S4B in the online Data Supplement). Median *HLCS* concentrations were 2300 copies/mL (IQR, 1900–2700 copies/mL) and 2100 copies/mL (IQR, 1700–2400 copies/mL) for mock-digested pre- and postdelivery plasma samples, respectively.

Downloaded from https://academic.oup.com/clinchem/article/56/1/90/5622561 by U.S. Department of Justice user on 16 August 2022

Fig. 1. Cloning and bisulfite sequencing results of *HLCS* region B2.

Individual CpG sites are numbered down the last column, with nucleotide positions defined relative to the transcription start site (position 0).The first CpG site (−232) corresponds to chr21: 37275031 (reverse strand) of the Human March 2006 (hg18) assembly of the UCSC Genome Browser. Each column of circles depicts the methylation status across the CpG sites in a single DNA molecule studied by cloning. Filled and unfilled circles represent methylated and unmethylated CpG sites, respectively. T21 PLN, Normal PLN 1st, and Normal PLN 3rd indicate clones from T21, normal first-trimester, and normal third-trimester placental tissue samples, respectively; MBC indicates clones from maternal blood cells. Placenta and maternal blood cells from different pregnant individuals are identified by the case number.

MICROFLUIDICS DIGITAL PCR ANALYSIS OF MSRE-DIGESTED DNA

We compared chromosome dosages for the chromosome 21 and reference chromosome markers on a microfluidics digital PCR platform. The chromosome 21 marker was hypermethylated *HLCS*, and the reference markers on chromosome 3 and chromosome Y were hypermethylated *RASSF1A* and male-specific *ZFY*, respectively.

Two pairs of placental tissue and maternal blood cell samples were used to check the performance of the *HLCS* and *RASSF1A* assays with and without enzyme digestion. The *ZFX/ZFY* duplex assay also was performed [GenBank accession nos. NM_003410 (*ZFX*) and NM_003411 (*ZFY*)]. Because there is no *Bst*UI site within the amplified regions of *ZFX* and *ZFY*, we expected the copy number not to be affected by the enzyme digestion. Two postdelivery maternal plasma samples were used to test the postpartum clearance of the digestion-resistant *HLCS* and *RASSF1A* molecules.

One microfluidics digital PCR panel with 765 reaction wells was used for each assay of each of the genomic DNA samples. For the postdelivery maternal plasma samples, enzyme-digested DNA was distributed into 2 panels for the scoring of any wells with positive signals. Results for the genomic DNA and plasma DNA samples are summarized in Tables S2A and S2B, respectively, in the online Data Supplement. We counted the number of wells positive for each target locus. The actual number of molecules distributed into the panel followed the Poisson distribution and was corrected with the following equation: Target =

Downloaded from https://academic.oup.com/clinchem/article/56/1/90/5622561 by U.S. Department of Justice user on 16 August 2022

$-\ln(E/N) \times N$, where Target is the Poisson-corrected counts of the target molecules, E is the number of negative (empty) wells, and N is the total number of digital PCRs in the experiment.

Results from the *Bst*UI-digested samples of placental DNA showed that about 60%–70% of the *HLCS* and *RASSF1A* molecules remained detectable, compared with the mock-digested samples. In the samples of maternal blood DNA, the hypomethylated *RASSF1A* molecules were completely digested by the *Bst*UI treatment, whereas a few *HLCS* molecules remained detectable in the samples. For the *ZFY* and *ZFX* assays, there was no change in the copy numbers in the mock- or *Bst*UI-digested DNA samples (see Table S2A in the online Data Supplement).

Analysis of postdelivery maternal plasma revealed extremely low levels of both the *HLCS* and *RASSF1A* molecules after *Bst*UI digestion (Table S2B in the online Data Supplement).

CHROMOSOME-DOSAGE ANALYSIS OF PLACENTAL DNA SAMPLES

The *HLCS* and *RASSF1A* assays were applied to 10 euploid and 12 T21 placental DNA samples from pregnancies with male fetuses. Four digital PCR panels were counted for each assay (Fig. 2A). The ratios of the numbers of positive *HLCS* and *RASSF1A* amplifications in the euploid and T21 samples were significantly different ($P = 0.005$, Mann–Whitney rank sum test); however, the 2 groups showed a large degree of overlap (Fig. 3A). We calculated a reference interval, defined as the mean ratio of *HLCS* to *RASSF1A* $\pm$ 1.96 SD, for the euploid samples as 0.86–1.63. More than half of the T21 cases fell within the reference interval. The heterogeneity in the methylation density of the 2 loci across different samples may have contributed to the large interindividual variation in the *HLCS*/*RASSF1A* ratio. Solving this precision problem would require a more stable baseline for comparing the dosages of the euploid and T21 DNA samples.

We then turned to a fetal-specific genetic marker, the *ZFY* locus, to serve as a baseline for comparing chromosome dosages in pregnancies involving a male fetus. We called this strategy the EGG approach.

The same restriction enzyme–digested placental DNA samples used for the *HLCS* and *RASSF1A* comparison were subjected to the *ZFY* digital PCR analysis (Fig. 2A). The total copy number from 4 panels was used as the reference baseline for the total copy number of the *HLCS* molecules obtained above. A reference interval, defined as the mean ratio of *HLCS* to *ZFY* $\pm$ 1.96 SD, was calculated from the euploid samples as 1.08–1.62. All of the T21 samples had a ratio greater than the upper reference limit ($P < 0.001$, Mann–Whitney rank sum test; Fig. 3B).

EGG ANALYSIS OF MATERNAL PLASMA DNA

We obtained 8 maternal plasma samples from each of the 3 trimesters among pregnancies involving euploid fetuses, for a total of 24 samples. After *Bst*UI digestion, each DNA sample was analyzed with the *HLCS* and *ZFY* assays in at least 6 panels on the microfluidics chips (Fig. 2B). The total number of Poisson-corrected positive wells was used to calculate the ratio of *HLCS* to *ZFY* in each sample.

A reference interval of 1.49–2.88 was calculated for the 24 maternal plasma samples from euploid pregnancies. The ratio of 1 euploid DNA sample fell outside the reference interval. The fact that this sample dried up during SpeedVac concentration and had to be reconstituted with water during sample preparation might explain this apparent outlier. In contrast, all 5 maternal plasma samples from T21 pregnancies had a ratio of *HLCS* to *ZFY* that was greater than the upper reference limit ($P < 0.001$, Mann–Whitney rank sum test; Fig. 4). The data showed that the EGG approach can detect fetal T21 noninvasively in maternal plasma samples.

Discussion

In this study, we used COBRA mainly to screen for promoter regions on chromosome 21 that are not associated with CGIs. This approach differs from that of a previous study, which mainly targeted CGIs on chromosome 21 in a search for loci that were differentially methylated in placental tissues and blood cells *(19)*. In the first part of this study, we successfully identified a new hypermethylated fetal DNA marker on chromosome 21, the promoter region of the *HLCS* gene. The hypermethylated nature of this marker allows it to be detected relatively easily in maternal plasma with MSRE digestion and PCR amplification.

We then used *HLCS* to demonstrate that the novel EGG approach is a feasible method for analyzing fetal chromosome dosage. The main advantage of the EGG method over the previously described epigenetic allelic-ratio analysis *(12)* is that we have bypassed the requirement of the latter method for both the epigenetic target and the genetic target (i.e., the SNP) to be present in the same locus and within a short distance of each other. In this proof-of-concept study, we used the *ZFY* gene on the Y chromosome as a model for the fetal-specific genetic target. Additional Y-chromosomal markers can also be included. Indeed, any fetal-specific genetic target (e.g., a SNP allele or a gene that the fetus has inherited from the father but is absent in the pregnant mother) can be used. We envision the relatively easy development of a panel of such markers that would ensure broad population coverage

Downloaded from https://academic.oup.com/clinchem/article/56/1/90/5622561 by U.S. Department of Justice user on 16 August 2022

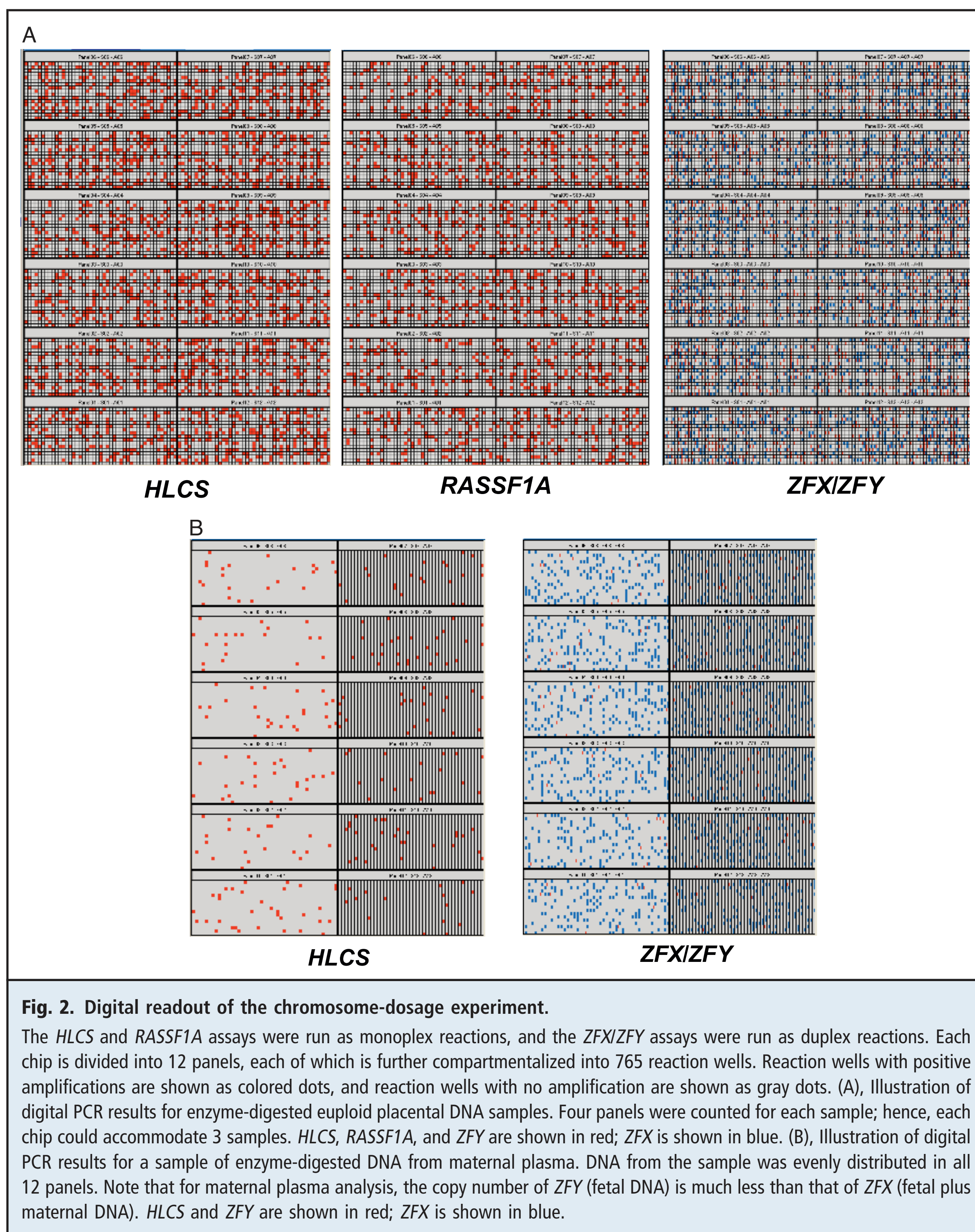

Fig. 2. Digital readout of the chromosome-dosage experiment.

The *HLCS* and *RASSF1A* assays were run as monoplex reactions, and the *ZFX/ZFY* assays were run as duplex reactions. Each chip is divided into 12 panels, each of which is further compartmentalized into 765 reaction wells. Reaction wells with positive amplifications are shown as colored dots, and reaction wells with no amplification are shown as gray dots. (A), Illustration of digital PCR results for enzyme-digested euploid placental DNA samples. Four panels were counted for each sample; hence, each chip could accommodate 3 samples. *HLCS*, *RASSF1A*, and *ZFY* are shown in red; *ZFX* is shown in blue. (B), Illustration of digital PCR results for a sample of enzyme-digested DNA from maternal plasma. DNA from the sample was evenly distributed in all 12 panels. Note that for maternal plasma analysis, the copy number of *ZFY* (fetal DNA) is much less than that of *ZFX* (fetal plus maternal DNA). *HLCS* and *ZFY* are shown in red; *ZFX* is shown in blue.

of this approach and allow pregnancies with female fetuses to be analyzed.

We also demonstrated that the EGG approach has greater power to distinguish T21 than an approach based purely on epigenetic markers, e.g., the ratio of hypermethylated *HLCS* to hypermethylated *RASSF1A*. We believe that one possible explanation for the suboptimal performance of the latter approach is the vari-

Downloaded from https://academic.oup.com/clinchem/article/56/1/90/5622561 by U.S. Department of Justice user on 16 August 2022

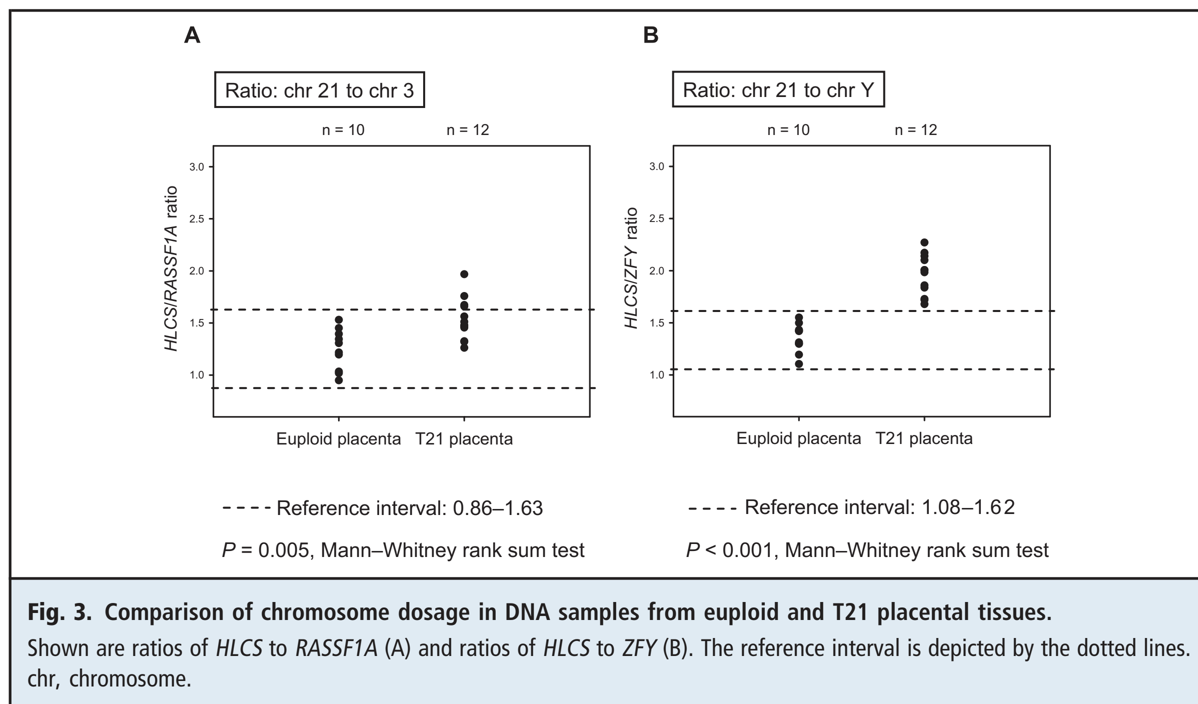

Fig. 3. Comparison of chromosome dosage in DNA samples from euploid and T21 placental tissues.
Shown are ratios of *HLCS* to *RASSF1A* (A) and ratios of *HLCS* to *ZFY* (B). The reference interval is depicted by the dotted lines. chr, chromosome.

ability in the level of DNA methylation of individual fetal-derived *HLCS* and *RASSF1A* molecules, as can be seen in Fig. 1 and in previously published bisulfite sequencing results *(18)*. Thus, one would expect that a ratio determined from these 2 potentially varying parameters would have a wider reference interval than a ratio in which one of the parameters is a relatively stable genetic marker (as in the EGG approach).

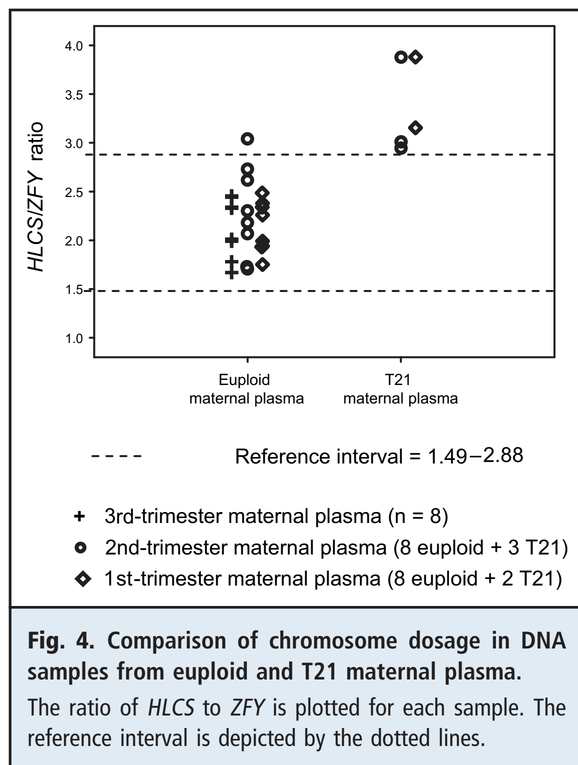

Fig. 4. Comparison of chromosome dosage in DNA samples from euploid and T21 maternal plasma.
The ratio of *HLCS* to *ZFY* is plotted for each sample. The reference interval is depicted by the dotted lines.

In this study, we have used microfluidics digital PCR as the detection and measurement platform because previous results have shown it to be a highly precise analytical method *(25, 29)*. We envision that the EGG approach can also be implemented in variations of this molecular-counting theme, e.g., by targeted sequencing with a "next-generation" sequencer. In fact, because both *HLCS* and *ZFY* are fetal-specific markers in maternal plasma, it should be possible to detect fetal aneuploidy in maternal plasma by EGG analysis with nondigital platforms, such as conventional real-time quantitative PCR.

The development of fetal epigenetic markers on the other chromosomes important for prenatal screening (e.g., chromosomes 18 and 13) would further broaden the usefulness of the EGG approach.

Author Contributions: *All authors confirmed they have contributed to the intellectual content of this paper and have met the following 3 requirements: (a) significant contributions to the conception and design, acquisition of data, or analysis and interpretation of data; (b) drafting or revising the article for intellectual content; and (c) final approval of the published article.*

Authors' Disclosures of Potential Conflicts of Interest: *Upon manuscript submission, all authors completed the Disclosures of Potential Conflict of Interest form. Potential conflicts of interest:*

Downloaded from https://academic.oup.com/clinchem/article/56/1/90/5622561 by U.S. Department of Justice user on 16 August 2022

Employment or Leadership: None declared.
Consultant or Advisory Role: Y.M.D. Lo, Sequenom.
Stock Ownership: Y.M.D. Lo, Sequenom.
Honoraria: None declared.
Research Funding: R.W.K. Chiu, University Grants Committee of the Government of the Hong Kong Special Administration Region, China—Areas of Excellence Scheme (AoE/M-04/06); Y.M.D. Lo, Sequenom.
Expert Testimony: None declared.
Other: Y.K. Tong, patent applications filed on the detection of fetal nucleic acids in maternal plasma for noninvasive prenatal diagnosis; (other remuneration) R.W.K. Chiu, patent application filed.

Role of Sponsor: The funding organizations played no role in the design of study, choice of enrolled patients, review and interpretation of data, or preparation or approval of manuscript.

References

1. Wapner R, Thom E, Simpson JL, Pergament E, Silver R, Filkins K, et al. First-trimester screening for trisomies 21 and 18. N Engl J Med 2003;349: 1405–13.
2. Malone FD, Canick JA, Ball RH, Nyberg DA, Comstock CH, Bukowski R, et al. First-trimester or second-trimester screening, or both, for Down's syndrome. N Engl J Med 2005;353:2001–11.
3. Tabor A, Philip J, Madsen M, Bang J, Obel EB, Norgaard-Pedersen B. Randomised controlled trial of genetic amniocentesis in 4606 low-risk women. Lancet 1986;1:1287–93.
4. Lau TK, Leung TN, Fung TY, Chan LW, Sahota DS, Leung TN. Outcome of 1355 consecutive transabdominal chorionic villus samplings in 1351 patients. Chin Med J 2005;118:1675–81.
5. Kong CW, Leung TN, Leung TY, Chan LW, Sahota DS, Fung TY, Lau TK. Risk factors for procedure-related fetal losses after mid-trimester genetic amniocentesis. Prenat Diagn 2006;26:925–30.
6. Lo YMD, Corbetta N, Chamberlain PF, Rai V, Sargent IL, Redman CW, Wainscoat JS. Presence of fetal DNA in maternal plasma and serum. Lancet 1997;350:485–7.
7. Lo YMD, Chiu RWK. Prenatal diagnosis: progress through plasma nucleic acids. Nat Rev Genet 2007;8:71–7.
8. Lo YMD, Hjelm NM, Fidler C, Sargent IL, Murphy MF, Chamberlain PF, et al. Prenatal diagnosis of fetal RhD status by molecular analysis of maternal plasma. N Engl J Med 1998;339:1734–8.
9. Finning K, Martin P, Summers J, Massey E, Poole G, Daniels G. Effect of high throughput RHD typing of fetal DNA in maternal plasma on use of anti-RhD immunoglobulin in RhD negative pregnant women: prospective feasibility study. BMJ 2008;336:816–8.
10. Costa JM, Benachi A, Gautier E. New strategy for prenatal diagnosis of X-linked disorders. N Engl J Med 2002;346:1502.
11. Lo YMD, Tsui NBY, Chiu RWK, Lau TK, Leung TN, Heung MMS, et al. Plasma placental RNA allelic ratio permits noninvasive prenatal chromosomal aneuploidy detection. Nat Med 2007;13:218–23.
12. Tong YK, Ding C, Chiu RWK, Gerovassili A, Chim SSC, 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.
13. Chiu RWK, Cantor CR, Lo YMD. Non-invasive prenatal diagnosis by single molecule counting technologies. Trends Genet 2009;25:324–31.
14. Lo YMD, Lun FMF, Chan KCA, Tsui NBY, Chong KC, Lau TK, et al. Digital PCR for the molecular detection of fetal chromosomal aneuploidy. Proc Natl Acad Sci U S A 2007;104:13116–21.
15. Chiu RWK, Chan KCA, Gao Y, Lau VYM, 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 U S A 2008;105: 20458–63.
16. 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 U S A 2008;105: 16266–71.
17. Chan KCA, Ding C, Gerovassili A, Yeung SW, Chiu RWK, Leung TN, et al. Hypermethylated RASSF1A in maternal plasma: a universal fetal DNA marker that improves the reliability of noninvasive prenatal diagnosis. Clin Chem 2006;52:2211–8.
18. Chiu RWK, Chim SSC, Wong IH, Wong CSC, Lee WS, To KF, et al. Hypermethylation of RASSF1A in human and rhesus placentas. Am J Pathol 2007;170:941–50.
19. Chim SSC, Jin S, Lee TYH, Lun FMF, Lee WS, Chan LYS, 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.
20. Old RW, Crea F, Puszyk W, Hulten MA. Candidate epigenetic biomarkers for non-invasive prenatal diagnosis of Down syndrome. Reprod Biomed Online 2007;15:227–35.
21. 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.
22. Xiong Z, Laird PW. COBRA: a sensitive and quantitative DNA methylation assay. Nucleic Acids Res 1997;25:2532–4.
23. Ottesen EA, Hong JW, Quake SR, Leadbetter JR. Microfluidic digital PCR enables multigene analysis of individual environmental bacteria. Science 2006;314:1464–7.
24. Warren L, Bryder D, Weissman IL, Quake SR. Transcription factor profiling in individual hematopoietic progenitors by digital RT-PCR. Proc Natl Acad Sci U S A 2006;103:17807–12.
25. Lun FMF, Chiu RWK, Chan KCA, Leung TY, Lau TK, Lo YMD. Microfluidics digital PCR reveals a higher than expected fraction of fetal DNA in maternal plasma. Clin Chem 2008;54:1664–72.
26. Yamada Y, Watanabe H, Miura F, Soejima H, Uchiyama M, Iwasaka T, et al. A comprehensive analysis of allelic methylation status of CpG islands on human chromosome 21q. Genome Res 2004;14:247–66.
27. Bianchi DW. Circulating fetal DNA: its origin and diagnostic potential—a review. Placenta 2004; 25(Suppl A):S93–101.
28. Lui YYN, Chik KW, Chiu RWK, Ho CY, Lam CW, Lo YMD. Predominant hematopoietic origin of cell-free DNA in plasma and serum after sex-mismatched bone marrow transplantation. Clin Chem 2002;48:421–7.
29. Lun FMF, Tsui NBY, Chan KCA, Leung TY, Lau TK, Charoenkwan P, et al. Noninvasive prenatal diagnosis of monogenic diseases by digital size selection and relative mutation dosage on DNA in maternal plasma. Proc Natl Acad Sci U S A 2008; 105:19920–5.

Downloaded from https://academic.oup.com/clinchem/article/56/1/90/5622561 by U.S. Department of Justice user on 16 August 2022