Noninvasive Prenatal Molecular Karyotyping from Maternal Plasma

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Abstract

Fetal DNA is present in the plasma of pregnant women. Massively parallel sequencing of maternal plasma DNA has been used to detect fetal trisomies 21, 18, 13 and selected sex chromosomal aneuploidies noninvasively. Case reports describing the detection of fetal microdeletions from maternal plasma using massively parallel sequencing have been reported. However, these previous reports were either polymorphism-dependent or used statistical analyses which were confined to one or a small number of selected parts of the genome. In this report, we have described a procedure for performing noninvasive prenatal karyotyping at 3 Mb resolution across the whole genome through the massively parallel sequencing of maternal plasma DNA. This method has been used to analyze the plasma obtained from 6 cases. In three cases, fetal microdeletions have been detected successfully from maternal plasma. In two cases, fetal microduplications have been detected successfully from maternal plasma. In the remaining case, the plasma DNA sequencing result was consistent with the pregnant mother being a carrier of a microduplication. Simulation analyses were performed to determine the number of plasma DNA molecules that would need to be sequenced and aligned for enhancing the diagnostic resolution of noninvasive prenatal karyotyping to 2 Mb and 1 Mb. In conclusion, noninvasive prenatal molecular karyotyping from maternal plasma by massively parallel sequencing is feasible and would enhance the diagnostic spectrum of noninvasive prenatal testing.

Introduction

The presence of fetal DNA in maternal plasma has opened up exciting possibilities for noninvasive prenatal testing [1,2]. Recently, there has been much interest in the use of massively parallel sequencing (MPS) for analyzing circulating fetal DNA for prenatal testing purposes. Thus, fetal trisomies 21, 13, 18 and selected sex chromosomal aneuploidies have been detected using MPS on maternal plasma DNA [3–7] and have been rapidly introduced into clinical service.

Apart from abnormalities due to copy number changes involving a whole chromosome, it would be important to evaluate whether the MPS-based analysis of maternal plasma might be sensitive enough for detecting subchromosomal deletions or duplications. In this regard, Peters et al reported the detection of a 4.2 Mb deletion on chromosome 12 in a maternal plasma sample obtained at the 35th week of gestation [8]. Jensen et al reported the detection of a 3 Mb deletion on chromosome 22 in maternal plasma samples obtained from two pregnant women at the 19th and 20th weeks of gestation [9]. Apart from the deleted region, Peters et al also performed statistical analysis on another region on chromosome 12, as well as 20 nonoverlapping 4 Mb regions on chromosome 14 [8]. Jensen et al, on the other hand, only focused their statistical analysis on the deleted region on chromosome 22 [9]. Thus, from the data presented by Peters et al and Jensen et al, it is not clear if the approach would be robust enough for a genomewide survey of microdeletions or microduplications, or indeed for the noninvasive determination of a fetal karyotype.

Lo et al reported that fetal single nucleotide polymorphisms (SNPs) can be genotyped in a genomewide scale using maternal plasma DNA sequencing [10]. In particular, these investigators have demonstrated that SNP alleles and mutations for single gene disorders that are inherited by a fetus from its mother can be elucidated by a process called relative haplotype dosage analysis [10]. Fan et al confirmed the robustness of relative haplotype
Materials and Methods

Ethical Statement

The study was approved by the Joint Chinese University of Hong Kong – Hospital Authority New Territories East Cluster Clinical Research Ethics Committee. We recruited pregnant women with written informed consent from the Prince of Wales Hospital, the Kwong Wah Hospital and the Tsan Yuk Hospital in Hong Kong, and the Asan Medical Center in Seoul.

Sample Collection

For cases 01, 02, and 03, maternal peripheral blood samples were collected into EDTA-containing tubes after invasive procedures (Table 1). For cases 04, 05 and 06, maternal peripheral blood samples were collected before performing any invasive procedures. Maternal blood samples were drawn at 12 3/7 to 28 4/7 weeks of gestation (Table 1).

Among the six test samples, there were three cases (cases 01, 02 and 03) of fetal de novo 22q11.2 microdeletion, one case (case 04) of fetal de novo 22q11.2 microduplication (2.4 Mb) and one case (case 05) of maternally-inherited 22q11.2 microduplication (2.4 Mb). There was also one case (case 06) in which the mother had a balanced translocation of t(3;4)(q29;q32) and the fetus was found to have 3q29 microduplication (5.1 Mb) and 4q32.1-q35.2 deletion (32.9 Mb). Full karyotyping was performed and the fetal karyotypes were further ascertained by array comparative genomic hybridization (array CGH) [16], fluorescence in situ hybridization (FISH) or a combination of quantitative fluorescence PCR (QF-PCR) and FISH.

In addition, we collected a group of eight singleton pregnant women with normal fetal karyotypes as reference controls for downstream data analysis.

Sample Processing and DNA Extraction

Peripheral blood samples were centrifuged at 1600 g for 10 min at 4°C and the plasma portion was re-centrifuged at 16000 g for 10 min at 4°C [17]. We extracted cell-free DNA from 1.8 to 8.4 mL of maternal plasma with the QIAamp DSP DNA Blood Mini Kit (Qiagen) as described previously [3]. The extracted plasma DNA was quantified by a real-time PCR assay targeting the leptin (LEP) gene as described previously [18].

Plasma DNA Sequencing

We prepared sequencing libraries of plasma DNA with the Paired-End Sequencing Sample Preparation Kit (Illumina) as described previously [19]. Due to the variable volume of maternal plasma available, we aimed to have a relatively consistent amount of plasma DNA input for library preparation. We thus used 13 to 20 ng of the extracted plasma DNA for library preparation which corresponded to the amount extracted from 1.5 to 2.2 mL of maternal plasma. The adaptor-ligated plasma DNA was enriched by a 12-cycle PCR. We performed cluster generation on a cBot clonal amplification system (Illumina) with the TruSeq PE Cluster Generation Kit v3 (Illumina). Each library (both test and reference samples) was sequenced with one lane of a flow cell on a HiSeq 2000 sequencing system (Illumina) in a paired-end format of 50-bp x2. Sequence data have been deposited at the European Genome-Phenome Archive (EGA, http://www.ebi.ac.uk/ega/), which is hosted by the European Bioinformatics Institute (EBI), under the accession number EGAS00001000439.

Sequence Alignment and Filtering

Paired-end reads were aligned to the non-repeat masked human reference genome (NCBI Build 36.1/hg18) using the Short Oligonucleotide Alignment Program 2 (SOAP2) (http://soap.genomics.org.cn/). We allowed up to two nucleotide mismatches for each member of the paired-end reads. Only paired-end reads with both ends aligned to the same chromosome with the correct orientation, spanning an insert size ≤600 bp were included in downstream analysis. We also removed duplicated reads which were defined as paired-end reads showing identical start and end positions in the human genome.

Calculation of the Genomic Representation

We first divided each chromosome into 100-kb bins and performed locally weighted scatterplot smoothing (LOESS) to correct for GC-associated bias on the sequenced read counts [20]. All the calculations below were based on the GC-corrected read counts.

Table 1. Sample information.

| Case no. | Gestational age at plasma collection (weeks) | Plasma sampling relative to invasive procedure | Invasive procedure | Chromosomal aberration | Methods used to confirm karyotype |
|----------|-------------------------------------------|-----------------------------------------------|-------------------|------------------------|----------------------------------|
| 01       | 24 1/7                                    | Post-invasive                                 | Cordocentesis     | 22q11.2 microdeletion  | FISH                             |
| 02       | 28 4/7                                    | Post-invasive                                 | Cordocentesis     | 22q11.2 microdeletion  | FISH                             |
| 03       | 22 5/7                                    | Post-invasive                                 | Aminocentesis     | 22q11.2 microdeletion  | QF-PCR and FISH                  |
| 04       | 12 3/7                                    | Pre-invasive                                  | Chorionic villus sampling | 22q11.2 microduplication (2.4 Mb) | Array CGH                        |
| 05       | 20 2/7                                    | Pre-invasive                                  | Aminocentesis     | 22q11.2 microduplication (2.4 Mb) | Array CGH                        |
| 06       | 21 4/7                                    | Pre-invasive                                  | Aminocentesis     | 3q29 microduplication (5.1 Mb); 4q32.1-q35.2 microdeletion (32.9 Mb) | Array CGH                        |

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For the detection of subchromosomal aberrations, we merged the 100-kb bins into 1-Mb bins and calculated the genomic representation of each 1-Mb bin \((GR_{x-y})\), where \(x\) and \(y\) denote the start and end genomic coordinates of the 1-Mb bin. We determined the number of sequence reads originated from each 1-Mb bin and calculated the \(GR_{x-y}\) using this equation [20]:

\[
GR_{x-y} = \frac{RC_{x-y}}{RC_{total}}
\]

where \(RC_{x-y}\) is the read counts for the 1-Mb bin; and \(RC_{total}\) is the total read counts.

**Calculation of z-scores**

We used the group of eight singleton pregnant cases with normal fetal karyotypes as the reference for the analysis of subchromosomal copy number aberrations.

We determined the mean and the standard deviation of the genomic representation of each 1-Mb bin \((GR_{x-y})\) of the reference group and calculated the z-score for each 1-Mb bin of the test.
sample using this equation:

\[ z_{\text{score}_{GR_{x-y}}} = \frac{GR_{x-y_{\text{test}}} - meanGR_{x-y_{\text{reference}}}}{SD_{x-y_{\text{reference}}}} \]

where \( GR_{x-y_{\text{test}}} \) is the genomic representation of the 1-Mb bin in the test sample; \( meanGR_{x-y_{\text{reference}}} \) and \( SD_{x-y_{\text{reference}}} \) are the mean and the standard deviation of the genomic representation of the 1-Mb bin of the reference samples. To minimize the systematic inter-sample variations between different chromosomes, we performed median correction for each chromosome. Thus, the median genomic representation of all the bins on a particular chromosome was used as a baseline. For all bins located on that particular chromosome, the difference from this baseline value was used for the calculation of the z-score.
Calculation of Fetal DNA Percentage

The extent of under- or overrepresentation of a particular 1-Mb bin in the maternal plasma is linearly correlated with the fetal DNA percentage (fetal %) in cases with fetal de novo copy number aberration involving that bin [4]. Hence, we calculated the fetal % based on the plasma genomic representations of the regions showing copy number aberrations in the test case using this equation:

\[
\text{Fetal} \% = \left( \frac{\text{GR}_{y_{\text{test}}} - \text{meanGR}_{y_{\text{reference}}}}{\text{meanGR}_{y_{\text{reference}}}} \right) \times 2 \times 100\% 
\]

Only those 1-Mb bins which were completely covered by the region showing copy number aberration were included in the calculation of fetal %.

Table 2. The fetal DNA percentage estimated by the alterations of the genomic representation of the regions affected by microdeletion/microduplication, and the proportions of chromosome Y sequences in the maternal plasma.

| Case | Fetal sex | By genomic representation of the affected chromosomal region(s) | By chr Y approach* |
|------|-----------|---------------------------------------------------------------|-------------------|
| 01   | F         | 10.5%                                                         | –                 |
| 02   | M         | 17.4%                                                         | 21.5%             |
| 03   | M         | 9.2%                                                          | 13.7%             |
| 04   | M         | 17.8%                                                         | 20.3%             |
| 05b  | F–        | –                                                             | –                 |
| 06   | F         | 10.9%/13.4%                                                   | –                 |

*The chr Y approach is only applicable for those cases with a male fetus.

The sensitivity and specificity of detecting a microdeletion or a microduplication were affected by different parameters including the fetal % in the sample, the number of plasma DNA molecules sequenced and aligned, and the size of the aberration. Therefore, we performed computer simulation analyses to determine 1) the sensitivity of detecting a 3 Mb microdeletion/microduplication with the existing sequencing depth; and 2) the number of molecules needed to be analyzed to achieve a 95%/99% sensitivity when the fetal % was 5%.

This simulation represented an ideal situation when all analytical biases were minimized. In each simulation analysis, the whole genome (3,000 Mb) was divided into bins of equal size according to the desired resolution, which in the first instance was 3 Mb. For the detection of a subchromosomal aberration, we required three consecutive bins having genomic representation of >3 standard deviations (either over- or underrepresentation) away from the mean of the reference group in the same direction. Therefore, the bin size would be equal to 1/3 of the desired diagnostic resolution. For example, if we aim to detect aberrations of 3 Mb, the bin size would be 1 Mb. We assumed that the three bins covered by the microdeletion/microduplication would have an abnormal genomic representation resulting from the contribution of the minority population of fetal DNA. In the plasma, the expected proportion of total molecules (E) falling into a bin within an affected region can be calculated as:

\[
E = \left(1 + \frac{f}{2} \times d\right) \times \frac{1}{T}
\]

where f is the fetal DNA percentage in plasma,

\(d\) is the change in the chromosome number in the aberration (d equals to −1 for microdeletion and +1 for microduplication), and

\(T\) is the total number of bins for the whole genome.

Simulations of 1,000 normal cases and 1,000 affected cases were performed assuming a binomial distribution of the plasma DNA molecules with the expected plasma representations as calculated above. The fetal %, the bin size and the total number of molecules being analyzed were changed to achieve the desired purpose. The simulation was conducted using the rbinom function in R (http://www.r-project.org/).
Table 3. Number of molecules required to be sequenced and aligned to achieve different diagnostic resolutions and diagnostic sensitivities assuming that the fetal DNA percentage is 5%.

| Diagnostic sensitivity | Diagnostic resolution | 3 Mb    | 2 Mb    | 1 Mb    |
|------------------------|-----------------------|---------|---------|---------|
| 95%                    | No. of molecules required in each 1-Mb bin | 42,000  | 42,000  | 42,000  |
|                        | Total no. of bins for the whole genome | 3,000   | 4,500   | 9,000   |
|                        | Total no. of molecules required for the whole genome | 125 million | 192 million | 380 million |
| 99%                    | No. of molecules required in each 1-Mb bin | 53,000  | 53,000  | 53,000  |
|                        | Total no. of bins for the whole genome | 3,000   | 4,500   | 9,000   |
|                        | Total no. of molecules required for the whole genome | 160 million | 240 million | 480 million |

*In this theoretical analysis, the diagnostic specificity is >99.9% for all cases based on the criteria that three consecutive bins having genomic representations >3SD (for either over- or underrepresentation) from the mean of the references in the same direction.

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Results

Framework for Data Analysis

We used one lane of a flow cell on an Illumina HiSeq 2000 sequencer to analyze each maternal plasma sample of the six test cases and the eight controls. A mean of 211 million (range: 177 million to 236 million) DNA fragments were sequenced from each plasma DNA sample. Such sequencing resulted in a mean of 144 million (range: 96 million to 180 million) alignable and non-duplicated sequenced reads per case which was equivalent to 4.81 folds of the haploid human genome.

To obtain a plasma karyotype, the entire genome was divided into 2,687 1-Mb bins. We compared the genomic representation for each 1-Mb bin of the test sample with that of the reference group. For regions with normal genomic representation, the expected distributions of z-scores of all 1-Mb bins would be close to zero. We defined a reference interval as a z-score from −3 to 3. Such a criterion is robust to deviations from normal expectation, as less than 0.3% of the bins would fall outside of this interval just by chance. As 2,687 bins were analyzed, we would on average expect that 8 bins would fall outside of the reference interval just by chance. To reduce false-positive calls, we therefore included an additional criterion of calling a copy number aberration only if three consecutive 1-Mb bins exhibited a z-score outside of the reference interval and in the same direction.

Detection of Subchromosomal Copy Number Aberrations

The z-scores of all 1-Mb bins across the entire genome for each case were plotted using Circos plots [21] (Figure 1). In the test samples, 94.9%–98.7% of the 1-Mb bins showed normal representation. With the above-mentioned criterion of calling a copy number aberration only if three consecutive bins showed the same aberration, we correctly identified the copy number aberrations in all cases with no false-positives.

Figure 2 shows the z-scores of all 1-Mb bins of the chromosome(s) showing copy number aberrations for each case. For cases 01, 02 and 03, we detected underrepresentation in three consecutive 1-Mb bins on the q arm of chromosome 22. These were the three cases with de novo 22q11.2 microdeletion. For cases 04 and 05, we detected overrepresentation in three consecutive 1-Mb bins of chromosome 22q. Case 04 was a case with a de novo 22q11.2 microduplication of 2.4 Mb. Case 05 was a case with a maternally-inherited microduplication in the same region. For case 05, since the mother herself harbored the microduplication, we could easily detect the aberration in the maternal plasma. This was supported by the extremely high z-score values (range, 59.7 to 71.7) for the three consecutive bins. Further exploration of noninvasive prenatal testing of the fetus could proceed with the use of SNP-based methods, namely relative mutation dosage or relative haplotype dosage analysis [10,11,22]. For case 06, we detected five consecutive 1-Mb bins with overrepresentation on the q arm of chromosome 3 and thirty-one consecutive 1-Mb bins with underrepresentation on the q arm of chromosome 4, which corresponded to a 5-Mb duplication on 3q and a 31-Mb deletion on 4q. For all cases, the copy number aberrations detected had sizes comparable to those confirmed by array CGH, FISH and/or QF-PCR. For case 05, the microduplication carried by the mother was confirmed by array CGH.

Fetal DNA Percentage

In this report, we used the DNA sequences from the regions showing under- or overrepresentation to estimate the fetal % in maternal plasma (Table 2). We validated this approach by comparing the fetal % calculated using this method and that using the chr Y-based method [4] for the three cases carrying male fetuses (i.e., cases 02, 03 and 04). The fetal % values agreed well between the two methods (Table 2). For the five cases with fetal de novo copy number aberrations, the fetal % ranged from 9.2% to 17.8%. For case 05, the fetal % estimated by the genomic representation of the microduplication was 96.7%, suggesting that almost all of the circulating DNA would harbor this change. This result is consistent with the fact that the mother carried the aberration.

Simulation Analysis for Diagnostic Sensitivity

We used computer simulation to determine the diagnostic sensitivity of shotgun MPS-based noninvasive prenatal molecular
that is performed by at least one commercial provider of trisomy testing. At the current depth of sequencing and its resultant diagnostic resolution of 3 Mb, the current protocol could cover approximately 20% of the known pathogenic copy number variants [23]. We have predicted that 240 million and 480 million plasma DNA molecules would need to be sequenced and aligned to extend the diagnostic resolution to 2 Mb and 1 Mb, respectively, with a 99% sensitivity. At these diagnostic resolutions, shotgun MPS of maternal plasma DNA would be expected to cover approximately 50% and 80%, respectively, of the known pathogenic copy number variants [23]. With a continual increase in throughput of massively parallel sequencers and the concomitant reduction in sequencing costs, it is likely that the costs associated with such sequencing depths will reach a level that would be acceptable to healthcare providers in a few years’ time. The amount of sequencing required by this approach is already a significant reduction over our previously reported fetus-derived single nucleotide variation detection method which was performed using billions of sequenced reads per sample [10]. Further reduction in costs could come from targeted sequencing of genomic regions harboring pathogenic copy number variants, similar to what has been achieved for fetus-derived single nucleotide variation detection from maternal plasma [24,25]. Finally, the advent of single molecule sequencing would also be expected to further improve the diagnostic accuracy of this approach as amplification process, which might distort the genomic representation of the sequenced molecules, is not needed [26].

In summary, we have demonstrated that it might be feasible to obtain a noninvasive prenatal molecular karyotype by shotgun MPS of maternal plasma DNA. We have shown that our method can detect fetal de novo copy number changes, unbalanced translocations and maternal copy number changes. Future studies could be designed to address the efficacy of the present approach for detecting a wider spectrum of subchromosomal copy number changes. These results have further expanded the diagnostic spectrum of noninvasive prenatal diagnosis. In conclusion, methods based on MPS analysis of maternal plasma DNA have been developed for the prenatal detection of whole chromosome aneuploidies [3–7], subchromosomal copy number changes and fetal mutations for single gene disorders [10]. This array of noninvasive tests could in the first instance be applied for screening of fetal genomic and chromosomal abnormalities. Abnormalities revealed by the noninvasive maternal plasma DNA tests could be further confirmed by conventional invasive prenatal testing. Upon validation by large-scale prospective studies, it is envisioned that noninvasive maternal plasma DNA sequencing could provide prenatal assessment of a large spectrum of fetal genomic and chromosomal abnormalities and provide safer prenatal assessments.

**Author Contributions**

Review manuscript: KWC HSW WCL ETL MHYT TYL. Conceived and designed the experiments: SCYY PJ KCAC YMDL RWKC. Performed the experiments: SCYY. Analyzed the data: PJ KCAC KWC TYL YMDL RWKC. Contributed reagents/materials/analysis tools: KWC HSW WCL ETL MHYT TYL. Wrote the paper: SCYY PJ KCAC YMDL RWKC.

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