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Noninvasive prenatal testing of hereditary colorectal cancer syndromes using cell-free DNA in maternal plasma

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Abstract

Objective: This study aimed to establish a practical protocol for early noninvasive prenatal testing (NIPT) for fetuses at risk of Peutz-Jeghers syndrome or familial adenomatous polyposis, two classical types of hereditary colorectal cancer syndromes, for risk evaluation and whole-life monitoring.

Method: Target enrichment was performed using hybridization probes coordinating the *serine-threonine kinase 11* gene region and *APC* gene region, with 1458 highly heterozygous Single Nucleotide Polymorphisms included. Semitarget amplification random sequencing was used for large fragment deletion detection. For relative haplotype dosage (RHDO) analysis, haplotype construction was performed by segmented haplotype estimation and imputation tool software, the circular binary segmentation algorithm was used for recombination event calculation, and Bayes factor was used for the determination of whether the fetus was affected.

Results: Haplotypes were successfully constructed in the nine recruited families with different pedigree characteristics, and the results for the RHDO analysis were consistent with the amniocentesis sampling detection results. The cell-free fetal DNA fraction can be detected as low as 2% in maternal plasma.

Conclusion: This is the first NIPT assay on hereditary colorectal cancer syndromes based upon RHDO analysis.

Highlights

What's already known about this topic?

- Maternal cell-free DNA (cfDNA) can be used for noninvasive prenatal testing (NIPT) in monogenic hereditary diseases.
- There are few reports on NIPT of hereditary cancers, which are considered collectively to contribute substantially to morbidity and mortality.

What does this study add?

- This study provides methodological steps needed to achieve cfDNA assessment of monogenic diseases and an NIPT assay is established for hereditary colorectal syndromes based on relative haplotype dosage (RHDO) analysis feasible in sophisticated clinical situations.

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1 | INTRODUCTION

The validation of the presence of cell-free fetal DNA (cffDNA) in maternal plasma is a milestone for noninvasive prenatal testing (NIPT).[1] Compared with maternal-derived cell-free DNA (cfDNA), cffDNA is relatively shorter in length,[2,3] and the concentration in maternal plasma is 0.39%–11.9% at gestational ages from 11 to 17 weeks[4] and approximately 10%–20% in the last weeks of gestation.[5] Meanwhile, with the blossom of massive parallel sequencing, NIPT based upon cffDNA and next-generation sequencing (NGS) makes screening fetal status, such as fetal chromosome abnormalities and monogenic hereditary diseases through maternal plasma within the first gestational trimester possible, and is becoming a first-tier test for high-risk pregnancies in many areas.[6]

Peutz-Jeghers syndrome (PJS, Online Mendelian Inheritance in Man [OMIM]: 175200) and familial adenomatous polyposis (FAP, OMIM: 175100), two classical types of hereditary colorectal cancer syndromes, are both autosomal dominant monogenic hereditary disorders caused by germline mutations in serine-threonine kinase 11 (*STK11*) and APC regulator of WNT signaling pathway (*APC*), respectively. The patients always come in before decreased life expectancy, physical and mental burden and financial pressure along with the detection, screening and treatment of the diseases. According to the newest European Hereditary Tumor Group Guideline for the management of PJS, it is of strong recommendation that PJS can be taken as an indication for prenatal genetic diagnosis and preimplantation genetic diagnosis and these options should be discussed with PJS patients in whom a *STK11* pathogenic variant has been identified.[7] Close relatives and the members of the FAP affected families should resort to genetic testing as recommended by the American College of Gastroenterology clinical guideline.[8] Meanwhile, there are no hotspot variations in *STK11* and *APC*, and the detected pathogenic variations vary, including various types, such as small deletions/insertions, single-base substitutions, and loss of exons or even the whole gene. Therefore, a complete set of detection and analysis systems is needed for these patients planning for children to identify the pathogenic variation before pregnancy and for families at risk to execute prenatal diagnosis and genetic counseling for risk evaluation. Considering the hereditary mode of PJS and FAP, there is a 50% chance for the offspring to be influenced by either of the parents. In this way, NIPT is a reasonable choice for at-risk families.

Since the entire fetal genome was demonstrated to be present in a constant relative proportion to maternal DNA in maternal plasma,[3] relative haplotype dosage analysis (RHDO analysis) using target sequencing data was reported to be applicable in monogenetic diseases,[9] such as Duchenne and Becker muscular dystrophies,[10] spinal muscular atrophy,[11] congenital adrenal hyperplasia,[12] and β-thalassemia.[9,13] However, to date, there have been few reports of the prenatal diagnosis of hereditary cancers, and the vast majority of the reports involved invasive tests.[14] Therefore, in this study, we aimed to construct a complete set of NIPT assays for PJS and FAP in the early stage of pregnancy using targeted enrichment sequencing and RHDO analysis for sophisticated clinical conditions.

2 | METHODS

2.1 | Patient recruitment

In this study, we recruited nine families at risk of PJS or FAP. This study was in accordance with the Helsinki Declaration and was confirmed by the ethnic committee of Changhai Hospital. Written consent was obtained.

2.2 | Sample collection and processing

For the pregnant women, 9 ml peripheral blood samples were collected using anticoagulant tubes with ethylenediaminetetraacetic acid (EDTA) added at the 9th–14th gestational week. The peripheral blood samples were centrifuged at 2000 × *g* for 20 min at 4°C and divided into three layers, namely, the erythrocyte layer, buffy coat layer and plasma layer. The plasma layer was separated and recentrifuged at 4000 × *g* for 20 min at 4°C, and the supernatant was used for maternal cfDNA extraction. The buffy coat was used for maternal genomic DNA extraction. At the gestational age of the 16th–20th week, amniotic fluid puncture was performed, and fetal genomic DNA was extracted from the amniotic fluid cells. In addition, 2 ml peripheral blood samples were collected from the other family members, and genomic DNA samples were obtained from the buffy coat separated following the method mentioned above. For family P8, the affected relative's genomic DNA sample was the whole genome amplification (WGA) product of the embryonic trophoblast cell for preimplantation genetic testing. All the maternal cfDNA and genomic DNA extraction processes mentioned above were performed using the Nucleic Acid Extraction and Purification Kit (NaHai™) following the manufacturer's instructions. The DNA samples obtained were stored at −80°C for further processing.

2.3 | Target capture probe design

We designed probes covering approximately 220.87 kb of the targeted genomic regions. A set of probes was designed for all exons, untranslated regions, splicing regions of the *STK11* gene, and 743 highly heterozygous Single Nucleotide Polymorphisms (SNPs) (minor allele frequency [MAF] > 0.3 in 1000 G) within the 1 Mb genome region upstream and downstream of the *STK11* gene (chr19: 245,275-1,881,977, Hg19). The other set of probes was designed for the corresponding regions for the *APC* gene and 715 highly heterozygous SNPs (MAF > 0.3 in 1000 G) within the 1 Mb genome region upstream and downstream of the *APC* gene (chr5: 111,129,372-112,893,227, Hg19). Details are shown in Figure S1.

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2.4 | DNA library preparation, target enrichment and sequencing

The buffy coat-derived genomic DNA and WGA-derived DNA were fragmented by restriction endonuclease, and the plasma-derived cfDNA was end paired. Then, these two sources of DNA were ligated with compatible barcoded adapters and amplified by polymerase chain reaction (PCR). Then, target DNA capture was performed using the probes designed above. After incubation with the probes, the captured DNA libraries were set for another 12 cycles of PCR amplification, which were then sequenced on the Ion Proton platform using the Ion PI™ Hi-Q™ Sequencing 200 Kit (Thermo Fisher) after the cleanup process.

2.5 | In silico analysis of variations

The single-end sequencing reads were mapped to the human reference genome (Hg19, GRCh37) using Ion Torrent's mapping program (v5.2.25). Polymerase chain reaction duplications and multiple-aligned reads were removed. Single nucleotide variations and small indels were detected by Torrent Variation Caller (TVC, v5.2-25). Copy number variation (CNV) calling was performed using an in-house algorithm. Pathogenic variations were classified according to the American College of Medical Genetics and Genomics and the Association for Molecular Pathology guidelines.[15] The transcription versions used in this study are NM_000455.5 (*STK11*) and NM_000038.5 (*APC*).

2.6 | Variation validation

For pathogenic or likely pathogenic variations detected by NGS of peripheral blood genomic DNA and WGA DNA, Sanger sequencing was used for variation validation. Sanger sequencing was also used to confirm the NIPT results using fetal genomic DNA extracted from amniotic fluid cells.

For large fragment deletion involving the whole *STK11* gene in family P3, a rough region of the breakpoint could be obtained from the variant allele frequency of SNPs combined with sequencing depths based on peripheral blood genomic DNA targeted sequencing data. Then, the exact breakpoint was located by a self-dependent innovation method called semitarget amplification followed by random sequencing (STARS). The general principles for STARS are as follows: (a) fragmented gDNA with a length of 1–2 kb was ligated to universal adapters at both ends; (b) DNA sequences within the breakpoint were enriched by semitargeted multiplex PCR amplification with a set of forward primers spanning the upstream and downstream of the breakpoint and the universal reverse primer; and (c) the products were fragmented again and into an ordinary NGS workflow, and the breakpoint position was obtained from the Compact Idiosyncratic Gapped Alignment Report string information after genome mapping. According to the STARS results, PCR amplification and agarose gel electrophoresis were performed on maternal peripheral blood genomic DNA and fetal DNA for validation.

2.7 | Pathogenic haplotype construction

In this study, segmented haplotype estimation and imputation tool[16] was used to construct haplotypes for each family. The informative SNPs are defined as SNPs that are heterozygous in the affected parent, but homozygous in the unaffected parent. The two haplotypes of the affected parent are called Hap 1 and Hap 2, representing pathogenic haplotype and wild-type haplotype, respectively. In maternal plasma, type 1 and type 2 SNPs are defined as informative SNPs correlate to the dosage increase when the fetus inherited Hap 1 and Hap 2, respectively. Specifically, in maternal inheritance, type 1 SNPs are the informative SNPs on Hap 1 and identical to the SNP alleles from the paternal side, while a type 2 SNP is one in which the allele inherited from the paternal side is identical to the allele on Hap 2; in paternal inheritance, type 1 SNPs are the informative SNPs on Hap 1 and different from the alleles from the maternal side, while a type 2 SNPs are the informative SNPs on Hap 2 and different from the alleles from the maternal side. If the paternal sample was absent, maternal heterozygous SNPs were taken as the informative SNPs. And the informative SNPs on Hap 1 and Hap 2 were taken as type 1 SNPs and type 2 SNPs, respectively, and used for dosage change (DC) calculation (Figure S2).

2.8 | Fetal DNA concentration measurement

The homozygous SNPs in both parents but for different alleles were used for the calculation of the fetal fraction.[9,12] The equation is as follows: $f = \Sigma 2d_b / \Sigma(d_b + d_a)$, where f means the fractional fetal DNA concentration in maternal plasma, d_b means the read count of the fetal alleles inherited from the father, and d_a means read count of the alleles shared by the fetus and the mother in the maternal cfDNA.[9,12]

2.9 | RHDO analysis

The RHDO analysis is based upon the DC of the informative SNPs. For a given informative SNP (informative SNP^i), the equation for the DC calculation is as follows:

$$DC_i = HAF^i_{\mathrm{cfDNA}} - HAF^i_{\mathrm{gDNA}} \quad (i = 1,\ 2\ldots,\ N)$$

N is the number of informative SNPs. HAF^i_{cfDNA} and HAF^i_{gDNA} represent the haplotype allele frequency of informative SNP^i in maternal cfDNA and gDNA, respectively.

Then, the function “segmentByCBS” in the R package “paired parent-specific circular binary segmentation”[17] was used to detect

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the recombination event, which segmented informative SNPs using the circular binary segmentation algorithm based on the DC_i. If more than one segment was calculated, a recombination event existed, and segment j (j = 1, 2..., N) represented each calculated segment. The Bayes factor (BF) was used to predict whether the fetus inherited the pathogenic haplotype in segment j as follows:

$$BF = \frac{P\left(DC^{j}_{\text{type 1}} - DC^{j}_{\text{type 2}} \middle| H1\right)}{P\left(DC^{j}_{\text{type 1}} - DC^{j}_{\text{type 2}} \middle| H2\right)}$$

$DC^{j}_{\text{type 1}}$ and $DC^{j}_{\text{type 2}}$ represent the calculated statistical DC values for type 1 SNPs and type 2 SNPs in segment j. H1 and H2 correspond to the hypothesis that the fetus inherits Hap 1 or Hap 2 from the affected parent, respectively. The difference in DC^j between type 1 and 2 SNPs was used to judge the inherited haplotype of the fetus (Table S1). $P\left(DC^{j}_{\text{type 1}} - DC^{j}_{\text{type 2}} \middle| H_k\right)$ $(k = 1,\ 2)$ denotes the possibility of the observed difference in DC^j under the hypothesis that Hap1 or Hap2 was inherited by the fetus. Bayes factor is the ratio of the two hypotheses; when $BF > 1$, the observed value shows a higher possibility in H1 than in H2, and the opposite consequence can be obtained when $BF < 1$. A threshold set was defined to obtain a reliable result: when $BF \geq 10$, the fetus inherited the pathogenic haplotype (Hap 1); when $BF \leq 0.1$, the fetus was classified as unaffected; and an unclear result was defined when BF[18] ranged from 0.1 to 10.

2.10 | Fetal DNA concentration influence evaluation

To evaluate the influence of the fetal fraction on the detection, we mixed one of the maternal genomic DNA and counterpart genomic DNA of the child for the participant family at gradients of 100:0, 98:2, 96:4, and 92:8 and fragmented through sonication to simulate plasma samples with 0%, 2%, 4%, and 8% fetal fractions. Three replicates were carried out for each fetal DNA concentration. Target enrichment sequencing and RHDO analysis were performed on all 12 spike DNA samples.

2.11 | Variation direct detection in paternal inheritance families

For the families with paternal inheritance, we took variation direct detection in maternal plasma as an additional validation for RHDO analysis. Based on the obtained paternal variants and the frequencies of the variants detected in the maternal cfDNA and genomic DNA, when the variant frequencies met the following requirements, the fetus was considered to be affected: the frequency of variation in maternal cfDNA was more than five times higher than the frequency of variation in the gDNA sample, and the frequencies in variation in the maternal cfDNA were in concordance with the fetal fractions in maternal plasma.

3 | RESULTS

3.1 | Clinical cases

We recruited nine families in this study including eight PJS families and one FAP family, and the pedigrees and clinical details are shown in Figure 1 and Table 1. For families P1, P2, P4, P6, P7, and P9 with reference to NM_000455.5 (*STK11*), the pathogenic variations were c.825_831del, c.375-2A>G, c.738C>A, c.766G>T, c.862+1G>A, and c.565_1108+737del, respectively, and for families P1, P2, P4, and P6, each family had an affected living offspring, while the living offspring of family P7 was unaffected. For family P8, the pathogenic variation was NC_000019.9: g.1198763_1213243, and the affected embryo was used for haplotype construction. For family P5, the pathogenic variation is NM_000038.5 (*APC*): c.3340C>T. For families P1, P4, P5 and P9, the variations were paternally inherited, while in family P2, P6, P7, and P8, the pregnant woman was the carrier of the variation. For family P3, the pregnant woman was the proband with a de novo heterozygous large deletion containing the whole *STK11*, and the exact deletion region spanned chr19: 1,158,705-1,241,129. Blood samples were extracted from the pregnant women at gestational ages of approximately 9–14 weeks. Among the fetuses analyzed, the fetuses of families P2 and P6 inherited the variation from the mother, while the others were unaffected. The noninvasive diagnosis results were consistent with the results of amniocentesis sampling detection (Figure S3, Figure S4).

3.2 | Effectiveness of target enrichment sequencing evaluation

For the PJS and FAP families, genomic DNA and cfDNA were enriched by the probes described in the materials and methods. All the sequencing data met the analysis requirements, and the average depth, uniformity and capture ratio for genomic DNA and cfDNA are shown in Table S2.

3.3 | The influence of fetal DNA concentration on detection

The fetal DNA concentrations in the P1-P9 families were 4.06%, 5.56%, 10.52%, 8.37%, 4.40%, 8.31%, 8.22%, 5.60%, and 8.19%, respectively. The average BF for the three repeated simulated samples with the expected fractional fetal DNA concentrations of 0%, 2%, 4%, and 8% was 3.47, 1.22E+10, 6.66E+34, and 1.98E+119, respectively. The correlation coefficient for the polynomial regression was 0.97, indicating that the BF and fetal fraction obey an obvious positive relationship (Figure 2). The sequencing depth of artificial samples ranged from 1086 to 1645. The calculated fetal fraction was consistent with expectation and highly stable among replications. For the BF, a value smaller than 0.1 or greater than 10

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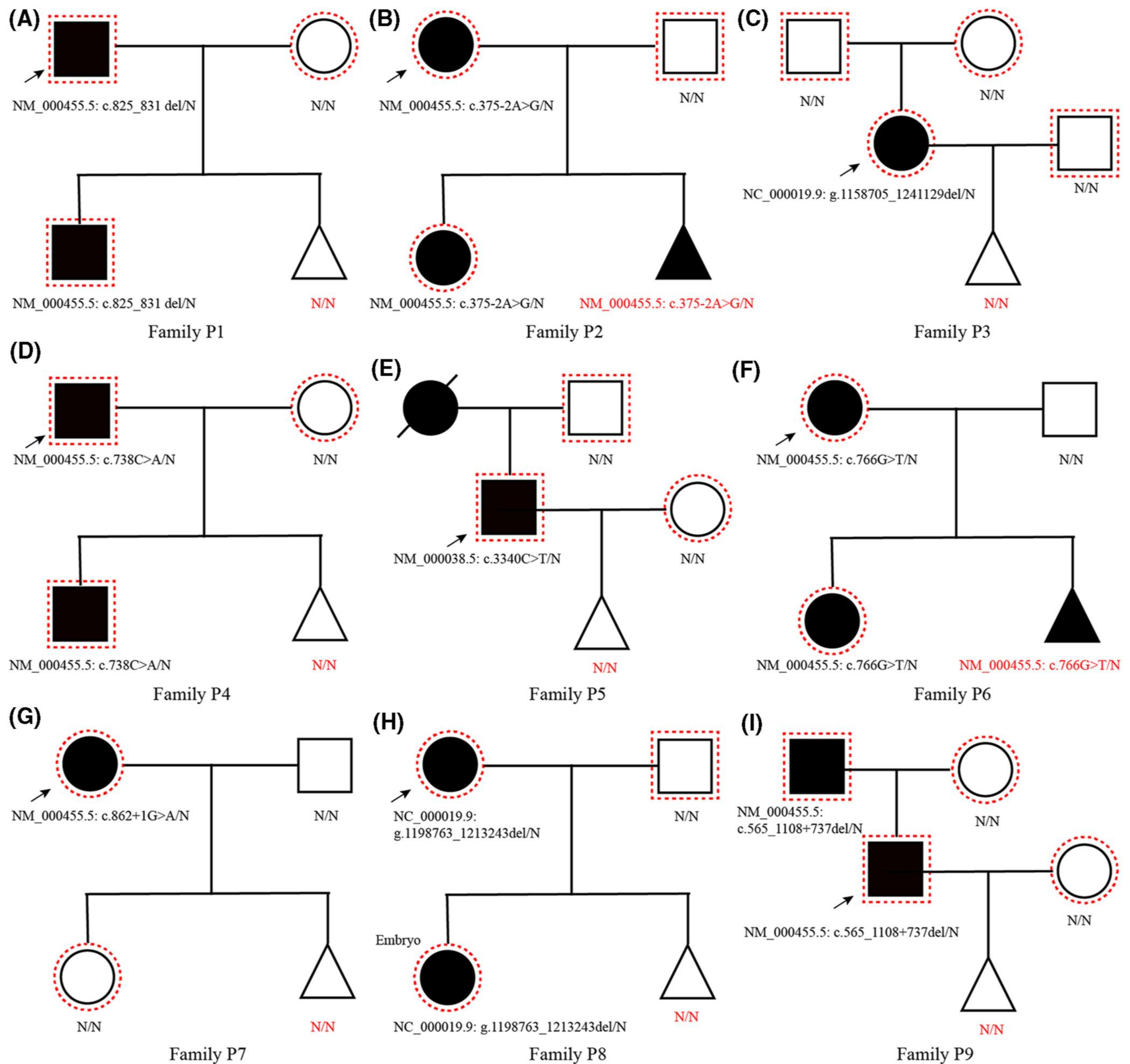

FIGURE 1 Pedigree for nine families that underwent target enrichment sequencing and haplotype analysis. Families P1-P4 and P6-P8 were at risk of Peutz-Jeghers syndrome (PJS), and family P5 was at risk of familial adenomatous polyposis (FAP). The pathogenic variations are shown in the figure. Families P1, P4, P5, and P9 are in paternal inheritance; in families P2, P3, and P6-P8, the pregnant women are patients. The family members denoted with red dotted lines were used for haplotype construction [Colour figure can be viewed at wileyonlinelibrary.com]

indicates that the fetus inherits the wild-type haplotype or pathogenic haplotype.[20] That is, the haplotypes of all the simulated samples excluding samples with a 0% fetal fraction can be detected even at a fetal fraction lower than 2%. The details for spike sample target enrichment sequencing and RHDO analysis are shown in Table S3 and Table S4.

3.4 | Family P3 analysis

For family P3, the proband was the pregnant woman, and the pathogenic change was a de novo large deletion containing the whole gene of *STK11* discovered by copy number analysis. The exact breakpoint on the chromosome was confirmed by STARS. The remaining SNPs at the disease locus for the mother naturally formed the haplotype because the counter-part haplotype was deleted. And the homozygous SNP alleles in the deletion region of the proband and her parents were used to deduce the pathogenic haplotype. Briefly, the homozygous SNP alleles in the proband were completely consistent with the homozygous SNP alleles in her mother (10/10), while the consistency rate was 27.59% (8/29) when compared with the father of the proband. That is, the pathogenic haplotype was linked with the father of the proband, which was then used for fetal haplotype deduction (Figure 3). A total of 99 type 1 SNPs and 79 type 2 SNPs were

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TABLE 1 Non-invasive prenatal diagnosis results for the five families

Family	Spouse with PJS/FAP	Disease	Gestational weeks for NIPT	Pathogenic variations	RHDO Type 1[a]	RHDO Type 2[b]	FF[c]	BF[d]	Result	Amniocentesis sampling
P1	Husband	PJS	10W + 2	NM_000455.5: c.825_831del	130	176	4.06%	1e−300	Unaffected	Unaffected
P2	Wife	PJS	14W + 1	NM_000455.5: c.375-2A>G	173	131	5.56%	1.6e+124	Affected	Affected
P3	Wife	PJS	10W + 0	NC_000019.9: g.1158705_1241129del	78	63	10.52%	2.1e−68	Unaffected	Unaffected
P4	Husband	PJS	9W + 5	NM_000455.5: c.738C>A	148	119	8.37%	1e−300	Unaffected	Unaffected
P5	Husband	FAP	8W + 6	NM_000038.5: c.3340C>T	84	38	4.40%	1.8e−300	Unaffected	Unaffected
P6	Wife	PJS	14W + 0	NM_000455.5: c.766G>T	306	306	8.31%	1.00e+300	Affected	Affected
P7	Wife	PJS	13W + 4	NM_000455.5: c.862+1G>A	401	401	8.22%	2.00e−300	Unaffected	Unaffected
P8	Wife	PJS	8W + 3	NC_000019.9: g.1198763_1213243del	110	84	5.60%	5.50e−172	Unaffected	Unaffected
P9	Husband	PJS	9W + 3	NM_000455.5: c.565_1108+737del	82	31	8.19%	8.20e−288	Unaffected	Unaffected

[a]The number of informative alleles on pathogenic haplotype (Hap 1).
[b]The number of informative alleles on the wild-type haplotype (Hap 2).
[c]Fetal Fraction.
[d]Bayes factor.

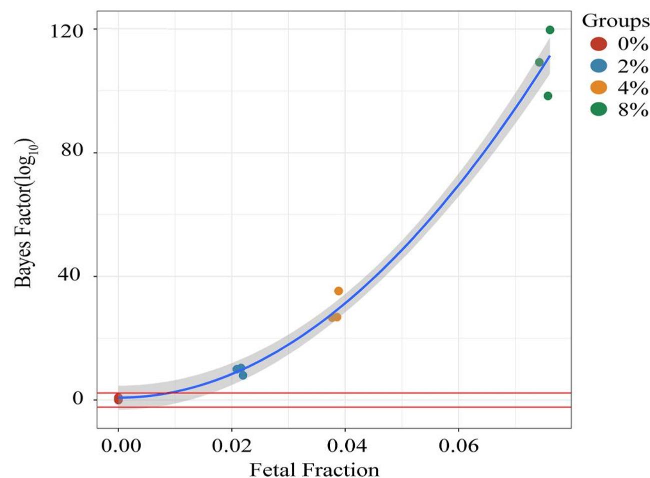

FIGURE 2 Relationship between the fetal fraction and Bayes factor (BF). The relationship between the fetal fraction in maternal plasma and BF is illustrated in the figure. The *x*-axis represents the concentration ratio of the fetal fraction in maternal plasma, and the *y*-axis represents the value of the BF after the transformation of the logarithm of 10. The correlation coefficient for the polynomial regression is 0.97. The different fetal genomic DNA ratios of simulative DNA samples are illustrated by circles in different colors, and 3 repeats for each fetal fraction were tested. The horizontal red lines with a value of 10 and −1 denote the threshold to distinguish the positive, no call and negative results [Colour figure can be viewed at wileyonlinelibrary.com]

identified, with a BF of 2.1E−68 indicating that the fetus was unaffected (Figure 4).

3.5 | Families P1, P2, P4, P8 and P9 analysis

For families P1, P2, P4, and P8, the parents and the affected offspring constructed trios for pathogenic haplotype analysis. A recombination event was found in family P1, as predicted between chr19: 610,035-618,158 (Figure 4). The Bayes factors for families P1, P4 and P8 were 1.0E−300, 1.0E−300, and 5.5E−172, respectively, indicating that the fetuses of the families were not affected. For family P9, the pathogenic haplotype construction was based upon the father and grandparents of the fetus. Considering that the pathogenic variations in families P1, P4 and P9 were all paternally derived, an additional direct analysis was also performed for the validation of NIPT results, and the same results were deduced (Table S5, Figure S5). For family P2, the BF was 1.6E+124, and the increase in the pathogenic haplotype (Hap 1) was consistent with the fetal DNA fraction, which meant that the fetus was affected (Figure 4).

3.6 | Families P6 and P7 analysis

For families P6 and P7, the paternal blood samples were not obtained, the haplotype construction was based upon the blood samples from the pregnant women and their living offspring. In these two

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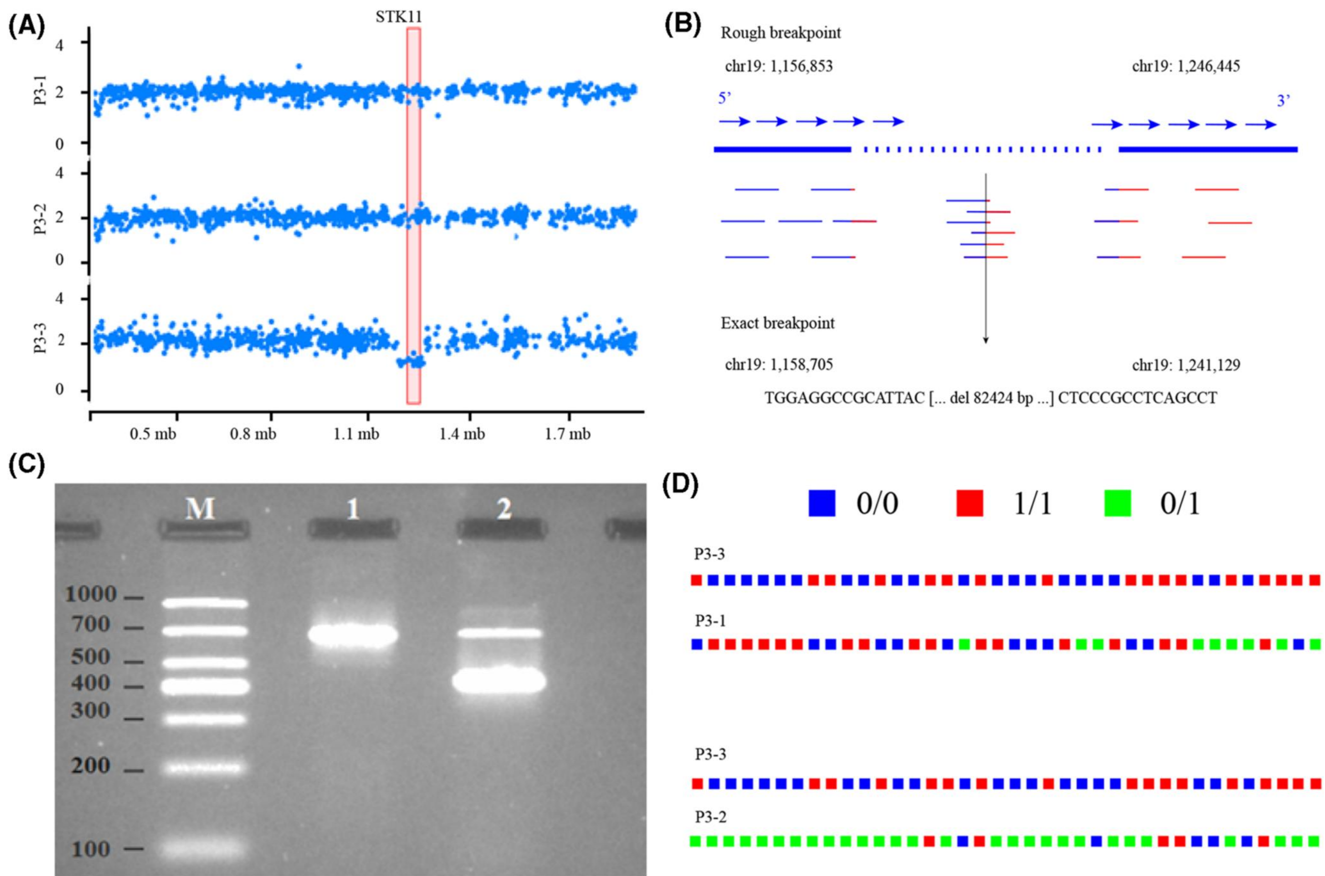

FIGURE 3 Genetic analysis of family P3. (A) Copy number analysis for the pregnant woman (P3-3) and her parents (P3-1 and P3-2). The *x*-axis represents loci in the panel, and the *y*-axis indicates the copy number. A de novo large deletion including the whole serine-threonine kinase 11 (STK11) gene was detected in the pregnant woman. (B) Schematic illustration of the detection of the exact breakpoint of P3-3 using semitarget amplification followed by random sequencing (STARS). A set of primers (blue right arrow, listed in Table S5) covering the upstream, intra- and downstream regions of the rough deletion region (chr19: 1,156,853-1,246,445) detected by copy number variation (CNV) analysis was used for polymerase chain reaction (PCR) amplification to enrich reads spanning the breakpoint. DNA sequences aligned to the upstream region are denoted by short blue lines, while those aligned to the downstream region are marked by short red lines. An exact deletion region spanning chr19: 1,158,705-1,241,129 (82,424 bp) was defined. (C) PCR product agarose gel electrophoresis (AGE) results for the validation of the large deletion in P3-3. Lane M: DL1000 DNA marker; Lane 1: unaffected control; Lane 2: P3-3, and a heterozygous deletion was defined. (D) Pathogenic haplotype deduction of P3-3. The colored squares represent different genotypes, namely, 0/0 and blue represent homozygous reference alleles, 1/1 and red represent homozygous alternative alleles, 0/1 and green represent heterozygous alleles. Except for the heterozygous SNPs in the parental sample, the genotype consistency between the patient (P3-3) and her mother (P3-2) was 100% (10/10), but for the father (P3-1), the genotype consistency was 27.59% (8/29). Therefore, the haplotype of the patient was deduced to be paternally derived [Colour figure can be viewed at wileyonlinelibrary.com]

cases, when the haplotype of the mother transmitted to the fetus, the DC value of the related SNP alleles will increase (the SNP alleles identical to those paternal transmitted SNP alleles) or not change (the SNPs different from those paternal transmitted SNP alleles), meanwhile the DC value of the SNP alleles related to the other haplotype which is not passed on to the fetus will not change or decrease. After calculation, the BF of families P6 and P7 was 1.00E+300 and 2.00E−300, respectively. That is, the fetus of family P6 was affected, and the fetus of family P7 was unaffected (Figure 4).

3.7 | Family P5 analysis

The father of the fetus in family P5 suffered from FAP, and the proband was the grandmother of the fetus although she had been dead for years. The pathogenic haplotype was deduced by exclusion of nonpathogenic haplotypes using the sequencing data of the father and grandfather of the fetus. The counts of informative SNPs identified were 84 for type 1 SNPs and 38 for type 2 SNPs. The calculated Bayer factor was 1.8E−300, indicating that the fetus did not inherit the disease (Figure 4). Because of paternal inheritance, the result was also validated by direct detection of paternal variation in maternal plasma (Table S5, Figure S5).

4 | DISCUSSION

In this study, we report cases of NIPT in pregnancies at risk of PJS and FAP, two classical hereditary colorectal cancer syndromes, based upon target enrichment sequencing and RHDO analysis. Although

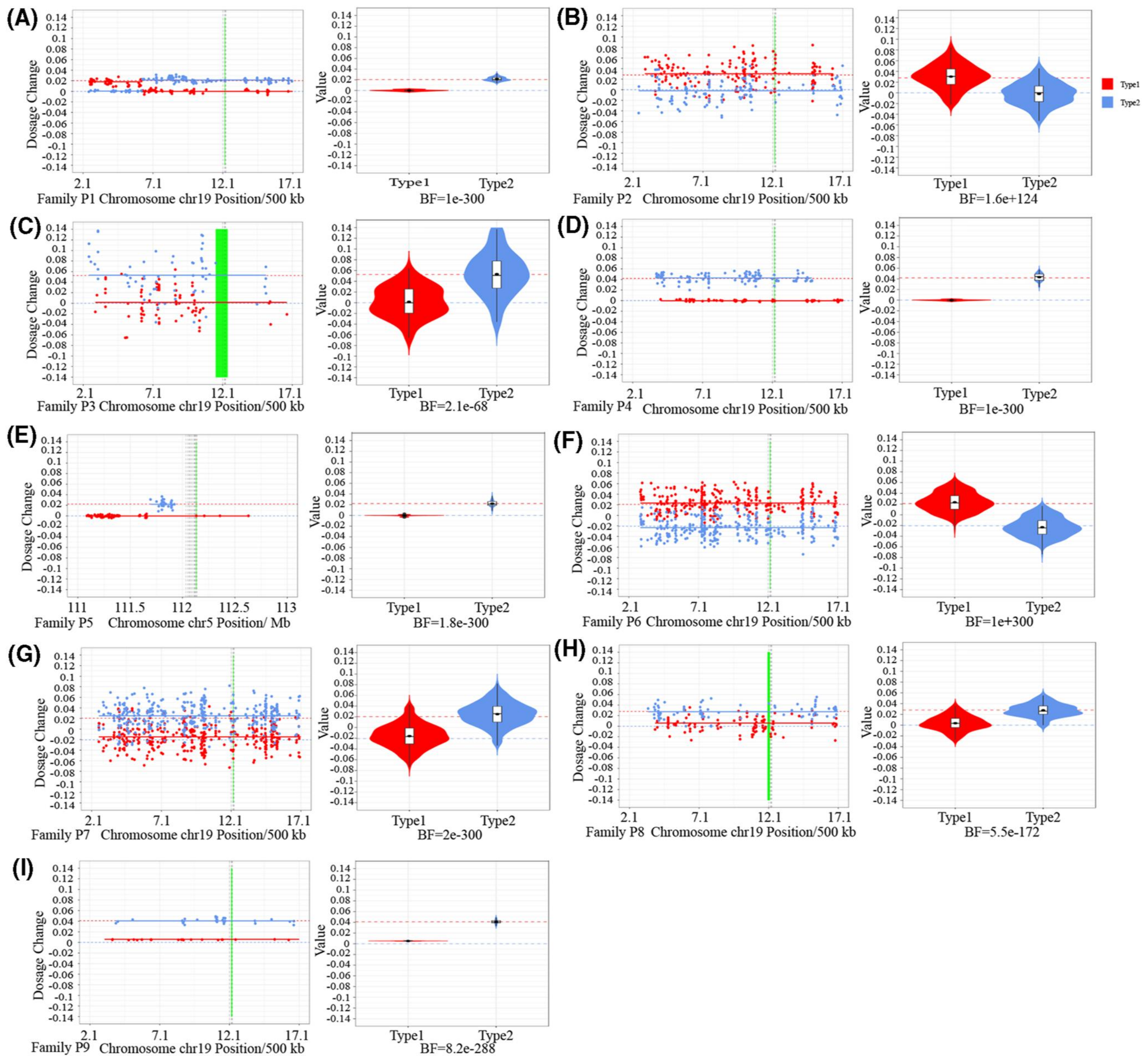

FIGURE 4 Relative haplotype dosage (RHDO) analysis results. (A–I) (left). Scatter plot of the dosage change (DC) value of each allele. The *x*-axis represents the genomic coordinates, and the *y*-axis represents the DC value. The green line indicates the position of the pathogenic variation, and the gray vertical dashed line marks the position of the serine-threonine kinase 11 (STK11) exons ([A–D] [left] and [F–I] [left]) or the APC exons (E) (left). The red dots denote the DC of the type 1 allele, while the blue dots are the DC of the type 2 allele. The red and blue horizontal lines are the center of the DC returned by the circular binary segmentation (CBS) algorithm. In Figure 4A (left), both lines crossing over at the switch site indicates that a recombination event exists. (A–I) (right). Violin plot of the DC. The shape around each box illustrates the distribution of DC. The red and blue dashed lines indicate the expected DC value for type 1 and type 2 alleles under the assumption that the fetus inherits the pathogenic haplotype. With the calculated Bayes factor (BF), the fetuses of families P2 and P6 were affected (B, F) [Colour figure can be viewed at wileyonlinelibrary.com]

hereditary cancer is relatively rare in individuals, when considered collectively, hereditary cancer contributes substantially to morbidity and mortality. The penetrance of the two diseases is almost 100%,[8,19] and the life-time cancer risk of PJS is 55%–85%[7] and that of FAP is almost 100%.[8] Though the ages of cancer diagnosis vary, PJS and FAP patients suffer a much higher incidence of tumors not only colorectal cancer but also tumors in other systems of the body. If the clinical diagnostic criteria for PJS are met, genetic germline screening of the *STK11* gene is warranted regardless of age, a baseline oesophagogastroduodenoscopy and colonoscopy is recommended at the age of 8 years in asymptomatic individuals with PJS.[7] For the children at-risk of FAP, they should take sigmoidoscopy beginning at 10–12 years of age every 1- to 2-year, and colonoscopy should be done as the first examination for the children initially screened at an older age.[8] And statistical data have shown that early screening and prevention can decrease the incidence of colorectal cancer.[20,21]

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Therefore, NIPT at early gestational age is of great significance for at-risk families both in terms of genetic counseling and whole-life monitoring.

For PJS patients, the large fragment deletion occurrence rate is approximately 30%,[22,23] which cannot be efficiently detected only by NGS. Loss of heterozygosity can be detected on chr5 in approximately 30%–40% of colorectal cancer cases,[24] and in the cohort of *APC* mutation-negative patients, approximately 4%–33%[25–27] of the patients had partial or entire *APC* gene deletions. In our study, a de novo large fragment deletion on chromosome 19, including the whole gene *STK11*, was detected by CNV analysis on the trio of the pregnant woman and their parents of family P3. The exact breakpoint was determined by STARS. The haplotype was constructed based on the trio of the pregnant woman and their parents without probands. Herein, loss of heterozygosity should be routinely analyzed in such diseases, and based on the methods we developed, NIPT by RHDO analysis can be initiated without probands in such conditions.

According to recent studies, the vast majority of pathogenic haplotype construction depends on the data from parents and their ailing offspring for sufficient informative SNPs in the fetus status judgment. While in the clinical practice, the conditions vary. For family P5, the grandmother of the fetus as the proband had been dead for years, and then the analysis of the pathogenic haplotype was based on the ailing father and healthy grandfather of the fetus. Compared with the haplotype construction based upon the information of the first-degree relatives of the fetus, there were fewer informative SNPs when using data from second-degree relatives. Families P6 and P7 were cases in which the pregnant women were probands and the paternal haplotypes were not known. The informative SNPs on Hap 1 and Hap 2 were taken as type 1 SNPs and type 2 SNPs, respectively, and used for DC calculation. For such cases, RHDO analysis can't be done when special conditions exit, namely, the differences between type 1 and type 2 allele frequencies will be zero or trend to zero, then no significant value of BF will be obtained when the vast majority of the SNP alleles from the parents transmitted to the fetus are different.

In this study, to address the sophisticated situations in fetal inheritance judgment, BF was used, which took an overall consideration of the sequencing depth, informative SNP number, fetal DNA fraction and the standard deviation of DC. Moreover, the interference of subjective judgment can be avoided, and the requirements for the analysts can be reduced, which makes standard operation, analysis and feasible clinical application easy.

The fetal DNA fraction plays a vital role in the process of NIPT based upon RHDO analysis, regardless of DNA extraction, target sequencing or RHDO analysis. For at-risk families resorting to NIPT, early detection and diagnosis are of significance, while a lower fetal fraction makes NIPT in early gestational age challenging. In our study, the fetal fraction in maternal plasma tested ranged from 4.06% to 10.52%, with 8 weeks and 6 days as the minimum gestational age. To further evaluate the influence of fetal DNA concentration on the results, artificial pregnancy samples with fetal DNA in gradient concentrations were tested, and the results showed that the fetal genotype could be successfully judged at a 2% fetal fraction. According to the above results and taking the turn-around time of five working days into account, the examining report could be provided in the first trimester of gestational age.

We recommend RHDO analysis as the first-tier test for NIPT as far as condition permits, while direct detection of paternally inherited variations in maternal plasma as additional validation. The reasons are as follows: firstly, RHDO approach is more accurate, it combines the statistical power of counting dozens to hundreds of informative SNPs located in neighboring genomic areas.[3,28] Because sequencing errors or PCR replication errors during library preparation of NGS, the inaccuracies will accumulate in low fetal fractions in early gestational stages by direct targeted NGS testing.[28–30] Secondly, RHDO approach is not limited to variation types. As there are no hotspot variations in gene STK11 and APC, the detected pathogenic variations vary, about 30%[22,23] of pathogenic variations are large fragment deletion for PJS, when the breakpoints locate in introns, the exact breakpoints needed for the direct analysis can't be obtained using the NGS method based on exon detection. Additionally, non-paternity condition exit. According to literature, paternal information is not always obtained, and it is estimated that the non-paternity incidence is between 3% and 10%,[31,32] which makes the doctor-patient communication and detection more delicate.[33]

Our study has some limitations. It is a relatively small sample size including eight families of PJS and one family of FAP due to the incidence of the diseases and some objective factors, such as family birth inclinations and ages of diagnosis, and more families should be detected to further evaluate our method.

5 | CONCLUSIONS

We report an NIPT assay for PJS and FAP using target sequencing on cfDNA in maternal plasma, which is the first NIPT report on hereditary colorectal cancer syndromes. Although the number of pedigree samples was limited, all the above results indicate that the NIPT strategy based on target sequencing of cfDNA in maternal plasma and RHDO analysis is accurate and feasible in sophisticated clinical situations.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author on reasonable request.

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REFERENCES

1. Lo YM, Corbetta N, Chamberlain PF, et al. Presence of fetal DNA in maternal plasma and serum. *Lancet*. 1997;350(9076):485-487.
2. Li Y, Zimmermann B, Rusterholz C, Kang A, Holzgreve W, Hahn S. Size separation of circulatory DNA in maternal plasma permits ready detection of fetal DNA polymorphisms. *Clin Chem*. 2004;50(6):1002-1011.
3. Lo YM, Chan KC, Sun H, et al. Maternal plasma DNA sequencing reveals the genome-wide genetic and mutational profile of the fetus. *Sci Transl Med*. 2010;2(61):61ra91.
4. Lo YM, Tein MS, Lau TK, et al. Quantitative analysis of fetal DNA in maternal plasma and serum: implications for noninvasive prenatal diagnosis. *Am J Hum Genet*. 1998;62(4):768-775.
5. Lun FM, Chiu RW, Chan KC, Yeung Leung T, Kin Lau T, Dennis Lo YM. Microfluidics digital PCR reveals a higher than expected fraction of fetal DNA in maternal plasma. *Clin Chem*. 2008;54(10):1664-1672.
6. Bianchi DW, Chiu RWK. Sequencing of circulating cell-free DNA during pregnancy. *N Engl J Med*. 2018;379(5):464-473.
7. Wagner A, Aretz S, Auranen A, et al. The Management of Peutz-Jeghers Syndrome: European Hereditary Tumour Group (EHTG) guideline. *J Clin Med*. 2021;10(3):473.
8. Syngal S, Brand RE, Church JM, Giardiello FM, Hampel HL, Burt RW. ACG clinical guideline: genetic testing and management of hereditary gastrointestinal cancer syndromes. *Am J Gastroenterol*. 2015;110(2):223-262; quiz 263.
9. Lam KW, Jiang P, Liao GJ, et al. Noninvasive prenatal diagnosis of monogenic diseases by targeted massively parallel sequencing of maternal plasma: application to beta-thalassemia. *Clin Chem*. 2012;58(10):1467-1475.
10. Parks M, Court S, Cleary S, et al. Non-invasive prenatal diagnosis of Duchenne and Becker muscular dystrophies by relative haplotype dosage. *Prenat Diagn*. 2016;36(4):312-320.
11. Parks M, Court S, Bowns B, et al. Non-invasive prenatal diagnosis of spinal muscular atrophy by relative haplotype dosage. *Eur J Hum Genet*. 2017;25(4):416-422.
12. New MI, Tong YK, Yuen T, et al. Noninvasive prenatal diagnosis of congenital adrenal hyperplasia using cell-free fetal DNA in maternal plasma. *J Clin Endocrinol Metab*. 2014;99(6):E1022-E1030.
13. Li H, Du B, Jiang F, et al. Noninvasive prenatal diagnosis of beta-thalassemia by relative haplotype dosage without analyzing proband. *Mol Genet Genomic Med*. 2019;7(11):e963.
14. Wang Z, Liu S, Liu S, Wang Y, Chen J, Wu B. Prenatal diagnosis in a hereditary Peutz-Jeghers syndrome family with high cancer risk. *BMC Med Genet*. 2018;19(1):66.
15. Richards S, Aziz N, Bale S, et al. Standards and guidelines for the interpretation of sequence variants: a joint consensus recommendation of the American College of Medical Genetics and Genomics and the Association for Molecular Pathology. *Genet Med*. 2015;17(5):405-424.
16. Delaneau O, Marchini J, Zagury JF. A linear complexity phasing method for thousands of genomes. *Nat Methods*. 2011;9(2):179-181.
17. Olshen AB, Bengtsson H, Neuvial P, Spellman PT, Olshen RA, Seshan VE. Parent-specific copy number in paired tumor-normal studies using circular binary segmentation. *Bioinformatics*. 2011;27(15):2038-2046.
18. Jakobsen JC, Gluud C, Winkel P, Lange T, Wetterslev J. The thresholds for statistical and clinical significance—a five-step procedure for evaluation of intervention effects in randomised clinical trials. *BMC Med Res Methodol*. 2014;14:34.
19. Hes FJ, Nielsen M, Bik EC, et al. Somatic APC mosaicism: an underestimated cause of polyposis coli. *Gut*. 2008;57(1):71-76.
20. Dekker E, Rex DK. Advances in CRC prevention: screening and surveillance. *Gastroenterology*. 2018;154(7):1970-1984.
21. Akimoto N, Ugai T, Zhong R, et al. Rising incidence of early-onset colorectal cancer—a call to action. *Nat Rev Clin Oncol*. 2021;18(4):230-243.
22. Aretz S, Stienen D, Uhlhaas S, et al. High proportion of large genomic STK11 deletions in Peutz-Jeghers syndrome. *Hum Mutat*. 2005;26(6):513-519.
23. Volikos E, Robinson J, Aittomäki K, et al. LKB1 exonic and whole gene deletions are a common cause of Peutz-Jeghers syndrome. *J Med Genet*. 2006;43(5):e18.
24. Fearnhead NS, Britton MP, Bodmer WF. The ABC of APC. *Hum Mol Genet*. 2001;10(7):721-733.
25. Nielsen M, Bik E, Hes FJ, et al. Genotype-phenotype correlations in 19 Dutch cases with APC gene deletions and a literature review. *Eur J Hum Genet*. 2007;15(10):1034-1042.
26. Sieber OM, Lamlum H, Crabtree MD, et al. Whole-gene APC deletions cause classical familial adenomatous polyposis, but not attenuated polyposis or “multiple” colorectal adenomas. *Proc Natl Acad Sci U. S. A*. 2002;99(5):2954-2958.
27. Aretz S, Stienen D, Uhlhaas S, et al. Large submicroscopic genomic APC deletions are a common cause of typical familial adenomatous polyposis. *J Med Genet*. 2005;42(2):185-192.
28. Wong FC, Lo YM. Prenatal diagnosis innovation: genome sequencing of maternal plasma. *Annu Rev Med*. 2016;67:419-432.
29. Snyder MW, Simmons LE, Kitzman JO, et al. Noninvasive fetal genome sequencing: a primer. *Prenat Diagn*. 2013;33(6):547-554.
30. Kitzman JO, Snyder MW, Ventura M, et al. Noninvasive whole-genome sequencing of a human fetus. *Sci Transl Med*. 2012;4(137):137ra76.
31. Macintyre S, Sooman A. Non-paternity and prenatal genetic screening. *Lancet*. 1991;338(8771):869-871.
32. Bellis MA, Hughes K, Hughes S, Ashton JR. Measuring paternal discrepancy and its public health consequences. *J Epidemiol Community Health*. 2005;59(9):749-754.
33. Fan HC, Gu W, Wang J, Blumenfeld YJ, El-Sayed YY, Quake SR. Non-invasive prenatal measurement of the fetal genome. *Nature*. 2012;487(7407):320-324.

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