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Modified methylated DNA immunoprecipitation protocol for noninvasive prenatal diagnosis of Down syndrome

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Abstract

Aim: Methylated DNA immunoprecipitation real-time quantitative polymerase chain reaction (MeDIP-real-time qPCR) has been introduced as noninvasive prenatal test that has shown absolute detection rate in the screening of Down syndrome. Herein, we aimed to propose a novel modification of MeDIP-qPCR and assess its potential to alleviate the overall cost of the test, being used in very early weeks of pregnancy, and develop it to a noninvasive prenatal diagnosis biosensor in future researches.

Methods: Cell-free fetal DNA (cffDNA) isolated from 60 pregnant women, including 29 normal and 31 trisomy 21 pregnancies, were analyzed using proposed MeDIP protocol. Enriched methylated DNA sequences were amplified through real-time qPCR using eight fetal-specific primer pairs. The status of samples was determined through the calculation of D-value with the cutoff point of zero.

Results: The sensitivity and specificity of the MeDIP protocols using nanoparticles were 100% and 100%, respectively.

Conclusion: Remarkable decrease in the price of MeDIP test per each patient would be a reasonable factor to confirm it on larger sample size. Moreover, the high detection rate of screening and the availability of the required instruments around the world make satisfactory reasons to be tested in earlier weeks of pregnancy, thanks to the high sensitivity of gold shell nanoparticles.

Key words: down syndrome, nanoparticle, noninvasive.

Introduction

Down syndrome (DS) is the leading cause of intellectual disability with similar incidence in all populations around the world (1 per 700 child births).[1] In addition to intellectual disability, these patients usually suffer from other complications, which makes their prenatal screening even more critical.[2] Currently, there are two approaches available for prenatal DS screening and diagnosis: noninvasive and invasive tests. Invasive diagnostic tests are recommended to individuals determined to be high risk by noninvasive screening methods and are associated with high risk of abortion (0.5–3%) and other prenatal complications. Noninvasive methods can be classified into two major categories. The first category, which is also conventional, includes first and second trimester biochemical screening in addition to measuring nuchal translucency through ultrasound sonography in late first trimester.[3,4] The second category is based on the

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detection of cell-free fetal DNA (cffDNA) in maternal plasma, which is possible in early pregnancy. It is thought that successful cffDNA methodologies of prenatal screening may replace invasive screening methods in near future of obstetrics medicine.[3]

Several noninvasive prenatal screening tests have been reported in recent decades, which are based on cffDNA.[5] Enrichment of fetal DNA from maternal circulation is a major concern in using cffDNA in all noninvasive prenatal test (NIPT) methods. Most methods are based on the sequencing of cffDNA using next-generation sequencing (NGS) technologies.[6,7] However, they are dependent on expensive DNA-sequencing instruments, which are not readily available worldwide particularly in developing countries. Enrichment of DNA sequences, which are specifically methylated in the fetus, would be an available alternative to sequencing-based methods. Patsalis *et al*. have shown more than 99% accuracy in discriminating between normal and chromosomally defective fetuses using differentially methylated regions (DMR) found on chromosomes 13, 18, 21 and X.[8]

Herein, we aimed to introduce a novel methylated DNA immunoprecipitation (MeDIP) protocol using gold shell nanoparticles to trap fetal DNA in order to determine the DS status of 60 pregnancies.

Methods

Subject/Materials

In this study, we selected 60 stored plasma samples, including 31 trisomy 21 (T21) and 29 healthy fetuses from 10 000 high-risk women (10–12 weeks) aged 18–45 years who had been referred to the Niloo Laboratory between the years 2013 and 2015 based on first trimester screening. The selected women have filled the consent form according to local ethical committee and samples drawn after Chorionic Virus Sampling (CVS) procedure were excluded from further assessment. A volume of 10 mL of whole blood was drawn from every referred woman. All enrolled participants had confirmatory karyotype and quantitative fluorescent-polymerase chain reaction (QF-PCR) analysis and the true status of 21 samples was blind to our research group. DNA was isolated from samples using QIAamp DNA Blood Mini Kit (Qiagen) according to the manufacturer's instructions. Quality and quantity of purified DNA were examined by NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies) besides capillary electrophoresis according to our previously reported protocol.[9] Obtained DNA samples were stored at −80°C until further processing.

Methylated DNA immunoprecipitation

DNA samples were subjected to MeDIP via suggested novel protocol. The newly developed MeDIP method comprised two main steps: immunoprecipitation of methylated DNA and its purification from total isolated DNA.

Core-shell gold-coated magnetic nanoparticles (Fe_3O_4-Au-NP) were synthesized according to our previously reported protocol[10], with the size of 18 ± 2 nm. It was then conjugated with 5-methyl cytosine antibody (Abcam) according to our optimized protocol (Appendices S1 and S2, Supporting Information) and the conjugation was confirmed through enzyme-linked immunosorbent assay.[10] Gold nanoparticle (GNP)– antibody conjugate was then used to enrich the methylated DNA sequences based on the following steps:

1. DNA samples were denatured at 95°C for 2 min and were immediately placed on ice. A total of 800 ng of each DNA sample was left to be used as input DNA.
2. Every DNA sample was mixed with 50 μL reaction buffer 1 (Appendices S1 and S2) and then was added to 2 mg of GNP–antibody conjugate resuspended in 100 μL phosphate-buffered saline 0.01 M. The mixture was incubated at 4°C for 2 h on a shaker.
3. Double-stranded DNA bound to GNP–antibody conjugate was separated from the solution using a magnet, which is held on the lateral surface of the microtube containing the reaction solution.
4. The precipitate was exposed to 150 μL blocking buffer (Appendices S1 and S2) and was incubated at 4°C for 20 min.
5. The solution was washed twice with wash buffer (Appendices S1 and S2) and incubated at 4°C for 10 min on a shaker.
6. To separate and digest the conjugated antibody, 1 μL Proteinase K (20 mg/mL; Roche) was dissolved in 60 μL reaction buffer 2 (Appendices S1 and S2). The mixture was then added to the precipitated GNP–antibody–DNA complex and placed in water bath at 56°C for 1 h.
7. A volume of 100 μL of phenol/chloroform (1/1) was added to the sample reaction and centrifuged at 12 000 rpm for 10 min to separate methylated DNA in the upper aqueous phase. This step was repeated again.

8. Eventually, methylated DNA sequences were resuspended in 20 μL ddH_2O following double precipitation in 70% ethanol.

Real-time PCR

The real-time quantitative PCR was performed to amplify fetal-specific methylated DNA sequences in MeDIP product compared to input samples using eight primer pairs picked up from previously published study.[11] The quality and specificity of those primer pairs was confirmed again using Primer Blast in NCBI and Gene Runner software. Quantitative amplification reaction was included: 5 μL of 2× SYBR Premix Ex Taq Master Mix (TaKaRa Bio, Inc.), 5 ng of DNA, 10 μM of each primer adjusted with nuclease-free water up to total volume of 10 μL. Amplification was performed in a Rotor Gene 6000 Real Time Machine (Corbett) and the cycling conditions were optimized to be in two steps, including 10 s holding at 95°C, followed by 40 cycles of denaturation at 95°C for 10 s and polymerization and extension at 60°C. Melt curve analysis was performed following each run of amplification to confirm the specificity of the amplified products and absence of primer dimer. Melting curve analysis consisted of 57°C for 15 s, which was followed by temperature increase to 95°C for 15 s at the rate of 1°C per second with continuous reading of fluorescence. To ensure elimination of unmethylated sequences within the MeDIP procedure, two primer pairs specific for unmethylated fetal region on chromosome 22 and β-actin gene, which is unmethylated in maternal DNA, were used to amplify the MeDIP products. Hypermethylated region on fetal chromosome 18 was also used as positive control. Anti-mouse immunoglobulin G antibody and anti-bovine serum albumin (Abcam) were used instead of anti-5-methylcytosine antibody in proposed MeDIP-real time PCR protocol as negative control. Moreover, the optimized protocols were performed on purified DNA from maternal white blood cells separated from primary whole blood samples as another control experiment.

Statistical analysis

The statistical analyses were performed using SPSS statistical software (IBM SPSS Statistics V22.0). Obtained Ct value from immunoprecipitated (IP) and input DNA samples of each patient were used to determine at first to calculate the ΔCt in the SPSS software as following:

$$\Delta Ct^{Normal} = Ct_{Normal\ Input} - Ct_{Normal\ IP}$$

$$\Delta Ct^{T21} = Ct_{T21\ Input} - Ct_{T21\ IP}$$

Then, normalized ΔCt value was calculated using E number (normalized ΔCt value of T21/Normal = $E^{\Delta Ct\ value\ T21/Normal}$), wherein E was corresponded to E = 10[−1/slope].

For calculating the D value, ratio value of every 8 primer pair was calculated in SPSS software using the following formula for every T21 and normal sample:

Ratio value sample: DMR, Norm ΔCt Sample (Normal or T21)/Median (Norm ΔCt Normal), wherein the median of ΔCt Normal was calculated considering the Ct of all normal samples for every primer pair. Finally, D-value was derived from the formula D = −6.331 + 0.959XEP4 + 1.188XEP5 + 0.424XEP6 + 0.621XEP7 + 0.028XEP8 + 0.387XEP10 − 0.683XEP11+ 0.897XEP12, wherein XEPi was the ratio value of each studied DMRs. When the D-value was higher than zero, the fetus was determined to have T21 while it was 0 or lower than zero, the fetus was considered to be normal. The accuracy of nanoparticle-based MeDIP-real-time PCR technique was determined through cross tab in SPSS software (IBM SPSS Statistics V22.0).[8]

Results

Nanoparticle-based MeDIP protocol could confirm the true status of all T21 and normal samples including blind samples identified by asterisk (Table S1). Distribution of D-values obtained from both T21 and normal samples was represented in Figure 1. The sensitivity and specificity of nanoparticle MeDIP-real-time PCR protocols were 100% and 100%, respectively. The positive and negative predictive values were 100% and 100%, respectively. D-value was meaningfully different between two groups analyzed by novel protocol (P = 0.01). Of note, amplification of MeDIP products obtained from negative control experiments in the proposed MeDIP protocol was failed for all of the eight fetal-specific primers.

Discussion

The critical role of epigenetic mechanisms, especially DNA methylation, has been elucidated in fetal development.[12,13] There are some reports focused on

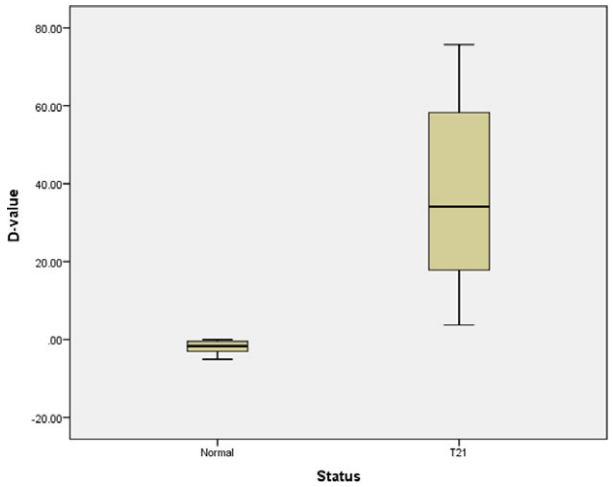

Figure 1 D-value distribution among trisomy 21 (T21) and normal samples.

finding DMRs in fetal DNA to generate a valuable tool in screening and diagnosis of fetal chromosomal abnormalities. Lim *et al.* achieved 77.8% sensitivity and 83.3% specificity in screening of T21 by studying 108 pregnancies, including 18 affected and 90 normal fetuses.[14] They determined the unmethylation index of PDE9A through quantitative methylation-specific PCR (qMSP-PCR). Methylation-specific PCR includes bisulfate modification of target DNA template, which is associated with high levels of DNA template degradation (>80%) and requires precise primer design to avoid nonspecific amplifications. Study on another fetal hypermethylated gene, holocarboxylase synthetase, through methylation-sensitive restriction endonuclease digestion and real-time PCR led to misclassification of one T21 and normal sample out of 14 T21 and 33 euploid samples, respectively.[15] Using methylation-sensitive restriction endonuclease usually suffers from the fact that most of the restriction enzymes are suppressed by their recognition sites. Moreover, this technique necessitates selection of sequences containing specific recognition sites to be recognized by restriction endonucleases.[16]

Affinity enrichment of methylated DNA sequences via 5-methyl cytosine antibody may be a better alternative choice in methylation studies. MeDIP-array study performed by Papageorgiou *et al.* was the largest methylation assay focused on seeking DMRs between maternal and fetal DNA.[17] It was followed by introduction of MeDIP-real-time PCR analysis with greater than 99% accuracy in discrimination between DS and normal pregnancies.[8] In recent decades, nanotechnology and nanoscience have opened new windows toward different aspects of biology and medicine. There are many medical products containing nanomaterial frequently used in medical investigations.[18] In the present study, we tried to enhance the efficacy of the MeDIP technique using Core-shell gold-coated magnetic nanoparticles conjugated with 5-methyl cytosine antibody to enrich eight DMRs, which have been previously evaluated.[11] To the best of our knowledge, this is the primary study to investigate the potential of nano MeDIP-real-time PCR in prenatal screening of DS with a novel modification in the MeDIP part of technique. It was demonstrated that we could attain 100% sensitivity, 100% specificity and 100% detection rate in identification of T21 cases using our optimized nanoparticle-based MeDIP-real-time PCR technique. The accuracy of proposed protocol was more than the same previously performed assay by Papageorgiou *et al*., which was determined to be greater than 99%. Following Papageorgiou *et al.*'s report, similar evaluation study was performed on three T21 and 18 normal samples. It was found that the sensitivity and specificity of MeDIP-real-time PCR test was 33% and 73%, respectively, which were significantly different relative to our proposed protocol and Papageorgiou *et al.*'s results.[19] In the other study, MeDIP-real-time PCR test was implicated to evaluate the status of six T21 and five normal samples using MagMeDIP-Diagenode commercial kit.[20] The sensitivity of the mentioned kit-based assay was shown to be lower than novel presented protocol (83%), whereas the specificity was similar to us (100%). It is noticeable that the sample size of the two previously mentioned studies was considerably lower than those of Papageorgiou *et al.* and the present assays. Either lower sensitivity or lower specificity in each replicated MeDIP-real-time PCR studies following Papageorgiou *et al.*'s report with perfect result may be indicating that in addition to the MeDIP protocol and used materials, the threshold and cutoff value needs to be modified. However, replication of perfect detection rate of previously described MeDIP protocol in the present study versus two mentioned studies may be due to proposed nanoparticle modification and its advantages in capturing methylated target sequences. Given the larger surface area of nanoparticles compared to larger particles as well as Dynabeads, which are frequently used in MeDIP protocols, nanoparticles have higher binding affinity to their specific target.[21] In addition, monoclonal antibodies tend to be self-associated in higher

concentrations, and therefore, using as lower as possible concentrations of antibody can decrease the chance of antibody aggregation and false-positive results.[22] Absolute elimination of unmethylated sequences from our tested samples in nanoparticle-based MeDIP protocol would be strong evidence on this claim. Of note, it was described that the affinity of antibody did not change after conjugation with nanoparticles.[23] Therefore, antibody–nanoparticle conjugate may have higher affinity to methylated DNA sequences in comparison with that using antibody alone and can improve the accuracy of MeDIP-real-time PCR test as revealed by the perfect detection rate of our nanoparticle-based protocol. According to Wilson's criteria, the best screening tests not only should be as cheap as possible but also should be easy to perform.[24] The estimated price of MeDIP-real-time PCR test based on our protocol is estimated to be 20€ per patient, which is definitely lower than previously determined cost (400€) and the minimum price paid for NGS test.[25] The most expensive material in every MeDIP reaction is antibody that was used in lower concentrations in the proposed protocol compared to every other present protocols besides it caused to higher detection of target cffDNA. Moreover, performing MeDIP-real-time PCR test does not require any special instruments as well as NGS and could be established in every diagnostic laboratory around the world. The other major advantage of our protocol is its potential to be modified and improved in order to be used in the structure of a novel home-based biosensor, which is our ongoing project. It is worth to note that implication of GNP in constructing NIPT biosensors based on colorimetric change upon binding to target molecules can dramatically enhance detection of very low cffDNA concentrations as less as 50 fM and may provide powerful screening method in very early weeks of pregnancy.[26]

Mosaicism is the major challenge and pitfall of all NIPT methods, including proposed methods, which should be considered in further modifications. In addition, the coincidence of maternal cancer, maternal copy number variations and placental mosaicism are the other limitations of the present protocol, which should be addressed in future assays.

In follow-up to this study, the accuracy of the nanoparticle-based MeDIP-real-time PCR protocol should be confirmed on a larger sample size to be implicated in constructing homemade nanobiosensor. The horizons to this application not only can alleviate the cost and time around conventional prenatal screening tests, but also may have significant effect on reducing parental anxiety due to achievement of results during very earlier weeks of pregnancy. By implicating nanotechnology, for the second time we could demonstrate that MeDIP-real-time PCR protocol has very high detection rate in NIPT of DS.

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Disclosure

None declared.

References

1. Petersen MBMM. Nondisjunction in trisomy 21: Origin and mechanisms. *Cytogenet Cell Genet* 2000; **91**: 199–203.
2. Epstein CJKJ, Anneren G, Antonarakis SE *et al*. Protocols to establish genotype-phenotype correlations in Down syndrome. *Am J Hum Genet* 1991; **49**: 207–235.
3. Tabor A, Alfirevic Z. Update on procedure-related risks for prenatal diagnosis techniques. *Fetal Diagn Ther* 2010; **27**: 1–7.
4. Crombag NM, Bensing JM, Iedema-Kuiper R, Schielen PC, Visser GH. Determinants affecting pregnant women's utilization of prenatal screening for Down syndrome: A review of the literature. *J Matern Fetal Neonatal Med* 2013; **26**: 1676–1681.
5. Gekas J, Langlois S, Ravitsky V *et al*. Identification of trisomy 18, trisomy 13, and Down syndrome from maternal plasma. *Appl Clin Genet* 2014; **7**: 127–131.
6. Guex N, Iseli C, Syngelaki A *et al*. A robust second-generation genome-wide test for fetal aneuploidy based on shotgun sequencing cell-free DNA in maternal blood. *Prenat Diagn* 2013; **33**: 707–710.
7. Fan HC, Quake SR. Sensitivity of noninvasive prenatal detection of fetal aneuploidy from maternal plasma using shotgun sequencing is limited only by counting statistics. *PLoS One* 2010; **5**: e10439.
8. Patsalis PC, Tsaliki E, Koumbaris G, Karagrigoriou A, Velissariou V, Papageorgiou EA. A new non-invasive prenatal diagnosis of Down syndrome through epigenetic markers and real-time qPCR. *Expert Opin Biol Ther* 2012; **12** (Suppl 1): S155–S161.
9. Karami F, Noori-Dalooii MR, Modarressi MH. Introducing a new and simple protocol for capillary electrophoresis of cell free fetal double stranded DNA. *J Sci I R Iran* 2014; **25**: 305–308.
10. Ahmadi A, Shirazi H, Pourbagher N, Akbarzadeh A, Omidfar K. An electrochemical immunosensor for digoxin using core-shell gold coated magnetic nanoparticles as labels. *Mol Biol Rep* 2014; **41**: 1659–1668.
11. Tsaliki E, Papageorgiou EA, Spyrou C, Koumbaris G, Kypri E, Kyriakou S *et al.* MeDIP real-time qPCR of maternal peripheral blood reliably identifies trisomy 21. *Prenat Diagn* 2012;**32**:996–1001.

12. Fagiolini M, Jensen CL, Champagne FA. Epigenetic influences on brain development and plasticity. *Curr Opin Neurobiol* 2009; **19**: 207–212.
13. Plasschaert RN, Bartolomei MS. Genomic imprinting in development, growth, behavior and stem cells. *Development* 2014; **141**: 1805–1813.
14. Lim JH, da Lee E, Park SY *et al*. Disease specific characteristics of fetal epigenetic markers for non-invasive prenatal testing of trisomy 21. *BMC Med Genomics* 2014; **7**: 1.
15. Tong YK, Chiu RW, Akolekar R *et al*. Epigenetic-genetic chromosome dosage approach for fetal trisomy 21 detection using an autosomal genetic reference marker. *PLoS One* 2010; **5**: e15244.
16. Ramsahoye BH, Burnett AK, Taylor C. Restriction endonuclease isoschizomers ItaI, BsoFI and Fsp4HI are characterised by differences in their sensitivities to CpG methylation. *Nucleic Acids Res* 1997; **25**: 3196–3198.
17. Papageorgiou EA, Karagrigoriou A, Tsaliki E, Velissariou V, Carter NP, Patsalis PC. Fetal-specific DNA methylation ratio permits noninvasive prenatal diagnosis of trisomy 21. *Nat Med* 2011; **17**: 510–513.
18. Chekman IS. Nanoparticles: Properties and application prospects. *Ukr Biokhim Zh* 2009; **81**: 122–129.
19. Tong YK, Chiu RW, Chan KC, Leung TY, Lo YM. Technical concerns about immunoprecipitation of methylated fetal DNA for noninvasive trisomy 21 diagnosis. *Nat Med* 2012; **18**: 1327–1328 author reply 1328–1329.
20. Gorduza EV, Popescu R, Caba L *et al*. Prenatal diagnosis of 21 trisomy by quantification of methylated fetal DNA in maternal blood: Study on 10 pregnancies. *Rev Romana Med Lab* 2013; **21**: 275–284.
21. Karra N, Nassar T, Ripin AN, Schwob O, Borlak J, Benita S. Antibody conjugated PLGA nanoparticles for targeted delivery of paclitaxel palmitate: Efficacy and biofate in a lung cancer mouse model. *Small* 2013; **9**: 4221–4236.
22. Liu J, Nguyen MD, Andya JD, Shire SJ. Reversible self-association increases the viscosity of a concentrated monoclonal antibody in aqueous solution. *J Pharm Sci* 2005; **94**: 1928–1940.
23. Xu Y, Baiu DC, Sherwood JA *et al*. Linker-free conjugation and specific cell targeting of antibody functionalized iron-oxide nanoparticles. *J Mater Chem B* 2014; **2**: 6198–6206.
24. Wilson JMG, Jungner G. *Principles and Practice of Screening for Disease*, Vol. **22**. *WHO Chronicle*. Geneva Switzerland: World Health Organization, 1968.
25. Patsalis PC. A new method for non-invasive prenatal diagnosis of Down syndrome using MeDIP real time qPCR. *Appl Transl Genom* 2012; **1**: 3–8.
26. Cao X, Ye Y, Liu S. Gold nanoparticle-based signal amplification for biosensing. *Anal Biochem* 2011; **417**: 1–16.

Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Appendix S1. Composition of used buffers.

Appendix S2. Conjugation protocol.

Table S1. D-Values obtained by novel MeDIP-Real time PCR protocol.