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Short report

Methylation-sensitive restriction enzyme nested real time PCR, a potential approach for sperm DNA identification

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ABSTRACT

Mammal H19 gene is an imprinting gene in which the paternal allele is silenced. On H19 imprinting control region (ICR), one of the mechanisms regulating the paternal allelic specific silence is DNA methylation in somatic cells throughout the individual's whole life. Nevertheless, this pattern of DNA methylation is erased and re-established in germline. As results, in mature sperm H19 ICR shows biallelic methylation instead of paternal specific methylation in somatic cells. Although the data were mainly from experiments on mice the same mechanisms are believed existing in human germline. We designed an experiment to probe the sperm DNA by methylation sensitive restriction enzyme based nested qPCR (MSRE-nested-qPCR). The genomic DNA digested/undigested by HhaI was amplified by outer primers encompassing four HhaI sites on H19 ICR. These PCR products were used as templates for second round real-time PCR to quantify the DNA methylation level. The results showed that DNA methylation level at H19 ICR were 55.27 ± 8.36% in 32 blood samples and 101.94 ± 11.66% in 31 semen samples. Based on our data sperm DNA could be identified if H19 ICR methylation level is over 78.62%.

1. Introduction

Human genomic imprinting refers to monoallelic expression caused by parental-specific gene marking. There are at least 90 imprinting genes in human genome. Many imprinting genes form clusters spanning 1 Mb or more. One of the clusters located on chromatin 11p15.5 is H19/Igf2 imprinting cluster. In this cluster H19 gene shows the maternal expression, whereas paternal allele is silenced by means of DNA methylation and histone modification.[1] Therefore, the paternal allele-specific methylation resided in a GC-rich region on H19 ICR is one of the characterizations of H19 imprinting markers.[2]

A pile of documents focused on parent-of-polymorphism in H19 gene ICR reveal to paternal specific allele methylated SNP or VNTR in samples originated from blood, skeletal muscle, skin, cerebrum, liver etc.[3–5] Based on Naito reports,[4] four HhaI sites are involved in paternal specific methylation located on human chromosome 11 2004477-2006247 (Supplementary data 1) on H19 ICR. That means only about half amount of DNA originated from somatic cells would be intact in this region after HhaI digestion. Whereas Jing-Yu Li et al. reported that H19 ICR retains both paternal and maternal methylation in mature sperm of mouse.[6] We presume that human mature sperm also shows such biallelic methylation status in this region, and thus the HhaI sites either from paternal allele or maternal allele are resistant to HhaI digestion. The differential methylation levels on H19 ICR between sperm and somatic cells prompt us to design an experiment to discriminate sperm DNA and somatic DNA.

The existence of semen in forensic samples usually couples to sexual assault crime. Until now there are many approaches to be employed to discover the semen by presumptive tests and confirmatory tests.[7] Seminal acid phosphatase test is often used to detect semen as presumptive test because of occurrence of phosphatase activity from semen as well as other tissues. Searching for sperm cells under microscope is a reliable method to identify the semen. However, the examination under microscope is a time-consuming task and needs the skilled personnel to perform. Albeit immunological assay can also be used to detect the seminal specific antigen such as

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prostate specific antigen(p30) and semenogenlin(Sg),[7,8] protein is less stable than DNA and degradation of protein might cause false-negative result for aged casework specimen examination.

Tissue specific mRNA profiling and miRNA signature are used to trace back to biological stain origin including semen in recent papers.[910] But in comparison with DNA, RNA molecules are far more easily degraded because of widely distribution of ribonuclease(RNnas) enzyme in environment.[11]

Saverio Giapaoli et al.[12] reported forFLUID kit testing results from eight laboratories for identification of vaginal fluids by analyzing the commensal bacteria genomes. The multiplex real-time PCR was used to probe genomic DNA from six microbes. The vaginal stains were identified by all eight laboratories with the simple and efficient multiplex real-time PCR. In some sexual abuse criminal cases it is useful evidence to find the vaginal stain on relevant matter of criminal suspect when semen is not discovery. However, sperm identification followed by STR profiling, in most cases, is more favorable evidence for court.

Recently, some groups developed new semen identification kits based on differential DNA methylation in human genomic DNA.[13–16] With aid of the methylation sensitive restriction enzyme the DNA fragments linked to differential DNA methylation markers are amplified and separated by capillary electrophoresis. Ja Hyun an et al.[13] tested four human tissue-specific differentially methylated regions(tDMRs) by MSRE-PCR and methylation SNaPshot. The sperm DNA can be identified from 40 semen samples, and furthermore the menstrual blood and vaginal fluids were dicriminated from blood and saliva samples by these four tDMRs. Adam Wasserstrom et al.[14] designed a practical semen proving system with adequate negative and positive control. In their semen identification profile the sperm DNA can be distinguished by MSRE-PCR-capillary electrophoresis from blood and saliva, the latter two samples are mostly mixed with semen in sexual abuse cases. The method of differential DNA methylation marker testing used by these two groups promises to automotive semen identification and avoids to the disadvantages of traditional semen identification ways. However, multiplex fluorescent primers for PCR and capillary electrophoresis of amplified products make testing kits being expensive. Here we recommend a rather simple and non-expensive approach to detect sperm DNA. Without figuring out the size of DNA fragments, The DNA is subjected to HhaI digestion, followed by two round of PCR. In our preliminary study, sperm DNA can be identified by this simple method.

2. Material and method

2.1. Experimental design

Fig. 1A and B shows H19 HhaI sites analyzed in this assay and MSRE-nested-qPCR workflow respectively.

2.2. Sample collection

Thirty-two blood samples were collected from male and female volunteers. Semen samples were from 31 healthy male volunteers. Seventeen abnormal semen samples from men suffered from oligoasthenoterazoospermia according to criteria of the World Health Organization[17] were gathered up from Second Hospital in Shanxi Medical University. The ways of sample collection were based on the procedures approved by the Ethics Committee of Shanxi Medical University.

Collections of samples including 100 blood and 8 semen samples were in the same way as mentioned above. These samples are for preliminary test. The sample collection procedure was obeyed the rule of Ethics Committee of Shanxi Medical University.

2.3. DNA extraction and MSRE-nested-PCR

The phenol-chloroform extraction protocol was used for DNA extraction from donor samples.[18] The composition of digestion and PCR mixture (50 μl) was as follows: two nanogram of human genomic DNA, 1.5 μl Quick Cut™ HhaI (Takara), 1.25 U *TaKaRa Ex Taq*® HS, 25 μl 2 × GC BufferI, 0.2 mM each dNTP, 0.2 μM of A-F and A-R primers and sterile water to a total volume of 50 μl.

The combined digestion and first round PCR cycling program was 37 °C for 15 min, 65 °C for 20 min, 95 °C for 5 min and thirty-five cycles of 94 °C for 30s, 63 °C for 30s and 72 °C for 2 min with last extension at 72 °C for 5 min in Life Touch Thermal Cycler (BIOER).

The second round of qPCR cocktail contained 1 μl of diluted first round PCR amplicons (1/30), 10 μl 2 × SYBR® *Premix Ex Taq*™ II(TaKaRa),1 μl inner primers (10 pmol/μl each) and 8 μl sterile water. After denaturing 95 °C for 30s, the PCR profile for 35 cycles was 95 °C for 10s, 55 °C for 20s and 72 °C for 20s in Real-Time QPCR System Mx3000P(*Stratagene*).

2.4. Subcloning of DNA fragment in human H19 upstream region (chrom 11 2004477-2006247)

All HhaI sites in plasmid DNA isolated from *E Coli*. can be cut by HhaI because of lacking of CpG MTases in *E Coli*. Therefore, we made a construct by subcloning DNA fragment in human H19 upstream region (chrom 11 2004477-2006247) into pGL3 plasmid to set up a non-methylation HhaI site control. DNA fragment to be inserted was amplified by PCR using primers sub-clone F and sub-clone R. The PCR products were digested by KpnIand XhoIand cloned into pGL3 vector (Promega) with KpnIand XhoIsites.

2.5. Evaluation of MSRE-nested-qPCR amplification bias

The DNA fragments in human H19 upstream region from plasmid with or without HhaI digestion were mixed by different ratios and subjected to two round PCR amplification. The ratios of amplicons between HhaI digestion and un-digestion were compared to ratios of DNA fragment mixture to evaluate PCR amplification bias.

Primers.

Outer primers for MSRE-Nested-qPCR analysis[4]:

A-F: 5′-GGGTCATTATAGACGCAATCG-3'

A-R: 5′-AGAACCTGTTGGGCGGTTAGA-3'

Inner primers for MSRE-Nested-qPCR analysis:

C-F: 5′-CTGGGAACACTGGGAAAG-3'

C-R: 5′-AGAAACTGGGCTGATTGG-3'

H19 ICR subcloning primers:

Sub-clone F: 5′-CAGTGGTACCGGGTCATTATAGACGCAATCG-3'

Sub-clone R: 5′-TACGCTCGAGAGAACCTGTTGGGCGGTTAGA-3'

2.6. Statistic analysis

SPSS17.0 software was used to analyze data. We used Shapiro-Wilk test and independent T-test to test the methylation measurement level distribution function and to compare methylation measurement level in blood samples and semen respectively. A cut-off corresponding to average methylation measurement level in sperm DNA minus 2 standard deviation(SD) was used to discriminate sperm DNA which was in biallelic methylation status.