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TSPY4 is a novel sperm-specific biomarker of semen exposure in human cervicovaginal fluids; potential use in HIV prevention and contraception studies

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Abstract

Background: Developing an objective, reliable method to determine semen exposure in cervicovaginal fluids is important for accurately studying the efficacy of vaginal microbicides and contraceptives. Y-chromosome biomarkers offer better stability, sensitivity, and specificity than protein biomarkers. TSPY4 belongs to the TSPY (testis-specific protein Y-encoded) family of homologous genes on the Y-chromosome. Using a multiplex PCR amplifying TSPY4, amelogenin, and Sex-determining region in the Y chromosome (SRY), our objective was to determine whether a gene in the TSPY family was a more sensitive marker of semen exposure in cervicovaginal fluids than SRY.

Study Design: The multiplex polymerase chain reaction (PCR) was developed using sperm and vaginal epithelial (female) DNA. Diluted sperm DNA and mixed male/female DNA was used to determine the sensitivity of the multiplex PCR. Potential interference of TSPY4 amplification by components in cervicovaginal and seminal fluids was determined. TSPY4 and SRY amplification was also investigated in women participating in a separate IRB-approved clinical study in which cervicovaginal swab DNA was collected before semen exposure and at various time points after exposure.

Results: TSPY4, SRY, and amelogenin were amplified in sperm DNA, but only amelogenin in female DNA. The limit of sperm DNA from which TSPY4 could be amplified was lower than SRY (4 pg vs 80 pg). TSPY4 could also be amplified from mixed male/female DNA. Amplification was not affected by cervicovaginal and seminal components. Using cervicovaginal swab DNA from three women before and after semen exposure, TSPY4 was detected up to 72 h post exposure while SRY detection was observed up to 24–48 h. TSPY4 was detected up to 7 days post exposure in one out of three women.

Conclusions: We have demonstrated that TSPY4 is a new sensitive, and sperm-specific biomarker. The multiplex PCR incorporating this new biomarker has potential to be an objective measure for determining semen exposure in clinical trials of vaginal products such as contraceptives and HIV pre/post-exposure prophylaxis agents.

Keywords: TSPY; Semen; HIV; Contraception; Y-chromosome

1. Introduction

Developing a reliable method to determine vaginal semen exposure after intercourse using cervicovaginal sampling has become an important requirement for those developing vaginal microbicides directed against sexually transmitted infections (STIs), including HIV, and new methods of contraception [1]. Biomarkers for this purpose have been based on either proteins in the seminal fluid or Y-chromosome-specific genes. The area of forensics has also used various Y-specific short tandem repeats (Y-STRs) of genomic DNA to identify semen exposure [2], while clinical studies to determine semen exposure in the vagina have employed seminal proteins such as prostate-specific antigen (PSA) [3,4] or semenogelin [5,6]. The goals of these types of determinations are varied. For instance, investigators have used these markers to evaluate condom integrity or to validate self reporting of sexual activity/protocol compliance [7–9]. Furthermore, PSA has been proposed as a surrogate of female condom efficacy [10,11]. However, these proteins

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have certain limitations. They can (although rarely) be produced by women, have a rapid decay curve, and cannot be amplified [12–15]. DNA-based biomarkers, such as the sex-determining region in the Y-chromosome (SRY), are more stable, likely more specific, and can be amplified by polymerase chain reaction (PCR). SRY has previously been used as a marker of semen exposure [16,17]. However, its rate of detection and half-life in cervicovaginal fluid is a limiting factor that can be improved upon. Since SRY is a single copy gene, it stands to reason that multi-copy Y-chromsomal genes would be more sensitive, especially in mixed DNA samples containing large amounts of female DNA such as those from cervicovaginal swabs. One such gene is TSPY4, which belongs to the TSPY (testis-specific protein Y-encoded) family of closely related or homologous genes that are embedded in a 20.4 kb repeat unit on the Y-chromosome ranging in copy numbers between 20 and 60 depending upon the individual man. TSPY genes contain 6 exons distributed among the large, tandemly repeated DNA units [18,19]. To date, the TSPY family is the largest identified tandem array encoding a protein expressed only in the testis [20] and has been used for sex determination [21,22]. Considering the high copy number of the TSPY family, we hypothesized that these genes could be a more reliable and sensitive marker for detecting male DNA in a predominantly female DNA sample. In this study, we demonstrated that TSPY4 is a more sensitive marker of sperm/semen exposure in cervicovaginal fluids than SRY.

2. Materials and methods

2.1. Sperm DNA isolation

Human semen was obtained from healthy, normozoospermic donors under an approved institutional review board (IRB) protocol (Eastern Virginia Medical School IRB, Norfolk, VA, USA). Semen was diluted to appropriate sperm concentrations with phosphate buffered saline (PBS). One milliliter (mL) of the suspension was spun down at 1200xg for 15 min at room temperature, and supernatant discarded. The procedure for isolating genomic DNA from the sperm pellet was based on a user-developed protocol provided by Qiagen® (Valencia, CA, USA) in conjunction with its QIAamp® DNA Mini Kit. One hundred microliters of PBS and 100 μL of a 2× lysis buffer containing 20 mM Tris−Cl, pH 8.0, 20 mM EDTA, 200 mM NaCl, 80 mM dithiothreitol, 4% Sodium dodecyl sulfate and 250 mcg/mL proteinase K was added to the sperm pellet. The samples were incubated in this lysis buffer for 1 h at 55°C. Two hundred microliters of Buffer AL (provided in the QIAamp® DNA Mini Kit) was added to each sample and incubated at 70°C for 10 min. Then, 200 μL of 100% ethanol was added before applying to the spin columns provided in the kit. Further purification of the DNA using the spin columns was performed according to the kit instructions and subsequently quantitated. DNA from VK2/E6E7 cells, a vaginal epithelial cell line [23], was used as a representative of female DNA. The cell line was a gift from Dr. Raina Fichorova (Brigham and Women's Hospital, Boston, MA, USA). These epithelial cells were isolated from vaginal mucosal tissue from a 32-year-old pre-menopausal woman undergoing anterior-posterior repair and have structural and functional properties similar to those of their parental primary cells. VK2/E6E7 cell DNA was isolated similarly to the above protocol.

2.2. Development of the multiplex PCR

The Qiagen® Multiplex PCR Plus kit was used to establish a multiplex PCR detecting TSPY4, SRY, and amelogenin simultaneously from one DNA sample. Amelogenin was included to serve as an internal control for detecting female DNA thereby confirming the general integrity of the DNA. Primer sequences are listed in Table 1. Primers for amelogenin and SRY were synthesized according to sequences previously published [24,25]. For amelogenin, the PCR products amplified were 114 base pairs (bp) (X-linked) and 120 bp (Y-linked). For SRY, the PCR product amplified was 93 bp. Multiple primers were designed to various sequences of the TSPY family using Primer design tool (www.ncbi.nlm.nih.gov/tools/primer-blast). The ones that showed a strict male specificity (did not amplify non-Y-linked and TSPX sequences as confirmed by sequencing and BLAST analysis) and best amplification in a multiplex format were those based on the TSPY4 sequence amplifying a product of 138 base pairs. Ten nanograms (ng) of sperm or VK2/E6E7 DNA was added to the master mix containing 0.2 μM of the above primers and Q-solution according to the manufacturer's protocol. The cycling conditions were as suggested by the manufacturer: 5 min at 95°C to activate the polymerase, 35 cycles of 30 s at 95°C, 90 s at 60°C, 30 s at 72°C, and a final extension of 10 min at 68°C. The PCR products were analyzed using the

Table 1
Primer sequences

Gene	Primer sequence	Reference
SRY-F	5′-ATAAGTATCGACCTCGTCGGAAG-3′	Santos et al., 1998 [24]
SRY-R	5′-GCACTTCGCTGCAGAGTACCGAAG-3′	
Amelogenin-F	5′-GTTTCTTCCCTGGGCTCTGTAAAGAATAGTG-3′	Morikawa et al., 2011 [25]
Amelogenin-R	5′-ATCAGAGCTTAAACTGGGAAGCTG-3′	
TSPY4-F	5′-TGGGCCCATGACCCCAGAGT-3′	NCBI Gene ID 728395
TSPY4-R	5′-TCTAGGTGGGGCTTGCGCCT-3′	