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Short communication

A model for data analysis of microRNA expression in forensic body fluid identification

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ABSTRACT

MicroRNAs (miRNAs, 18–25 bases in length) are small, non-coding RNAs that regulate gene expression at the post-transcriptional level. MiRNA expression patterns, including presence and relative abundance of particular miRNA species, provide cell- and tissue-specific information that can be used for body fluid identification. Recently, two published studies reported that a number of body fluid-specific miRNAs had been identified. However, the results were inconsistent when different technology platforms and statistical methods were applied. To further study the role of miRNAs in identification of body fluids, this study sets out to develop an accurate and reliable model for data analysis of miRNA expression. To that end, the relative expression levels of three miRNAs were studied using the mirVana™ miRNA Isolation Kit, high-specificity stem-loop reverse transcription (RT) and high-sensitivity hydrolysis probes (TaqMan) quantitative real-time polymerase chain reaction (qPCR) in forensically relevant biological fluids, including venous blood, vaginal secretions, menstrual blood, semen and saliva. Accurate quantification of miRNAs requires not only a highly sensitive and specific detection platform for experiment operation, but also a reproducible methodology with an adequate model for data analysis. In our study, the efficiency-calibrated model that incorporated the impact of the quantification cycle (Cq) values and PCR efficiencies of target and reference genes was developed to calculate the relative expression ratio of miRNAs in forensically relevant body fluids. Our results showed that venous blood was distinguished from other body fluids according to the relative expression ratio of miR16 using as little as 50 pg of total RNA, while the expression level of miR658 was unstable and that of miR205 was nonspecific among different body fluids. Collectively, the findings may constitute a basis for future miRNA-based research on body fluid identification and show miRNAs as a promising biomarker in forensic identification of body fluids.

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1. Introduction

In forensic casework, especially in cases of sexual assault and child sexual abuse, to uncover the criminal nature of an event requires not only identification of the DNA profile, but also confirmation of the origin of DNA. One of the challenges facing the forensic community is how to find a reliable biomarker and develop an accurate method for rapid identification of body fluids. Conventional serology-based methods for body fluid identification are prone to various limitations, such as sample consumption, intensive labor, time consumption, varying degrees of sensitivity and specificity, and no definitive tests for the presence of menstrual blood and vaginal secretions [1–5]. Numerous published studies have reported that some mRNAs are expressed in a tissue-specific manner and their expression patterns can confirm specific body fluids, even after long periods under controlled conditions [6–8]. However, heat, humidity, UV light and ubiquitous ribonucleases are detrimental to mRNA stability as a sensitive and specific biomarker for forensic applications [9,10].

MiRNAs belong to a class of small, non-coding RNA molecules that regulate gene expression at the post-transcriptional level [11–13]. Numerous published studies have demonstrated their important role in functions including embryonic development, proliferation, hematopoiesis and apoptosis, and in the pathogenesis of many human diseases such as viral infection, cancer and metabolic disorders [12–14]. Several studies revealed that many miRNAs were expressed in a tissue-specific manner [15,16]. The intrinsically short fragment and tissue-specific expression enable miRNA as an ideal biomarker for body fluid identification. Recently, two published studies reported their preliminary application in forensic identification of body fluids [17,18]. However, the results

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were inconsistent when different technology platforms and statistical methods were applied.

In this study, we used the mirVana™ miRNA Isolation Kit that optimized extraction of small nucleic acid molecules to enrich miRNAs. The expression abundance was detected using stem-loop reverse transcription (RT) followed by TaqMan quantitative real-time polymerase chain reaction (qPCR) analysis. We also utilized the gradient dilution cDNA method to test the amplification efficiencies of target and reference genes in body fluids [19–21]. The efficiency-calibrated method was used to calculate the relative expression ratio of target genes in body fluids [19]. To further explore its potential application in forensic cases, we used serial dilutions of total RNA for cDNA synthesis to establish the detection sensitivity of TaqMan RT-PCR assays in venous blood.

2. Materials and methods

2.1. Collection of body fluid samples

The body fluid samples were collected on sterile cotton swabs and dried at room temperature. Blood samples were collected by venipuncture without anticoagulation treatment and 50 μl aliquots were spotted onto sterile cotton swabs. Semen-free vaginal secretions and menstrual blood were collected from the vagina on sterile cotton swabs and dried at room temperature. Freshly ejaculated semen samples were provided in sealed plastic cups and dried onto sterile cotton swabs. Saliva samples were provided in sealed plastic tubes and dried onto sterile cotton swabs. Buccal samples were collected from donors using sterile swabs by swabbing the inside of mouth. Each body fluid sample was collected from 10 unrelated individuals (age: 18–47 years old) of the Chinese Han population living in Sichuan province. Written informed consent was obtained from all individuals. All the samples were stored at −80 °C for future use. A single cotton swab was used for RNA isolation.

2.2. RNA isolation and quantification

RNA was isolated using the mirVana™ miRNA Isolation Kit (Ambion, Austin, TX, USA) according to the manufacturer's instructions. The purity and quantity of RNA were assessed using the NanoDrop ND-1000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA). All the samples were diluted to a final concentration of 10 ng/μl. The samples were used immediately or stored at −80 °C for future use.

2.3. TaqMan RT-qPCR

The TaqMan® MicroRNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA) was used for preparation of cDNA. RT reactions were performed in a volume of 15 μl, and each reaction contained 10 ng of total RNA. RT reactions were performed on a GeneAmpPCR System 9600 (Applied Biosystems) with the following conditions: 16 °C for 30 min, 42 °C for 30 min, 85 °C for 5 min, and 4 °C on hold. Reactions without addition of reverse transcriptase (RT (−) controls) were performed alongside with cDNA synthesis of each sample and used in subsequent procedures to control the potential genomic DNA contamination. 1 μl of RT reaction product was added in the 20 μl qPCR reaction. All TaqMan assays were run in triplicate on an ABI Prism 7500 using TaqMan® Universal PCR Master Mix II without UNG (Applied Biosystems). Real-time PCR cycling conditions consisted of 95 °C for 10 min, followed by 50 cycles of 95 °C for 15 s and 60 °C for 1 min. The Cq is defined as the PCR cycle at which the fluorescent signal of the reporter dye crosses an arbitrarily placed threshold in the exponential phase. For our assays, the constant threshold value was set to 0.200 for easy comparison of results within and between assays. The sample with a Cq value over 40 or an amplification point not reaching the threshold was considered as invalid and was not useful for further analysis.

2.4. Data analysis

The three miRNAs assay was controlled with the snRNA (U6b) assay (Applied Biosystems, Supplemental Table 1) functioning as reference gene to evaluate Cq values and serving as an internal positive control if sample extraction and reverse transcription were performed successfully.

The relative expression ratio (R) is expressed as the target/reference ratio of each sample normalized by the target/reference ratio of the normalizer [19,22].

$$R = \frac{(E_{ref\,x}+1)^{Cqref\,x}}{(E_{tarx}+1)^{Cqtarx}} \div \frac{(E_{ref}+1)^{Cqref}}{(E_{tar}+1)^{Cqtar}}$$

The above equation shows the mathematical model of relative expression ratio in qPCR. The ratio of a target gene is expressed in a body fluid versus the normalizer (in this study, venous blood was designated as the normalizer) in comparison to the reference gene (U6b). E_{tarx} is the real-time PCR efficiency of target gene in x body fluids (x means vaginal secretions, menstrual blood, semen, saliva or buccal swabs); E_{refx} is the real-time PCR efficiency of reference gene in x body fluid; Cq_{tarx} is the Cq value of target gene in x body fluid; Cq_{refx} is the Cq value of reference gene in x body fluid; E_{tar} is the real-time PCR efficiency of target gene in venous blood; E_{ref} is the real-time PCR efficiency of reference gene in venous blood; Cq_{tar} is the Cq value of target gene in venous blood; and Cq_{ref} is the Cq value of reference gene in venous blood.

For calculation of R, the qPCR efficiencies and Cq values of investigated transcripts must be known. Serial dilutions of cDNA over a 100-fold range were prepared. According to linear regression equation and $E = 10^{[-1/\text{slope}]}$-1, the amplification efficiencies were calculated. $R > 1$ indicates higher abundance of the target gene in x body fluid than venous blood, while $R < 1$ suggests otherwise.

2.5. Analytical sensitivity of miRNA TaqMan assays

We used serial dilutions of total RNA (10 ng to 0.01 ng) isolated from venous blood as input for cDNA synthesis to establish the detection sensitivity of TaqMan RT-PCR assays.

3. Results

Relative abundance of three miRNAs relative to U6b among body fluids were presented and average Cq values of ten unrelated individuals were tabulated (Supplemental Table 2). The expression abundance of miR658 was lower than U6b in all body fluid samples. MiR658 was unstably expressed in the samples, especially in the venous blood (not detectable in 7 of 10 blood samples), and apparent inter-individual variation was observed in vaginal secretions. The expression of miR205 had much higher abundance in buccal swabs, vaginal secretions and menstrual blood compared with saliva, semen and venous blood, but it could not be distinguished from those in vaginal secretions and menstrual blood. The expression of miR16 varied significantly between the venous blood and other body fluids (Supplemental Fig. 1). Obvious expression difference (dCt∼6) was observed, though the abundance of miR16 was high in both the menstrual and venous blood. The RT (−) controls and no template controls were negative in all cases. Therefore, miR16 as a potential body fluid-specific miRNA was selected for further study.

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