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Screening and confirmation of microRNA markers for forensic body fluid identification

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ABSTRACT

MicroRNAs (miRNAs, ~22 nucleotides) are small, non-protein coding RNAs that regulate gene expression at the post-transcriptional level. MiRNAs can express in a tissue-specific manner, and have been introduced to forensic body fluid identification. In this study, we employed the qPCR-array (TaqMan® Array Human MicroRNA Cards) to screen the body fluid-specific miRNAs. Seven candidate miRNAs were identified as potentially body fluid-specific and could be used as forensically relevant body fluid markers: miR16 and miR486 for venous blood, miR888 and miR891a for semen, miR214 for menstrual blood, miR124a for vaginal secretions, and miR138-2 for saliva. The candidate miRNA markers were then validated via hydrolysis probes quantitative real-time polymerase chain reaction (TaqMan-qPCR). In addition, BestKeeper software was used to validate the expression stability of four genes, RNU44, RNU48, U6 and U6b, regularly used as reference genes (RGs) for studies involving forensic body fluids. The current study suggests that U6 could be used as a proper RG of miRNAs in forensic body fluid identification. The relative expression ratios (R) of miR486, miR888, miR214, miR16 and miR891a can differentiate the target body fluid from other body fluids that were tested in this study. The detection limit of TaqMan-qPCR of the five confirmed miRNA markers was 10 pg of total RNA. The effect of time-wise degradation of blood stains and semen stains for 1 month under normal laboratory conditions was tested and did not significantly affect the detection results. Herein, this study proposes five body fluid-specific miRNAs for the forensic identification of venous blood, semen, and menstrual blood, of which miR486, miR888, and miR214 may be used as new markers for body fluid identification. Additional work remains necessary in search for suitable miRNA markers and stable RGs for forensic body fluid identification.

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1. Introduction

Identifying the origin of body fluids left at a crime scene is important for crime scene reconstruction; however, 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 [1–5]. Several messenger RNA (mRNA) markers are expressed in a tissue-specific manner, and their expression patterns can confirm specific body fluids even after long periods of time under controlled conditions [6–9]. However, humidity, heat, UV light, and ubiquitous ribonucleases are detrimental to mRNA stability as a specific and sensitive biomarker for forensic applications [9,10].

MiRNAs belong to a class of small, non-coding RNA molecules containing 18–25 nucleotides that regulate gene expression at the post-transcriptional level [11–13]. By incorporating into the RNA-induced silencing complex (RISC) and hybridizing to the 3′UTR of specific mRNA targets, mature miRNA can cause translational repression and/or mRNA decay [14–16]. Recent studies have demonstrated the important role of miRNAs in physiological functions and pathogenesis, revealing that they can express in a tissue-specific manner [15–19]. Theoretically, the shorter fragment and tissue-specific expression of miRNA make it less susceptible to degradation caused by chemical and/or physical environmental strain, thus rendering it a useful biomarker for body fluid identification. Interestingly, it was reported that miRNA extracted from formalin-fixed paraffin-embedded (FFPE) tissue samples have obtained feasible and valid profiling results [20,21]. In several forensic laboratories, differentially expressed miRNAs have been investigated as a potential method for body fluid identification [22–26]. With an increasing number of miRNAs

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available on the chips, which are commonly used for screening, more specific miRNAs could be selected for the purpose of body fluid identification.

Previously, we presented a simple procedure for miRNA-based body fluid identification and an accurate model for data analysis [25]. In this study, we further searched potential body fluid-specific miRNAs through a qPCR-array (TaqMan® Array Human MicroRNA Cards) containing 754 known human miRNAs by using forensically relevant body fluid samples. We then confirmed five candidate miRNAs by the hydrolysis probes quantitative real-time polymerase chain reaction (TaqMan-qPCR). Additionally, to normalize qPCR data, four commonly used reference genes (RGs) were selected to validate the expression stability in forensically relevant body fluids. The relative expression ratios (R) of body fluid-specific miRNAs were calculated. We further tested body fluid-specific miRNA markers for degradation stability and assessed the sensitivity of marker-specific TaqMan assays.

2. Materials and methods

2.1. Collection of body fluid samples

Five forensically relevant body fluids were used for initial miRNA TaqMan® Array screening. Body fluid samples were collected on sterile cotton swabs and dried at room temperature. Venous blood was collected by venipuncture without anticoagulation treatment, and 50 μl aliquots were spotted onto sterile cotton swabs. Freshly ejaculated semen was provided in sealed plastic cups and dried onto sterile cotton swabs. Semen-free vaginal secretions and menstrual blood were collected from the vagina with sterile cotton swabs and dried at room temperature. Saliva samples were provided in sealed plastic tubes and dried onto sterile cotton swabs. Written informed consent was obtained from all sample donors (18–47 years old) of the Chinese Han population living in the Sichuan Province. All samples were stored at −80 °C following collection. RNA isolation was performed on individual cotton swabs.

Body fluid samples for TaqMan-qPCR validation (including those used for array screening) were collected from ten unrelated individuals. To observe the effect of natural environment on the miRNAs, cotton swabs were kept under normal laboratory conditions (approximately 15 °C and about 10-hour natural daylight exposure per day) for 1 month.

2.2. RNA isolation and quantification

Total RNA was extracted using the mirVana™ miRNA Isolation kit (Ambion, Austin, TX, USA) following the instructions of the manufacturer. Potential traces of genomic DNA were removed by DNase I digestion, performed with Turbo DNA-free™ kit (Ambion) according to manufacturer's protocol. Purity and quantity of RNA were assessed with the NanoDrop ND-1000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA). Equal quantities of total RNA from three donors were combined to produce pooled samples.

2.3. TaqMan® Array analysis and candidate selection

MiRNA profiling experiments using TaqMan® Array Human MicroRNA Cards (Cards A and B, Applied Biosystems, Foster City, CA, USA) for a total of 754 unique assays specific to human miRNAs (Comprehensive coverage of Sanger miRBase v14) were performed by Applied Biosystems. The sample of cDNA reverse-transcribed from saliva was pre-amplified according to the manufacturer's instructions. The Cq value is defined as the PCR cycle at which the fluorescent signal of the reporter dye crosses an arbitrarily placed threshold in the exponential phase. Since a quantification cycle (Cq) value of 35 represents single molecule template detection, Cq values > 35 were considered to be below the limit of detection [27]. Therefore, only the miRNAs with a Cq ≤ 35 were included in the analyses, carried out using DataAssist™ Software. Potential specific miRNA markers were selected primarily based on the absolute expression levels and large-magnitude fold-change of differential expression between body fluids. Preference was given to miRNA markers that were highly abundant (Cq ≤ 25) in the target body fluid and only minimally (Cq > 30) or not expressed in the non-target ones.

2.4. TaqMan RT-qPCR

cDNA was synthesized in a 15 μl reaction, containing 10 ng of total RNA, using TaqMan® MicroRNA Reverse Transcription Kit (Applied Biosystems). Reverse transcription (RT) reactions were performed on a GeneAmpPCR System 9600 (Applied Biosystems) under 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 used as controls for potential genomic DNA contamination. All TaqMan assays were run in triplicate on an ABI Prism 7500 using TaqMan® Universal PCR Master Mix II without UNG (Applied Biosystems). One μl of cDNA was used in the subsequent Real-time PCR reaction. Real-time PCR cycling conditions consisted of 95 °C for 10 min, followed by 40 cycles of 15 s at 95 °C and 1 min at 60 °C. Cq values were calculated using SDS software with an automatic baseline and a threshold of 0.2. Samples in which the fluorescent signal did not reach the threshold were considered invalid and were not used for further analysis.

2.5. Reference genes validation

RNU44, RNU48, U6 and U6b were chosen as RGs based on their high abundance (P/N: 044972, Applied Biosystems) and reported literatures about forensic body fluid identification [22–26]. We herein employed BestKeeper software to measure the expression stability of four putative RGs in different body fluids. The principle behind BestKeeper for identification of stably expressed RGs is that proper RGs should display a similar expression pattern [28].

2.6. Data analysis

Relative quantification of miRNA expression was performed by the relative expression ratio method [29,30]. The qPCR efficiency (E) was calculated according to the formula: $E = 10^{[-1/\text{slope}]} - 1$, as previously described [25,29]. Relative fold-change was calculated according to our previous study [25].

2.7. Analytical sensitivity of miRNA TaqMan assays

To evaluate the detection sensitivity of TaqMan RT-PCR assays for the confirmed specific miRNAs, serial dilutions of total RNA (ranging from 10 ng to 0.01 ng) were used as input for cDNA synthesis.

3. Results

3.1. TaqMan® Array data analysis

After removing the low-expression values across all body fluids from analysis, values of the remaining 177 of 754 (23.5%) miRNAs were normalized. Unsupervised hierarchical clustering of normalized Cq ($\Delta Cq = Cq_{miRNA} - Cq_{RG}$) values was carried out as bottom-up complete linkage clustering using the Euclidean distance as a measure. This revealed that different body fluids displayed distinct

miRNA expression signatures (Fig. 1). The numerical value of the ΔCq is inversely related to the amount of target miRNA in the reaction (i.e., the lower the ΔCq, the greater the amount of target miRNA). Only miR891a was identified as the truly body fluid-specific miRNA that showed expression of high-abundance ($Cq \leq 25$) in a single body fluid, and no expression ($Cq > 35$) in the other body fluids. Based on the absolute expression levels in target body fluid and fold change (at least 10-fold change) of differential expression between target fluid and other body fluids, we manually ascertained the most promising candidate markers and obtained six additional candidate markers for validation in TaqMan-based quantitative RT-PCR assays (Table 1).

3.2. Validation of reference genes

The results of the BestKeeper analysis show that the expressions of RNU44, RNU48, and U6b in different body fluids are unstable (Supplemental Fig. 1) and cannot be used as RG. One-way analysis of variance (ANOVA) suggests that the differences between the expression levels of U6 in five body fluids are not statistically significant. As such, U6 was subsequently used as the RG to calculate the relative expression ratios.

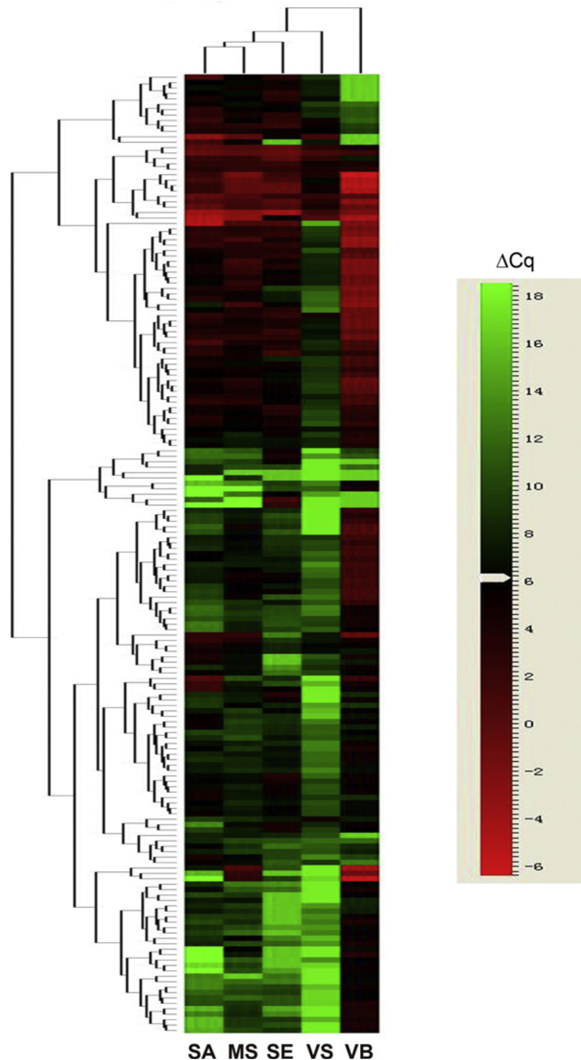

Fig. 1. Unsupervised hierarchical cluster map of 177 miRNAs from qPCR-array analysis of five forensically relevant body fluids, i.e., semen (SE), saliva (SA), vaginal secretions (VS), menstrual blood (MB), and venous blood (VB).

Table 1
Reported miRNA markers for forensic body fluid identification, and screened and confirmed miRNA markers by our group.

Groups	Hanson et al. [22]		Zubakov et al. [23]		Courts et al. [24]		Our group	
Body fluids	452 miRNAs	SYBR Green-qPCR	LNA™-modified Microarray (Sanger miRBase v10.1)	TaqMan-qPCR	Geniom® Biochips (Sanger miRBase v14)	SYBR Green-qPCR	qPCR-array (Sanger miRBase v14)	TaqMan-qPCR
Venous blood	miR451; miR16	miR451; miR16	miR20a; miR106a; miR185	miR20a; miR106a; miR185; miR144	miR126; miR150; miR451	miR126; miR150; miR451	miR486; miR16	miR486; miR16
Semen	miR135b; miR10b	miR135b; miR10b	miR943; miR135a; miR10a; miR507	miR943; miR135a; miR10a; miR507; miR891a			miR888; miR891a	miR888; miR891a
Menstrual blood	miR451; miR412	miR451; miR412	miR185*; miR144				miR214	miR214
Vaginal secretions	miR124a; miR372	miR124a; miR372	miR617; miR891a				miR124a	
Saliva	miR658; miR205	miR658; miR205	miR583; miR518c*; miR208b		miR200c; miR203; miR205	miR200c; miR203; miR205	miR138 -2	

3.3. Validation of candidate miRNA markers

Using the TaqMan® Array dataset, the seven more promising candidate markers were selected and validated with the TaqMan-based quantitative RT-PCR assays (Supplemental Table 1). Relative abundance values of these seven miRNAs to U6 were presented and average Cq values of ten unrelated individuals were tabulated (Supplemental Table 2).

Over-expression of candidate miRNAs was confirmed by both TaqMan® Array and TaqMan-qPCR in venous blood, semen, and menstrual blood. The abundance values show that miR16 and miR486 are over-expressed in both menstrual and venous blood; however, in venous blood, the expression levels of miR16 and miR486 afford an approximate 4-$\Delta\Delta$Cq ($\Delta\Delta Cq = \Delta Cq_{target\ body\ fluid} - \Delta Cq_{other\ body\ fluids}$) difference when compared to other body fluids. Two candidate miRNAs for semen are strongly over-expressed in the target body fluid; miR891a was below the detection level in all other body fluids, while miR888 was undetectable in both venous blood and vaginal secretions. In menstrual blood, miR214 had a 6-$\Delta\Delta$Cq difference when compared to other body fluids.

However, less concordance between the results of TaqMan® Array and TaqMan-qPCR was achieved in both vaginal secretions and saliva. The expression of miR124a, which was not detectable in semen, was higher (4-$\Delta\Delta$Cq) in vaginal secretions and menstrual blood than in saliva and venous blood, but only showed approximately 1-$\Delta\Delta$Cq difference between vaginal secretions and menstrual blood. The TaqMan® Array candidate marker for saliva, miR-138-2, showed non-specific and low expression across all body fluids in the qPCR results, which was inconsistent with the TaqMan® Array results.

Having attained these results, miR16, miR486, miR888, miR891a and miR214 were selected as potential body fluid-specific miRNAs for further study. The Cq values were plotted

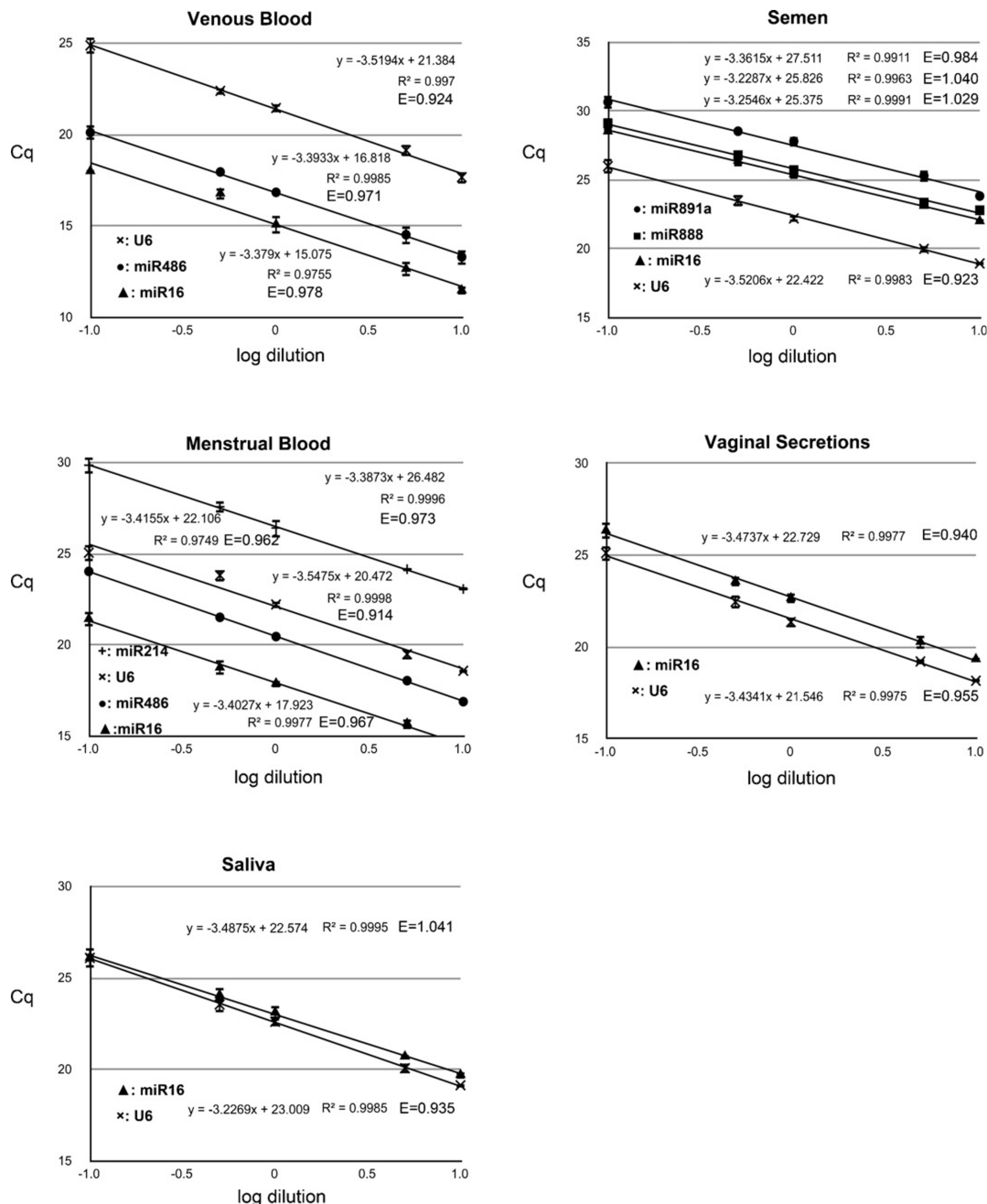

Fig. 2. Determination of qPCR efficiencies from the slopes of the calibration curve, according to the equation: $E = 10^{[-1/slope]} - 1$. Cq values versus cDNA concentration input (log scale) were plotted to calculate the slope (mean ± SD; $n = 3$).

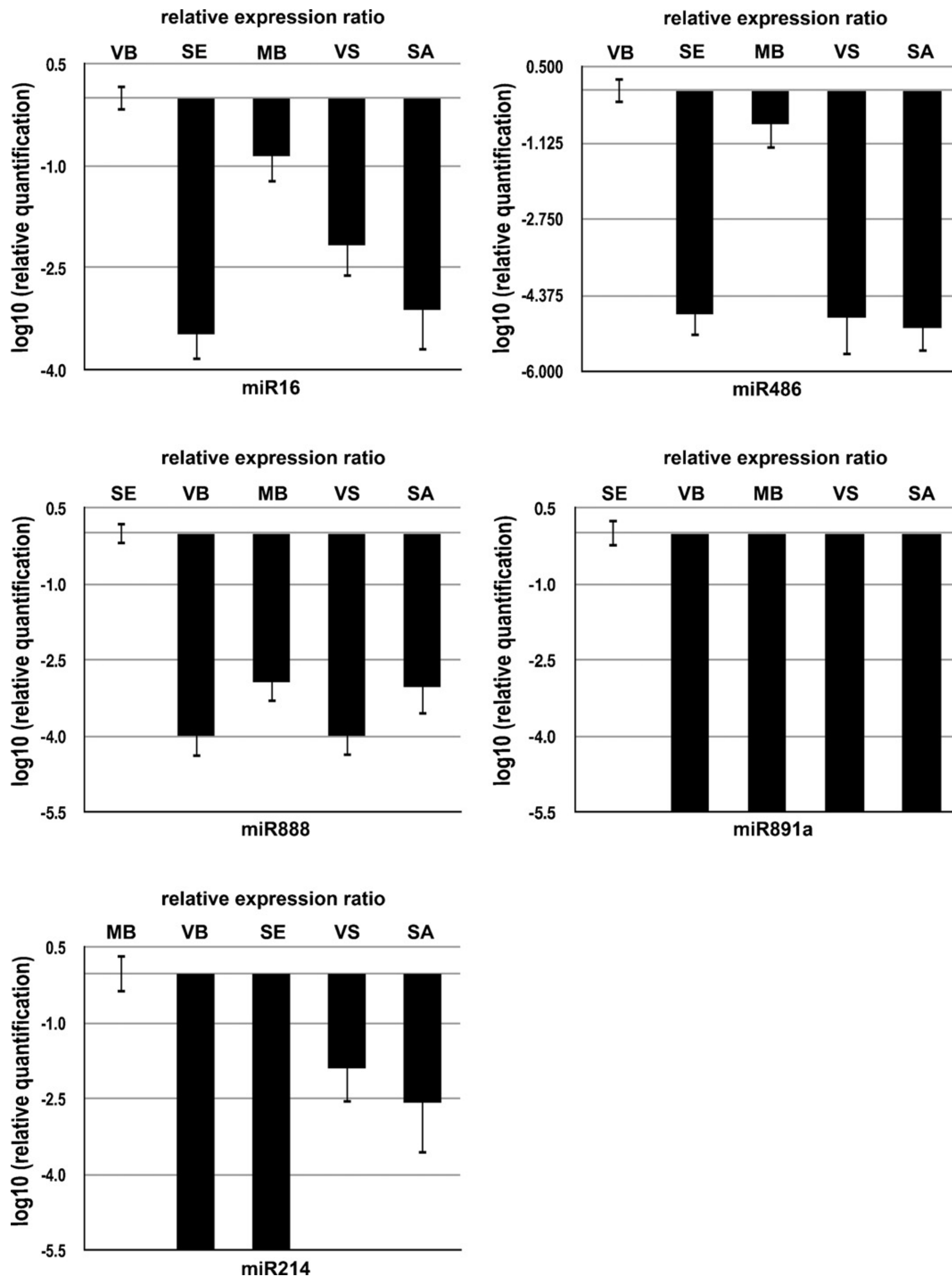

Fig. 3. Relative expression ratio of five miRNA markers in body fluids, i.e., venous blood (VB), vaginal secretions (VS), menstrual blood (MB), semen (SE), saliva (SA). Values underwent log10 transformation (error bars reflect variation between ten unrelated individuals).

against the log of the dilution cDNA, and linear regressions were performed, from which the mean E could be derived (Fig. 2). Calculation values of E from target and RG were between 0.924 and 1.041 in forensically relevant body fluid. The relative expression ratios of fluid-specific miRNAs in different body fluids were compared (Fig. 3). As shown in Fig. 3, these five miRNAs appeared over-expressed in the target body fluid relative to other body fluids.

3.4. Time-wise stability of miRNA markers

The effect of 1-month storage on the detection of miRNAs was tested by TaqMan-qPCR. Stability of miR16 and miR486 in venous blood, miR888 and miR891a in semen, and miR214 in menstrual blood were examined. Results revealed that absolute expression levels were slightly decreased (~2–3 Cq) in 1-month-old samples, while ΔCq ($Cq_{target} - Cq_{U6}$) values remained unchanged (Supplemental Fig. 2).

3.5. Sensitivity of miRNA TaqMan assays

Serial dilutions of total RNA were used as an input for cDNA synthesis to evaluate the detection sensitivity of TaqMan RT-PCR assays for the confirmed body fluid-specific miRNAs. As shown in Supplemental Fig. 3, all five markers were detectable in the target body fluid using an amount as low as 10 pg total RNA.

4. Discussion

Correct identification of the origin of the body fluid(s) left at crime scene can be significant to determining the nature of a crime. Characteristics of miRNA can overcome limitations of conventional serological and mRNA-based methods for body fluid identification [22–25]. Today, in the forensic community, differentially expressed miRNAs have been investigated as a means of body fluid identification. Two publications reported their preliminary application in forensic identification of body fluids [22,23]. But the body fluid specificity of miRNAs identified by the two teams did not overlap (Table 1). By employing the Geniom® Biochips, Courts et al. [24] identified six miRNAs in venous blood and saliva, but did not investigate miRNA markers for menstrual blood, vaginal secretions and semen. Notably, 3 out of 7 candidate markers were also reported by other groups: miR16 and miR124a, Hanson et al. [22], and miR891a, Zubakov et al. [23]. Given our problems in screening other reported miRNA markers for forensically relevant body fluids, we examined these reported miRNA markers in our qPCR-array experiment and demonstrated that most of them were over-expressed in target body fluids (Supplemental Fig. 4). There may be several explanations for the observed discrepancies between our and published findings. First, different groups utilized different screening platforms, which imply differing sets of miRNAs and capture probes. Second, the relatively small sample size for screening, especially in our study, does not rule out the natural variation existing between individuals. Third, candidate miRNAs selected from microarray expression data to qPCR validation always retains some degree of arbitrariness, and there are no fixed rules as to which criteria have to be met for a miRNA to be chosen as a candidate. In the current study, for the purpose of finding the best candidate miRNA, we focused on the highly abundant ($Cq \leq 25$), and thus, might have missed some moderately but specifically expressed miRNAs. All candidate miRNA markers selected from qPCR-array screening are the most over-expressed miRNAs in the respective body fluid (Supplemental Fig. 4), e.g., miR891a was only detectable in semen and was confirmed as a truly body fluid-specific miRNA by qPCR. This result is consistent with a research report that miR891a was present only in epididymis tissue and was practically absent from any other tissue analyzed [31].

Obviously, variant miRNA microarray platforms and verification methodologies might result in discrepant candidate markers and slightly different amplification efficiencies, and therefore differing results from comparable samples. To our knowledge, the critical steps of investigating miRNA expression in body fluid identification are microarray screening, RGs validation, and data analysis.

Microarrays have been widely used in miRNA profiling and can determine simultaneously the expression levels for large numbers of miRNAs in a single experiment [32]. However, the short length of miRNAs with inherently different melting temperatures (Tm) and the highly similar sequences between miRNA family members make probe design more difficult than mRNA arrays [33]. The current lineup of commercially available miRNA microarray systems fails to show a good inter-platform concordance. The data generated from different microarray platforms and probing chemistries are inconsistent with those obtained from TaqMan-qPCR, which is a golden standard of miRNA measurement [34,35]. Zubakov et al. explained that they did not succeed in confirming their miRNA candidates for saliva, menstrual blood, and vaginal secretions because they used LNA^{TM}-modified oligonucleotides as capture probes, which may not be able to discriminate mature and unprocessed miRNA [23]. Thus, in this study, we chose a TaqMan probe based-qPCR-array, which is based on the same founding principle of TaqMan-qPCR, to search potential body fluid-specific miRNAs candidates. Nevertheless, less concordance between qPCR-array and TaqMan-qPCR results was observed for saliva and vaginal secretions in this study. For example, miR138-2 was suggested as the best marker in saliva by the qPCR-Array (Supplemental Fig. 4E), but it cannot be validated by the TaqMan-qPCR. A possible explanation is that the saliva sample in array experiment underwent pre-amplification in our study. Although most of the miRNAs were uniformly pre-amplified, high variation associated with pre-amplification was observed for low abundant miRNAs [35]. Furthermore miR124a, the candidate marker for vaginal secretions, could not be effectively distinguished from menstrual blood according the TaqMan-qPCR results, possibly due to menstrual blood being a complex mixture containing products that also are detectable in vaginal secretions.

The accuracy and success of qPCR analysis depends on proper normalization of data, which minimizes potential variation that can exaggerate or mask biologically meaningful changes. Several normalization strategies have been proposed, but the use of one or more RGs is the currently preferred method [36]. While RGs constitute the best possible normalizers, these genes have no constant expression under all experimental conditions, which poses a major problem. With the increased sensitivity, reproducibility and large dynamic range of real-time RT-PCR methods, the requirements for a proper RG have become increasingly stringent. Researchers therefore need to carefully assess whether a particular RG is stably expressed in the experimental system under study. Recently, Hruz et al. [37] developed a tool named RefGenes for search RGs from a genome-wide background using microarray data. However, the data from our qPCR-array revealed that there were no miRNA RGs with universally stable expression across any type of body fluid. One possible explanation is that the sets of miRNA microarray data from forensically relevant body fluids were limited. Thus, we selected RNU44, RNU48, U6 and U6b as possible RGs due to their high abundance (P/N: 044972, Applied Biosystems) and literature reports [22–26]. Results from the BestKeeper software revealed that only U6 could be a proper RG in our study. However, we should keep in mind that, in order to remove the non-biological variation as much as possible, and to measure accurate expression levels, a joint use of multiple RGs is necessary [36]. The study of miRNAs in forensically relevant body fluids is still at an exploratory stage and more research for searching the generally accepted RGs for forensically relevant body fluids is intensively needed.

How to present the qPCR data is essential in miRNA expression study. One simple method to process qPCR, currently known as the $\Delta\Delta Cq$ method, is based solely on Cq values [38]. This method assumes that all amplification efficiencies are equal to 1, which does not take into consideration possible variations of amplification efficiencies. Different tissues exhibit different PCR efficiencies due to RT inhibitors, PCR inhibitors, and variations in the total extracted RNA fraction. Numerous published studies reported that the PCR efficiency has a major impact on the accuracy of calculated expression values [39,40], and similar results were found in our previous study [25]. However, its ease of use makes it ideal to screen changes rapidly in the expression level, after which a finer analysis on the genes displaying interesting expression profiles may be performed using a more accurate model. Soong et al. [30] proposed an efficiency-calibrated mathematical method for the relative expression ratio in qPCR. The advantage of the efficiency-calibrated method is that the PCR efficiencies of targets and RGs are included in the equation, making the data analysis more accurate [25,29,30]. In our study, we utilized the gradient dilution cDNA method to test the amplification efficiencies of target and RG in body fluids. Results

showed that the amplification efficiencies were not always equal to 1 (Fig. 2).

Currently, over 1000 mature miRNAs have been identified in the human genome (miRBase v18, November 2011). It remains necessary to search for suitable miRNA markers and stable RGs for forensically relevant body fluids. During the initial screening experiments of the current study, pooled samples were used to account for the possible inter-individual variation of miRNA expression, variations in the total extracted RNA fraction, and varied RT efficiency. However, it may not effectively rule out the natural variation between individuals and may lead to omission of some body fluid-specific miRNAs. Biological replicates array experiments will be required for further screening of body fluid-specific miRNAs (related research is in progress in our lab).

Biological stains from forensic casework are often challenged by ambient moisture and temperature, UV light, suboptimal environmental pH, affecting the detection of the aged samples. We established artificial environment to test degradation stability of miRNA markers in this study. We found that absolute expression levels of selected miRNAs were slightly decreased but their ΔCq values remained unchanged. Whether the aged samples contained small fragments degraded from large molecules affecting the amplification efficiency (inhibitors) or the miRNA degraded due to environmental factors, validation studies must still be performed.

Although this study failed to identify miRNA specific to saliva and vaginal secretions, we were able to screen and confirm five body fluid-specific miRNAs, three of which were new miRNA markers: miR486, miR888, and miR214.

5. Conclusions

With qPCR-array screening and subsequent TaqMan-qPCR validation, five body fluid-specific miRNAs were proposed for body fluid identification (miR16 and miR486 for venous blood, miR888 and miR891a for semen, and miR214 for menstrual blood) and their detection were highly sensitive. Our results highlight three new miRNA markers (miR486, miR888, and miR214) and a suitable RG (U6) for body fluid identification. Although current studies support the potential use of these miRNAs to identify the body fluid origin of forensic biological samples, we failed to identify saliva- and vaginal secretions-specific miRNAs. Additional work must be performed to further search for suitable miRNA markers and stable RGs for forensic body fluid identification. Moreover, a profound analysis of potential environmental impacts, such as humidity, UV radiation, and bacterial contamination, remains necessary.

Conflict of interest

The authors declare that they have no conflict of interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.fsigen.2012.07.006.

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