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Research paper

Differentiation of five body fluids from forensic samples by expression analysis of four microRNAs using quantitative PCR

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ABSTRACT

Applying molecular genetic approaches for the identification of forensically relevant body fluids, which often yield crucial information for the reconstruction of a potential crime, is a current topic of forensic research. Due to their body fluid specific expression patterns and stability against degradation, microRNAs (miRNA) emerged as a promising molecular species, with a range of candidate markers published. The analysis of miRNA via quantitative Real-Time PCR, however, should be based on a relevant strategy of normalization of non-biological variances to deliver reliable and biologically meaningful results. The herein presented work is the as yet most comprehensive study of forensic body fluid identification via miRNA expression analysis based on a thoroughly validated qPCR procedure and unbiased statistical decision making to identify single source samples.

1. Introduction

Regarding biological evidence found at the scene of a crime, it can be of crucial importance for the reconstruction of the events leading to its deposition to infer not only the DNA source, i.e. the person who deposited the stain, but also the bodily origin of the DNA, i.e. the body fluid(s) constituting the evidence material. Conventional methods of body fluid identification include immunological, chemical and enzymatic tests. These, however, vary greatly in terms of sensitivity and specificity and no reliable tests for e.g. vaginal secretion and menstrual blood are available. The comparably large amount of sample material required to perform these tests is another problematic aspect since the amount of evidential biological material is usually limited in forensic casework. In order to address these issues, a number of different molecular genetic approaches have been or are currently being explored by several groups (reviewed in Refs. [1–3]), including microRNA (miRNA) based body fluid identification.

Besides the essential prerequisite of cell type specific expression [4–6], these non-coding small RNAs exhibit certain characteristics that render them well suited to the challenging demands of the forensic setting: Firstly, due to their intrinsically small size of 18–25 nt, miRNAs are less prone to degradation caused by chemical and/or physical strains, secondly, miRNA is detected and quantified in its biologically active form so, in contrast to mRNA, no potential splice variants have to be differentiated, and thirdly, there is no gratuitous sample consumption as miRNA and DNA can be extracted simultaneously from the same specimen [7–9]. Previous studies have reported several body fluid specific miRNA markers that were identified using different methods [7–18] but only some of those markers were confirmed in more than one study. Also, most of these studies applied more or less arbitrary criteria to decide whether in a given sample a particular body fluid is present or not.

Quantitative PCR (qPCR) is widely considered as the gold standard for the quantification of miRNA expression, but for qPCR to deliver a reliable and biologically meaningful report of target molecule numbers an accurate and relevant normalization of non-biological variances is essential [19–22]. A robust normalization strategy that is appropriate for a particular experimental setup should be based on an individual and evidence based selection of one or a group of reference genes [19,23,24]. We therefore applied a normalization strategy specifically designed for body fluid identification in forensic type samples [25].

The aim of the herein presented study was to identify reliable miRNA markers for the statistically robust inference of the

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forensically relevant body fluids venous blood, saliva, semen, vaginal secretion and menstrual blood, and to develop an intuitive and easily conveyable approach for the prediction of the origin of unknown forensic samples.

2. Material and methods

2.1. Samples

Samples for each tested body fluid, i.e. venous blood, saliva, menstrual blood, vaginal secretion and semen, were collected from healthy volunteers. All volunteers provided informed consent and the study protocol was reviewed and approved of by the ethics committee of the Hospital of the University of Bonn.

Venous blood was collected by venipuncture using dry vacutainer tubes and spotted onto sterile cotton swabs. For collection of saliva via buccal swab, donors were asked to abstain from eating, smoking, drinking and oral hygiene at least 30 min prior to sampling. Samples of semen-free vaginal secretion were collected by the female donors themselves using sterile stemmed cotton swabs. Menstrual blood samples were obtained by the female donors using tampons. Male donors provided freshly ejaculated semen in sealed Falcon tubes that was then transferred onto sterile stemmed cotton swabs and dried by the researcher immediately after receipt. All samples used for the selection of body fluid specific miRNAs and the blinded specimens were dried at room temperature and processed for RNA extraction after 24 h. The samples used in mixtures (combinations of different body fluids) were stored frozen (−80 °C) after initial drying at room temperature for 24 h, then thawed and dried for another 24 h prior to combining and extraction. Aged samples were stored at room temperature for the period of time indicated in Table 1. A subset of the aged venous blood stains was made available from the Institute of Legal Medicine, Halle (Saale), Germany. These blood stains were either spotted on cotton fabric or presented as dried blood without a carrier substrate.

2.2. RNA extraction and quantification

All surfaces, devices and instruments utilized in the extraction procedure were thoroughly cleaned using RNase-Zap® (Ambion™, Austin, TX, USA) to remove ambient RNases and only RNase-free reagents and plastic consumables were used.

Total RNA was extracted using the mirVana™ miRNA Isolation Kit (Ambion) according to the manufacturer's protocol. Prior to extraction, samples (whole swab tip or approximately 2 cm^2 of the tampon or blood stain) were cut into pieces and incubated with 350 µl Lysis/Binding Buffer at 56 °C for 1 h. Forensic Filters (Macherey-Nagel, Düren, Germany) were used to separate lysate and substrate by centrifugation at 13,000 × *g* for 1 min. Total RNA eluates were stored at −80 °C until further processing.

Aged and blinded samples as well as mixture of different body fluids were treated likewise with the exception of an extended incubation time of 3 h in Lysis/Binding Buffer.

To remove potential traces of genomic DNA, subsequent DNase I digestion was performed with the Turbo DNA-free™ Kit (Ambion), following the manufacturer's protocol. Total RNA concentration and quality, represented by the RNA integrity number (RIN) [26], were determined using the Quant-iT™ RNA Assay Kit on a Qubit fluorometer (both Invitrogen™, Darmstadt,

Table 1
Specification of storage period and total RNA concentration per aged sample.

Body fluid	Sample name	Gender	Date of sampling	Date of extraction	Age		Total RNA concentration (ng/µl)
					years	days	
Venous blood	oldB1	M	13.01.2015	10.06.2015		148	3.8
	oldB2	F	25.07.2013	10.06.2015	1	320	2.6
	oldB3	F	09.05.2012	10.06.2015	3	32	6.4
	oldB4	M	14.09.2011	10.06.2015	3	269	3.6
	oldB5	F	14.09.2011	10.06.2015	3	269	2.7
	bloodHalle1	n.a.	09.01.1989	15.06.2015	26	157	3.7
	bloodHalle2	n.a.	09.01.1989	15.06.2015	26	157	4.3
	bloodHalle3	n.a.	22.09.1980	15.06.2015	34	266	1.0
	bloodHalle4	n.a.	08.09.1980	15.06.2015	34	280	1.5
	bloodHalle5	n.a.	1979[a]	15.06.2015	35	min.166	2.2
	bloodHalle6	n.a.	1979[a]	15.06.2015	35	min.166	2.2
	bloodHalle7	n.a.	01.10.1979	15.06.2015	35	257	2.1
Menstrual blood	oldMB1	F	30.03.2014	17.08.2015	1	140	30.5
	oldMB2	F	18.07.2012	17.08.2015	3	30	26.2
	oldMB3	F	12.10.2011	17.08.2015	3	309	22.8
Saliva	oldSA1	M	03.01.2015	17.08.2015		226	5.2
	oldSA2	F	23.05.2012	17.08.2015	3	86	6.7
	oldSA3	M	14.09.2011	17.08.2015	3	337	5.1
Semen	oldSE1	M	05.05.2014	17.08.2015	1	104	15.1
	oldSE2	M	05.05.2014	17.08.2015	1	104	5.7
	oldSE3	M	16.09.2011	17.08.2015	3	335	10.0
	oldSE4	M	15.09.2011	17.08.2015	3	336	0.9
Vaginal secretion	oldVS1	F	23.10.2014	17.08.2015		298	67.3
	oldVS2	F	11.06.2012	17.08.2015	3	67	56.6
	oldVS3	F	14.10.2011	17.08.2015	3	307	259.0

F—female; M—male; n.a.—gender information not available.
[a] Not otherwise specified.