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PAPER

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Specific Micro-RNA Signatures for the Detection of Saliva and Blood in Forensic Body-fluid Identification

ABSTRACT: Micro-RNAs (miRNAs) are a class of small noncoding RNA (ncRNA) molecules with a length of 18–24 nucleotides which play an essential regulative role for many cellular processes. Evidence suggests that the miRNome is a more precise and meaningful representation of a cell type and condition than the mRNA transcriptome. To identify miRNAs that are suitable for forensic body-fluid identification, a global screening by microarray analysis of *c.* 800 miRNAs of forensic blood and saliva samples was performed, and by bioinformatic processing, three differentially expressed candidate miRNAs for saliva and blood each were selected. The six candidates were then validated and confirmed via quantitative PCR. Herein, we present miRNA assays consisting of three differentially expressed miRNAs for the identification of blood (miR-126, miR-150, miR-451) and saliva (miR-200c, miR-203, miR-205), respectively. We conclude that miRNA extraction from forensic samples is possible and support a "proof of concept" that body-fluid identification by miRNA analysis may become a potent forensic technique.

KEYWORDS: forensic science, forensic genetics, body-fluid identification, micro-RNA, expression profiling, RNA extraction

Micro-RNAs (miRNAs) are a class of small noncoding RNA (ncRNA) molecules with a length of 18–24 nucleotides which play an essential regulative role for many cellular processes. MiRNAs can downregulate gene expression by base-pairing with the 3′ untranslated regions (3′UTR) of target messenger RNAs (mRNAs), and miRNA-mediated regulation is now acknowledged to represent a new instance of regulatory control over gene expression programs in many organisms. At the present day, more than 700 miRNAs have been identified for *Homo sapiens* and bioinformatic estimations predict the existence of 800–1000 miRNAs in the human genome (1,2). This approaches 2–3% of the number of all protein-coding genes (3,4). Newly discovered miRNAs are sequentially numbered and entered into the official "miRBase" registry which contains actual sequence data for both precursor and mature molecules as well as further information, for example, hints to putative functions (5). More than half of all mammalian mRNAs are under selective pressure to maintain the sequence integrity of their miRNA binding sites (6), and there may hardly be a biological process or function that is not influenced however subtly or selectively by it.

It is known that nearly all cells harbor a cell-type-specific transcriptome, that is, the entirety of mRNA at a given time, representing the dynamic correlate of the cells momentary need of gene expression environment. When miRNA entered the stage of biomedical research and after first associations of dysregulated miRNAs with disease had been discovered, miRNA profiling was brought into focus and methods for comprehensive and quantitative miRNome analysis were being devised (7–9). It soon became apparent that miRNA profiling, quantitating only about 200 miRNAs, performed better in classifying even weakly differentiated tumors than did mRNA profiling with several thousand mRNAs investigated (10,11), suggesting that the miRNome is a more precise and meaningful representation of a cell type and condition than the mRNA transcriptome. Another advantage of miRNA analysis is that because of their tiny size of about 22 nt, mature miRNAs are more stable than mRNAs which is of paramount importance in forensic settings as it renders mature miRNAs notably less susceptible against fractionation by chemical and/or physical environmental strain.

We therefore examined whether body-fluid-specific miRNA signatures for blood and saliva consisting of three miRNAs could be distilled from the global miRNome data of microarrays and confirmed by quantitative PCR to identify forensically relevant blood and saliva samples.

Materials and Methods

Samples

Body fluids for the microarray were collected from five healthy donors (four females, one male). Saliva was collected using sterile buccal swabs. Blood samples were obtained by vein puncture using dry vacutainer tubes.

Body fluids for quantitative PCR validation were collected as described earlier from five different healthy donors (three females, two males). Additionally, in one case, liquid saliva was collected in a reaction tube. The aged blood sample was generated by pipetting 50 μL freshly drawn blood on a sterile swab and storing it for 1 year at darkness and room temperature. The mixed sample consisted of 100 μL of liquid blood and 100 μL of liquid saliva.

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MiRNA Extraction

Before RNA extraction, all surfaces, devices, and machines used in the extraction procedure were thoroughly cleaned using RNase-Zap® (Ambion, Austin, TX) to remove all traces of ambient RNases. Only RNase-free liquids and plastic consumables were used.

Total RNA was extracted from liquid blood (50 μL), saliva swabs, mixtures of blood and saliva samples, liver, muscle and brain tissue and enriched for small RNAs including miRNAs using the miRNeasy Mini Kit and the RNeasy MinElute Cleanup Kit (Qiagen, Hilden, Germany), respectively, according to the manufacturers protocols. When miRNA was extracted from blood, a pretreatment to remove erythrocytes was performed, using RBC-Buffer (Qiagen). Briefly, 5 vol. of RBC-Buffer was added to the blood sample followed by incubation on ice for 15 min and centrifugation at 4°C and $400 \times g$ for 10 min. The supernatant was discarded, and 2 vol. of RBC-Buffer was added to the pellet which was then resuspended by vortexing and again centrifuged as described earlier. The supernatant was discarded, and the pellet was then introduced to the extraction procedure.

RNA concentration and quality, represented by the RNA integrity number (RIN), was determined using the Qubit fluorometer (Invitrogen, Darmstadt, Germany) and the 2100 Bioanalyzer (Agilent, Böblingen, Deutschland).

Microarray Analysis and Bioinformatics

Expression of about 800 miRNAs from blood and saliva samples of five individuals was analyzed using microarrays. For the analysis, Geniom® Biochips (Heidelberg, Germany) were employed. The probes are designed as the reverse complements of all major mature miRNAs and the mature sequences as published in the current Sanger miRBase release for *Homo sapiens* (version 14.0) (12). Signal distribution was evaluated by plotting the spatial distribution. An intensity gradient was adjusted for by carrying out a global background subtraction. All further analyses were performed using the local background corrected data. The data were normalized applying a probabilistic algorithm (change-of-variance function for dependent data or "function *h*") to remove the transcript variance dependency of their mean, thereby transforming the data to a generalized
log-scale. Microarray and bioinformatic analyses were performed by febit (Heidelberg, Germany) to whom we sent the extracted and enriched miRNA.

Candidate Selection

Three candidates for body-fluid identification were selected from the array data for blood and saliva, respectively. We chose miRNAs that were highly differentially expressed and showed strongly different expression values in saliva and blood, respectively. MiRNAs that had high expression values in blood samples were assigned to the blood assay "panel," whereas miRNAs highly expressed in saliva samples constituted the saliva assay panel (Table 1).

Evaluation by Quantitative RT-PCR

Expression of selected miRNA candidates for blood and saliva samples was performed by quantitative RT-PCR (qPCR) utilizing the miScript system (Qiagen). This system consists of the following components: miScript reverse transcription kit for single-step cDNA synthesis, miScript SYBR Green PCR Kit, which includes the miScript Universal Primer that allows detection of mature miRNAs in combination with an miRNA-specific primer and miScript Primer Assays.

MiRNA was extracted and quantified as described earlier, and 50 ng was then reversely transcribed using the miScript reverse transcription kit (Qiagen) according to the manufacturer's protocol: during the reverse transcription step, miRNAs are polyadenylated by poly(A) polymerase. Reverse transcriptase converts miRNA to cDNA, and the oligo-dT primers used in the reaction carry a universal tag sequence on the 5′ end which allows amplification in the following qPCR step. The cDNA from the reverse transcription step serves as the template for qPCR analysis using a specific miScript Primer Assay (Qiagen) (Table 1) in combination with the miScript SYBR Green PCR Kit (Qiagen) on an ABI Prism 7000 system (Applied Biosystems, Weiterstadt, Germany). Mature miRNAs were thus amplified using the miScript Universal Primer together with an miRNA-specific primer. Briefly, 1–3 ng of cDNA equivalent was used in a reaction volume of 50 μL.

For data normalization, expression of RNA 6b (RNU6b) was measured (Table 1). This spliceosomal RNA is highly conserved and ubiquitous and therefore well suited for normalization (23).

Bioinformatic Analysis

Hierarchical clustering was carried out as bottom-up complete linkage clustering using the Euclidean distance as a measure.

For variance-related barplots, miRNA expression was scored using the function

$$S(t) = \frac{\mathrm{var}(t)}{\sum_{i=1}^{g} \mathrm{var}(t,i)}$$

with var(t) meaning the complete variance of all samples for miRNA t and g representing the number of biological groups (i.e., 2) and var(t, i) representing the variance in biological group i. These were calculated by febit.

For qPCR normalization, RNU6b Ct values were subtracted for each sample from the Ct values of the respective miRNA, resulting in ΔCt:

$$\Delta\mathrm{Ct} = \mathrm{Ct}(\mathrm{miRNA}) - \mathrm{Ct}(\mathrm{RNU6b})$$

TABLE 1—*Body-fluid identification assay panels.*

Body-Fluid/Task	Assay Name	Sequence	References
Blood	miR-126	UCGUACCGUGAGUAAUAAUGCG	(4,13)
	miR-150	UCUCCCAACCCUUGUACCAGUG	(4,13)
	miR-451	AAACCGUUACCAUUACUGAGUU	(4,14,15)
Saliva	miR-200c	UAAUACUGCCGGGUAAUGAUGGA	(4,16–18)
	miR-203	GUGAAAUGUUUAGGACCACUAG	(4,19,20)
	miR-205	UCCUUCAUUCCACCGGAGUCUG	(4,18,19,21)
Normalization	RNU6b	CTGCGCAAGGATGACACGCAAATTCGTG AAGCGTTCCATATTTTT	(22,23)

As a measure of normalized miRNA expression $2^{-\Delta Ct}$ was used.

For relative quantification, normalized data were calibrated by using the other type of body fluid as calibrator, respectively, calculating ΔΔCt:

$$\Delta\Delta Ct = \Delta Ct\left(\mathrm{miRNA}_{\mathrm{body-fluid-1}}\right) - \Delta Ct\left(\mathrm{miRNA}_{\mathrm{body-fluid-2}}\right)$$

Relative fold change was then calculated, according to Livak and Schmittgen (24) as $2^{-\Delta\Delta Ct}$.

Results

MiRNAs Show Differential Expression in Saliva and Blood

The expression of about 800 miRNAs in blood and saliva samples was simultaneously assessed by microarray analysis. After background correction and normalization, a hierarchical cluster analysis of the 70 most differentially expressed miRNAs was performed. Clustering tries to identify samples that show a similar expression intensity pattern and thus are similar to each other. According to the similarity, a hierarchy of similarity clusters is built which is represented as a tree, a so-called dendrogram. Figure 1 shows a principal component analysis heatmap with a dendrogram on top. Differentially expressed miRNAs are strongly expressed in saliva (red) and weakly expressed in blood (green) and *vice versa*. The analysis showed high correlation within the sample groups blood and saliva, and each sample could be ordered to each group according to its miRNA expression profile.

Selection of Candidate miRNAs for Body-fluid-specific Signatures

Three miRNAs for blood and saliva, respectively, were selected from the most differentially expressed miRNAs as detected by microarray analysis. A volcano plot was generated to enable a visual identification of those miRNAs that display large-magnitude changes that are also statistically significant (Fig. 2). Subsequently, variance-related barplots were generated to confirm that the selected candidate groups of miRNAs showed low "intragroup" variance but strongly differed in their expression intensities between blood and saliva, respectively (Fig. 3). Table 1 shows the selected candidate miRNAs for saliva and blood.

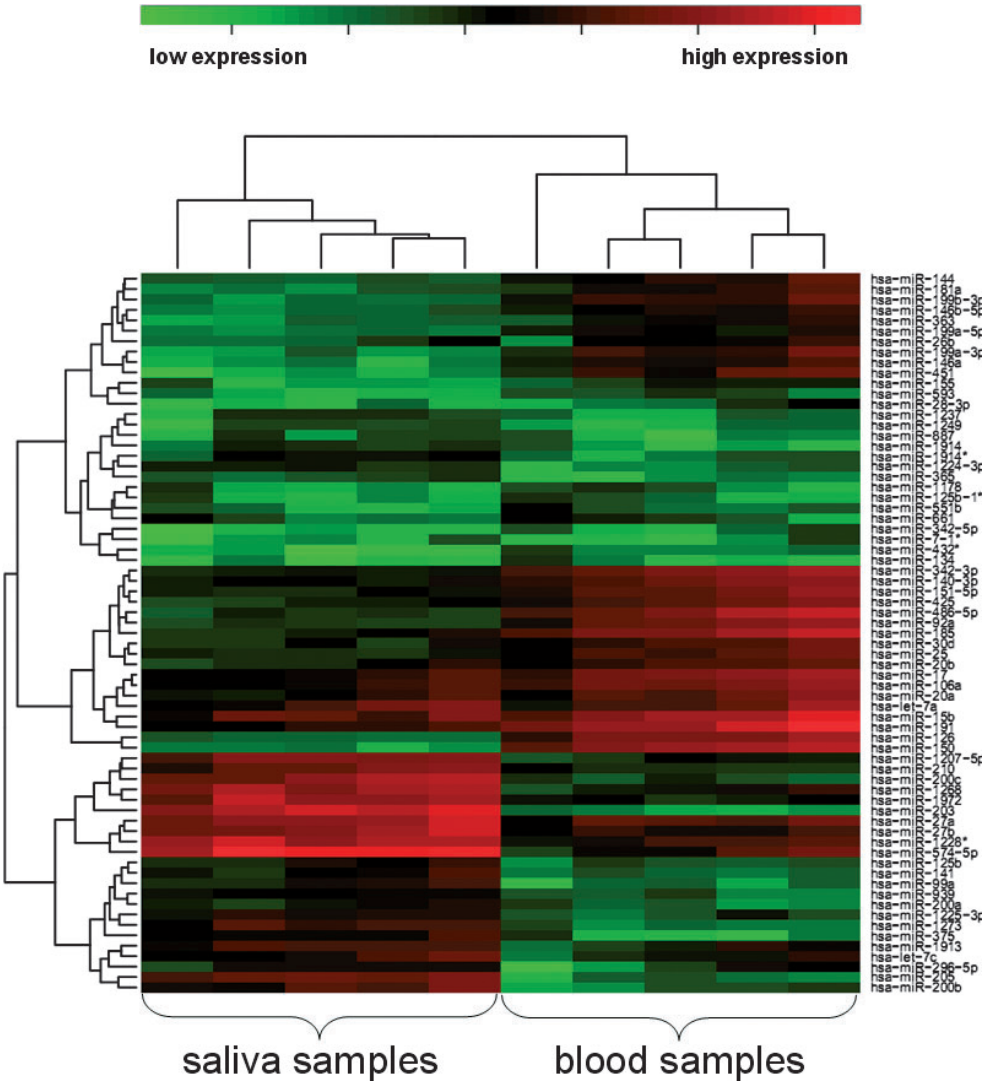

FIG. 1—*Hierarchical cluster heatmap of the most differentially expressed miRNAs. To detect possible clusters in rows (miRNA transcripts) and columns (samples) of the normalized expression matrix, hierarchical clustering was carried out. The dendrogram on top of the heatmap demonstrates that the sample of the two body fluids cluster together.*

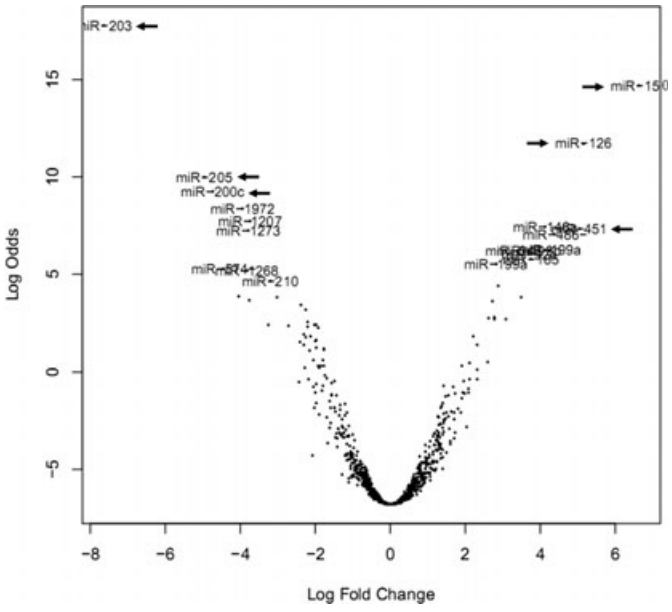

FIG. 2—*Volcano plot for candidate identification. This plot combines a statistical test (represented by odds) with the magnitude of the change. MiRNAs (as indicated by arrows) that are found toward the top of the plot that are far to either the left- or the right-hand side represent values that display large magnitude fold changes as well as high statistical significance which therefore were chosen as candidates.*

Evaluation and Confirmation of miRNA Candidates for the Identification of Blood and Saliva

To evaluate microarray analysis-mediated miRNA candidate selection, quantitative PCR was employed. QPCR results show a strong upregulation of "blood-miRNAs" in blood as compared to saliva and *vice versa* (Fig. 4, upper and lower panel), thereby confirming microarray data and legitimating the candidate selection. A similar result was obtained for a year-old blood stain, affirming that miRNA analysis from aged blood samples is possible and produces coherent results (Fig. 4, middle panel). Then the ability of the miRNA candidates to discriminate between saliva and blood, respectively, and other types of tissue was tested. For "blood-miRNAs" and "saliva-miRNAs," the average of the three candidate miRNA expression values was significantly higher in blood and saliva, respectively, than in all other tissues (Fig. 5). However, differences for "blood-miRNAs" were smaller than those for "saliva-miRNAs," and muscle tissue showed an average expression value of the three "blood-miRNAs" about half as large as that in blood. Analysis of the expression differences of the single "blood-miRNAs" in blood and other tissues showed that whereas miR-150 was nearly only expressed in blood, miR-126 was even stronger expressed in muscle and brain than in blood, and that miR-451 expression was about half as high in muscle as in blood (data not shown).

To test whether miRNA assays can detect components in mixtures of body fluids, liquid blood and liquid saliva were mixed together before RNA extraction and miRNA expression was assessed from the mixture. All six miRNAs showed strong expression comparable to their values in blood ("blood-miRNAs") or saliva ("saliva-miRNAs"), respectively (data not shown).

Discussion

Today, specific mRNA quantification has been extensively investigated in several forensic laboratories as a means of body fluid identification. Much work has been carried out to improve single aspects of this method and to detect and validate RNA signatures for different kinds of biological stains (25–30). The use of mRNA in forensic science has been reviewed in more detail elsewhere (31).

However, mRNA stability and susceptibility to degradation has always been an issue for mRNA-based gene expression analysis, and it was shown that impaired mRNA integrity, represented by the RIN (32), has an influence on the reproducibility of results by introducing a variable extent of bias (33). This is particularly complex for forensic routine applications using mRNA, because biological stains from forensic casework are often challenged by ambient moisture and temperature, UV light, suboptimal environmental pH, potentially degrading mRNA beyond usability. Although evidence concerning mRNA stability in forensic settings is conflicting, there can be no doubt that single-stranded RNA transcripts of considerable length are less stable and more susceptible to degradation by physical and chemical strain and especially by ubiquitous RNAses than for instance a DNA molecule of comparable length.

MiRNA profiling has serious advantages as compared to mRNA profiling. Owing to its small size of about 22 nt, mature miRNAs are much more stable than mRNAs which is of paramount importance in forensic settings as it renders mature miRNAs decidedly less susceptible to deterioration by chemical or physical actions. This also applies to formalin-fixed paraffin-embedded (FFPE) tissue samples that can be of pivotal importance to forensic casework, but in which intense nucleic acid fractionation occurs. Not only has miRNA recovery from FFPE tissues been shown to be feasible and to lead to valid profiling results (34–36), but it even outperforms FFPE tissue mRNA expression profiling by achieving a higher degree of resemblance to fresh tissue profiles and therefore significantly better correlation (37).

The aims of the present work were to confirm miRNA as a forensically relevant molecular species and prove the concept by extracting and enriching miRNA from forensic samples and identifying miRNA assays for reliable identification of blood and saliva that have the potential to easily and affordably be performed in a standard forensic laboratory.

To our knowledge, there are no more than two previous studies assessing the potential of miRNA expression analysis for body-fluid identification in forensic settings (38,39), and our study assays the most comprehensive and up-to-date set of human miRNAs for forensic applicability. The work by Hanson et al. (38) lacks microarray analysis and surveys a panel of only 452 miRNAs, whereas Zubakov et al. (39) did employ microarray analysis but used an out-dated version (10.1) of the miRNA registry (12) as basis for their array and did not succeed in confirming their miRNA candidates for saliva, menstrual blood, and vaginal secretion.

The critical step in the transition from microarray expression data to qPCR validation is candidate selection which always retains some degree of arbitrariness, as there are no fixed rules as to which criteria have to be met for an miRNA to be chosen as a candidate. Notably, given the fact that their miRNA set contained only roughly about half as many miRNAs, one of our candidate miRNAs for blood (miR-451) and for saliva (miR-205) has also been identified by Hanson et al. (38) for the identification of the respective body fluid, corroborating a reproducibly body-fluid indicative and differential expression of these candidates. The candidates, however, that Zubakov et al. (39) chose from their array were entirely different from those we and Hanson et al. chose with exception of the semen markers miR-135b and miR-10b, chosen by Zubakov et al., which are related (but not identical) to miR-135a and miR-10a chosen by Hanson et al. A possible reason for this discrepancy is the differing sets of miRNAs and the

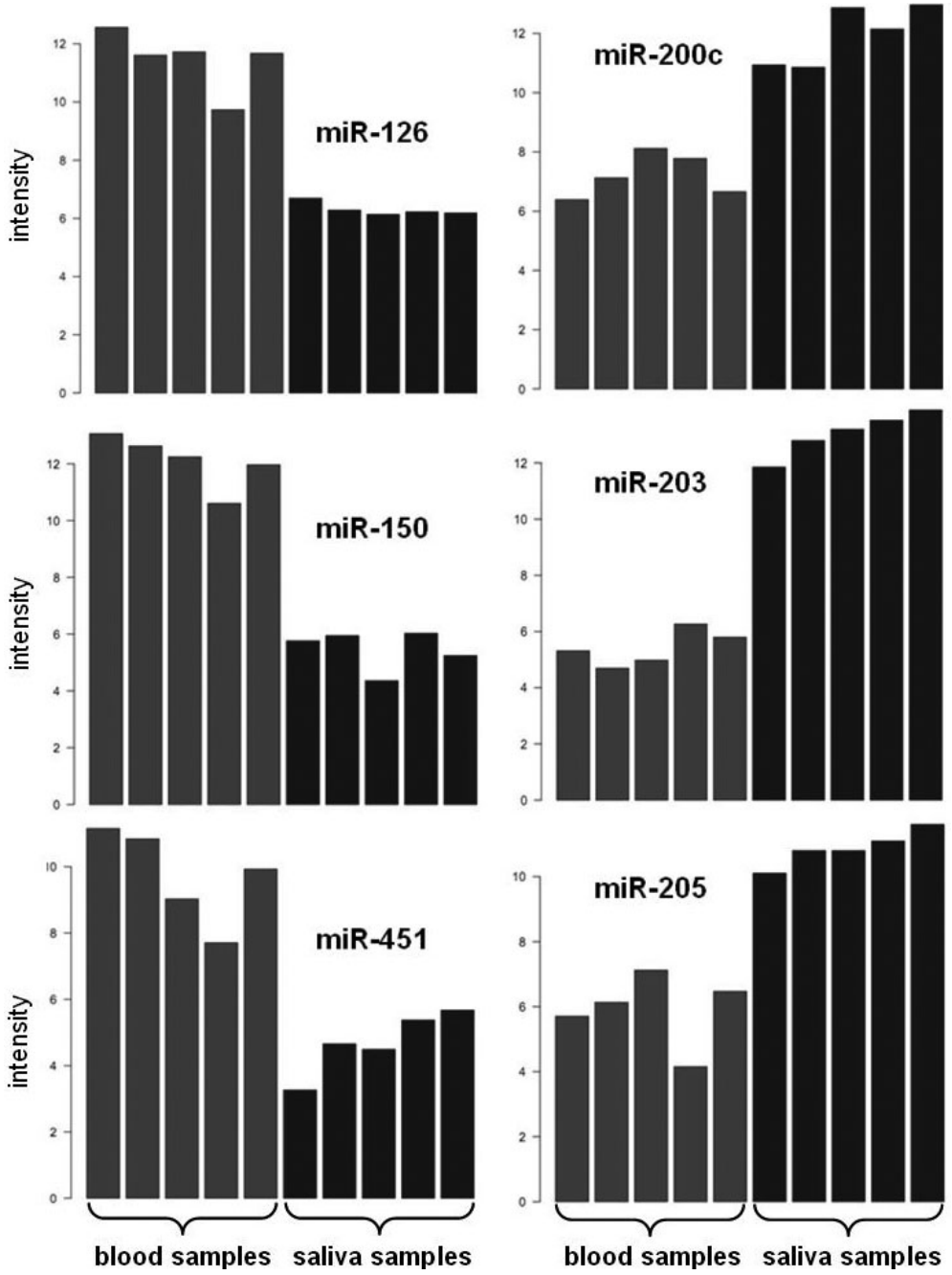

FIG. 3—*Variance-related barplots of differentially regulated miRNA candidates in blood and saliva. The miRNA candidates have low variance in their respective groups (blood and saliva) but have an overall high variance. Data are plotted on a logarithmic scale. Left panels: miRNA candidates for blood identification; right panels: miRNA candidates for saliva identification.*

different microarray platforms and probing chemistries used by our group and Zubakov et al.

In this study, we present miRNA assays consisting of three miRNAs for the identification of blood and/or saliva in forensic samples. These miRNAs were chosen based on comprehensive microarray data and validated using qPCR and were shown to be differentially regulated in "their" assigned type of body fluid and to be able to discriminate between "their" body fluid and another body fluid as well as several kinds of tissue. Moreover, the blood assay was successfully applied to an aged blood sample, confirming miRNA stability in aged samples. We hypothesized that although there is no single body-fluid-exclusive miRNA, only blood and saliva will show an upregulation of all three miRNAs in their respective assay. This was ratified by our results, in that only in blood and saliva, high expression of all three "blood-miRNAs" and "saliva-miRNAs" could be detected, respectively, and in case of blood, only the combination of the expression values of all "blood-miRNA" candidates selected for this body fluid was sufficient for its identification and discrimination from others.

Hanson et al. (38), probably due to their limited set size of 452 miRNAs, could identify only one candidate for vaginal secretions and menstrual blood which very probably is not sufficient for reliable identification and discrimination of these body fluids. We think that microarray analysis is necessary to simultaneously probe a sufficiently large set of miRNAs to enable a selection of at least three miRNA candidates per body fluid that can be combined to a

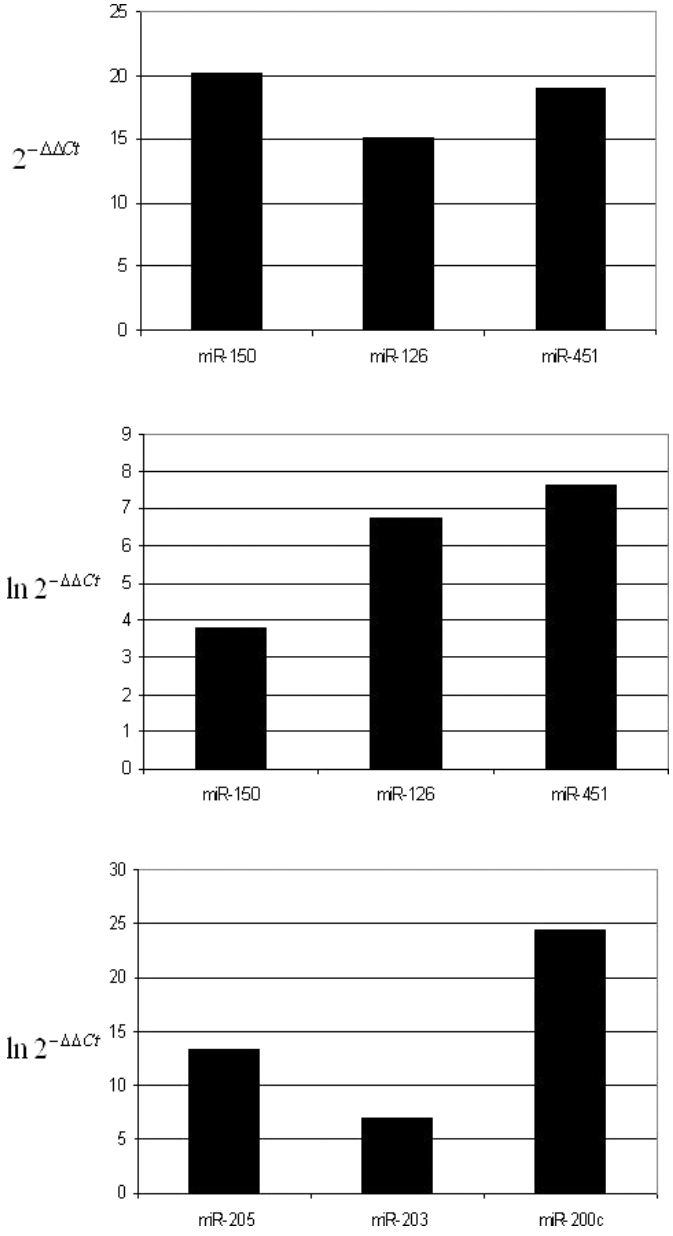

FIG. 4—*qPCR validation of candidate miRNAs for blood and saliva. Upper panel: relative expression of blood candidate miRNAs in blood compared to saliva, for which expression has been arbitrarily set to 0; Central panel: relative expression of blood candidate miRNAs in aged blood compared to saliva for which expression has been arbitrarily set to 0; Lower panel: relative expression of saliva candidate miRNAs in saliva compared to blood for which expression has been arbitrarily set to 0.*

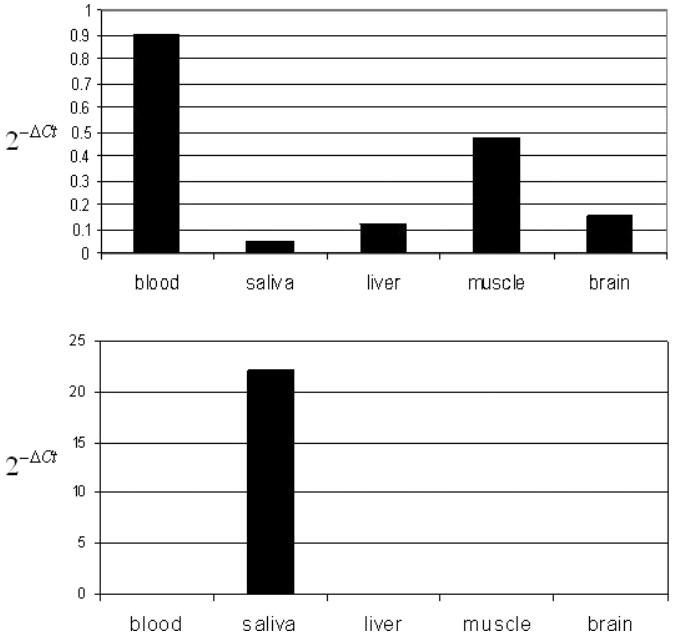

FIG. 5—*Expression of candidate miRNAs in different kinds of tissue. Upper panel: Normalized median expression of the three blood-miRNAs (miR-126, miR-150, miR-451) in blood, saliva, liver, muscle and brain Lower panel: Normalized median expression of the three saliva-miRNAs (miR-200c, miR-203, miR-205) in blood, saliva, liver, muscle and brain.*

body-fluid-specific assay panel. Such assays, consisting of three miRNAs, could easily be multiplexed in one qPCR using primers or fluorescent probes with three different fluorophores, making them accessible for forensic routine procedures.

A limitation of the present study is that only saliva and blood have been analyzed for miRNA expression, and it will be necessary to also investigate other forensically important body fluids like semen, vaginal secretions, and menstrual blood. MiRNA candidates selected for these body fluids will also have to be tested and validated and compared to saliva and blood and *vice versa*.

There are several unsolved issues concerning miRNA analysis that might prove obstacles for a broad acceptance of this analytic method in forensic science community.

The miRNome perhaps even more so than the transcriptome is highly context dependent, and it is conceivable that certain non-physiological or pathologic conditions might alter the expression level of miRNAs employed in assays for body-fluid identification, thereby distorting the results. It will therefore be necessary to test whether the expression of candidate miRNAs for body-fluid identification may be influenced by biological processes or conditions other than cell or tissue identity.

There are several different methods and devices for miRNA extraction, reverse transcription and quantitation which can be combined in many ways and might result in slightly different yields, miRNome compositions, transcription and amplification efficiencies and therefore produce differing results from comparable samples. Therefore, a standardized procedure, optimized for forensic needs and adapted to forensic settings, will be required and has to be thoroughly validated.

MiRNAs are just one of several classes of small, ncRNAs with regulatory functions (40), and there is no reason to exclude these RNAs from forensic analyses, so miRNA analysis may only be an interim strategy until more is known about other small RNAs, and comprehensive small-RNA analysis will come into reach and replace an miRNA-only analysis.

Forensic miRNA analysis is still at its very beginning and although this molecular species undoubtly has vast potential for forensic science, much effort will be required to settle these issues and to make miRNA quantitation a validated, standardized, and reliable technique for forensic casework routine.

Conclusion

By microarray and subsequent qPCR analysis, we selected and validated from a set of *c.* 800 miRNAs three candidates each for the forensically relevant body fluids blood and saliva. We combined the candidates to body-fluid-specific assay panels and successfully used these assays to identify blood and saliva and discriminate these body

fluids from other tissues. We thereby confirm the conclusion of two previous studies that miRNAs may be of great interest for the identification of body fluids and/or tissues in forensic analysis.

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References

1. Bentwich I, Avniel A, Karov Y, Aharonov R, Gilad S, Barad O, et al. Identification of hundreds of conserved and nonconserved human microRNAs. Nat Genet 2005;37(7):766–70.
2. Berezikov E, Guryev V, van de BJ, Wienholds E, Plasterk RH, Cuppen E. Phylogenetic shadowing and computational identification of human microRNA genes. Cell 2005;120(1):21–4.
3. Ruby JG, Jan C, Player C, Axtell MJ, Lee W, Nusbaum C, et al. Large-scale sequencing reveals 21U-RNAs and additional microRNAs and endogenous siRNAs in C. elegans. Cell 2006;127(6):1193–207.
4. Landgraf P, Rusu M, Sheridan R, Sewer A, Iovino N, Aravin A, et al. A mammalian microRNA expression atlas based on small RNA library sequencing. Cell 2007;129(7):1401–14.
5. Griffiths-Jones S, Saini HK, van Dongen S, Enright AJ. miRBase: tools for microRNA genomics. Nucleic Acids Res 2008;36(Data issue):D154–8.
6. Friedman RC, Farh KK, Burge CB, Bartel DP. Most mammalian mRNAs are conserved targets of microRNAs. Genome Res 2009;19(1): 92–105.
7. Liu CG, Calin GA, Volinia S, Croce CM. MicroRNA expression profiling using microarrays. Nat Protoc 2008;3(4):563–78.
8. Raymond CK, Roberts BS, Garrett-Engele P, Lim LP, Johnson JM. Simple, quantitative primer-extension PCR assay for direct monitoring of microRNAs and short-interfering RNAs. RNA 2005;11(11):1737–44.
9. Liu CG, Calin GA, Meloon B, Gamliel N, Sevignani C, Ferracin M, et al. An oligonucleotide microchip for genome-wide microRNA profiling in human and mouse tissues. Proc Natl Acad Sci USA 2004; 101(26):9740–4.
10. Lu J, Getz G, Miska EA, varez-Saavedra E, Lamb J, Peck D, et al. MicroRNA expression profiles classify human cancers. Nature 2005; 435(7043):834–8.
11. Jiang J, Lee EJ, Gusev Y, Schmittgen TD. Real-time expression profiling of microRNA precursors in human cancer cell lines. Nucleic Acids Res 2005;33(17):5394–403.
12. Griffiths-Jones S. The microRNA Registry. Nucleic Acids Res 2004;32(Data issue):D109–11.
13. Lagos-Quintana M, Rauhut R, Yalcin A, Meyer J, Lendeckel W, Tuschl T. Identification of tissue-specific microRNAs from mouse. Curr Biol 2002;12(9):735–9.
14. Altuvia Y, Landgraf P, Lithwick G, Elefant N, Pfeffer S, Aravin A, et al. Clustering and conservation patterns of human microRNAs. Nucleic Acids Res 2005;33(8):2697–706.
15. Sewer A, Paul N, Landgraf P, Aravin A, Pfeffer S, Brownstein MJ, et al. Identification of clustered microRNAs using an ab initio prediction method. BMC Bioinformatics 2005;6:267.
16. Michael MZ, O'Connor SM, van Holst Pellekaan NG, Young GP, James RJ. Reduced accumulation of specific microRNAs in colorectal neoplasia. Mol Cancer Res 2003;1(12):882–91.
17. Suh MR, Lee Y, Kim JY, Kim SK, Moon SH, Lee JY, et al. Human embryonic stem cells express a unique set of microRNAs. Dev Biol 2004;270(2):488–98.
18. Lui WO, Pourmand N, Patterson BK, Fire A. Patterns of known and novel small RNAs in human cervical cancer. Cancer Res 2007;67(13): 6031–43.
19. Lim LP, Glasner ME, Yekta S, Burge CB, Bartel DP. Vertebrate microRNA genes. Science 2003;299(5612):1540.
20. Sonkoly E, Wei T, Janson PC, Saaf A, Lundeberg L, Tengvall-Linder M, et al. MicroRNAs: novel regulators involved in the pathogenesis of psoriasis? PLoS One 2007;2(7):e610.
21. Zhu JY, Pfuhl T, Motsch N, Barth S, Nicholls J, Grasser F, et al. Identification of novel Epstein-Barr virus microRNA genes from nasopharyngeal carcinomas. J Virol 2009;83(7):3333–41.
22. Peltier HJ, Latham GJ. Normalization of microRNA expression levels in quantitative RT-PCR assays: identification of suitable reference RNA targets in normal and cancerous human solid tissues. RNA 2008;14(5): 844–52.
23. Brow DA, Guthrie C. Spliceosomal RNA U6 is remarkably conserved from yeast to mammals. Nature 1988;334(6179):213–8.
24. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 2001;25(4):402–8.
25. Bauer M, Patzelt D. Evaluation of mRNA markers for the identification of menstrual blood. J Forensic Sci 2002;47(6):1278–82.
26. Bauer M, Patzelt D. Protamine mRNA as molecular marker for spermatozoa in semen stains. Int J Legal Med 2003;117(3):175–9.
27. Bauer M, Patzelt D. Identification of menstrual blood by real time RT-PCR: technical improvements and the practical value of negative test results. Forensic Sci Int 2008;174(1):55–9.
28. Zubakov D, Kokshoorn M, Kloosterman A, Kayser M. New markers for old stains: stable mRNA markers for blood and saliva identification from up to 16-year-old stains. Int J Legal Med 2009;123(1):71–4.
29. Zubakov D, Hanekamp E, Kokshoorn M, van Ijcken W, Kayser M. Stable RNA markers for identification of blood and saliva stains revealed from whole genome expression analysis of time-wise degraded samples. Int J Legal Med 2008;122(2):135–42.
30. Setzer M, Juusola J, Ballantyne J. Recovery and stability of RNA in vaginal swabs and blood, semen, and saliva stains. J Forensic Sci 2008;53(2):296–305.
31. Bauer M. RNA in forensic science. Forensic Sci Int Genet 2007;1(1): 69–74.
32. Schroeder A, Mueller O, Stocker S, Salowsky R, Leiber M, Gassmann M, et al. The RIN: an RNA integrity number for assigning integrity values to RNA measurements. BMC Mol Biol 2006;7:3.
33. Auer H, Lyianarachchi S, Newsom D, Klisovic MI, Marcucci G, Kornacker K. Chipping away at the chip bias: RNA degradation in microarray analysis. Nat Genet 2003;35(4):292–3.
34. Andreasen D, Fog JU, Biggs W, Salomon J, Dahslveen IK, Baker A, et al. Improved microRNA quantification in total RNA from clinical samples. Methods 2010;50(4):S6–9.
35. Leite KR, Canavez JM, Reis ST, Tomiyama AH, Piantino CB, Sanudo A, et al. miRNA analysis of prostate cancer by quantitative real time PCR: comparison between formalin-fixed paraffin embedded and fresh-frozen tissue. Urol Oncol In press. 2009. doi:10.1016/j.urolonc.2009.05.008.
36. Hui AB, Shi W, Boutros PC, Miller N, Pintilie M, Fyles T, et al. Robust global micro-RNA profiling with formalin-fixed paraffin-embedded breast cancer tissues. Lab Invest 2009;89(5):597–606.
37. Liu A, Tetzlaff MT, Vanbelle P, Elder D, Feldman M, Tobias JW, et al. MicroRNA expression profiling outperforms mRNA expression profiling in formalin-fixed paraffin-embedded tissues. Int J Clin Exp Pathol 2009;2(6):519–27.
38. Hanson EK, Lubenow H, Ballantyne J. Identification of forensically relevant body fluids using a panel of differentially expressed microRNAs. Anal Biochem 2009;387(2):303–14.
39. Zubakov D, Boersma AW, Choi Y, van Kuijk PF, Wiemer EA, Kayser M. MicroRNA markers for forensic body fluid identification obtained from microarray screening and quantitative RT-PCR confirmation. Int J Legal Med 2010;124(3):217–26.
40. Ghildiyal M, Zamore PD. Small silencing RNAs: an expanding universe. Nat Rev Genet 2009;10(2):94–108.

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