Forensic Science International: Genetics 28 (2017) 99–110

Contents lists available at ScienceDirect

Forensic Science International: Genetics

journal homepage: www.elsevier.com/locate/fsig

Research paper

Identification of organ tissue types and skin from forensic samples by microRNA expression analysis

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ARTICLE INFO

Article history:
Received 20 September 2016
Received in revised form 26 January 2017
Accepted 3 February 2017
Available online 5 February 2017

Keywords:
Forensic genetics
MicroRNA
qPCR
Organ tissue identification

ABSTRACT

The identification of organ tissues in traces recovered from scenes and objects with regard to violent crimes involving serious injuries can be of considerable relevance in forensic investigations. Molecular genetic approaches are provably superior to histological and immunological assays in characterizing organ tissues, and micro-RNAs (miRNAs), due to their cell type specific expression patterns and stability against degradation, emerged as a promising molecular species for forensic analyses, with a range of tried and tested indicative markers.

Thus, herein we present the first miRNA based approach for the forensic identification of organ tissues. Using quantitative PCR employing an empirically derived strategy for data normalization and unbiased statistical decision making, we assessed the differential expression of 15 preselected miRNAs in tissues of brain, kidney, lung, liver, heart muscle, skeletal muscle and skin. We show that not only can miRNA expression profiling be used to reliably differentiate between organ tissues but also that this method, which is compatible with and complementary to forensic DNA analysis, is applicable to realistic forensic samples e.g. mixtures, aged and degraded material as well as traces generated by mock stabbings and experimental shootings at ballistic models.

1. Introduction

In the reconstruction of violent crimes, the identification of organ tissues can provide crucial information complementing the DNA based source attribution. Proving that a tissue sample recovered, for instance, from the blade of a knife or a bullet secured at a crime scene originates from an internal organ confirms that a serious injury has been inflicted with the weapon, while the mere presence of the victim's DNA established by standard STR profiling may be argued to result from a superficial cut or innocuous graze. Also, in cases involving post-mortal dismemberment to enable covert disposal of a corpse the identification of even mangled and degraded tissue recovered, for instance, from sewers or waste pipes may be required.

Conventional immunological, histological and/or enzymatic techniques for the assignment of tissue origin [1–4] may, however, pose problems in terms of specificity and sensitivity, especially when only trace amounts of material are present, and may interfere with DNA profiling. In recent years, molecular genetic approaches for the identification of cell types have been or are currently being explored by several groups, including messenger RNA- (mRNA) and microRNA- (miRNA) based methods for body fluid identification (BFI) as well as studies on the inference of organ tissue types using mRNA profiling [5,6] (extensively reviewed in [7–9]).

MiRNAs not only exhibit the prerequisite of cell type specific expression [10–18], but these small non-coding RNAs also feature certain characteristics that render them well suited to the challenging demands of the analysis of forensic samples. 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, miRNAs are detected and quantified in their biologically active form, so, in contrast to mRNAs, no potential splice variants have to be differentiated, and thirdly, there is no wasteful sample consumption as miRNA and DNA can be extracted simultaneously from the same specimen [19–21].

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http://dx.doi.org/10.1016/j.fsigen.2017.02.002
1872-4973/

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 to eliminate non-biological variance is essential [22–25]. 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 [26–28]. We therefore applied a normalization strategy specifically designed for the herein examined set of organ tissues [29].

The present study is, to the best of our knowledge, the first approach to employ miRNA markers for the identification of brain, kidney, liver, lung, skin, heart muscle and skeletal muscle in a forensic context.

2. Materials and methods

2.1. Samples

All samples were anonymized upon collection and discarded after use. The study design and experimental procedures were approved by the ethics committees of the University Hospital of Bonn and the University Medical Center of Schleswig-Holstein.

2.1.1. Native organ tissue samples and dried swabs for marker validation

Specimens of each of the seven examined human organs, i.e. brain, kidney, liver, lung, skin, heart muscle (hereinafter referred to as heart) and skeletal muscle, were obtained during medico-legal autopsies at the Institutes of Forensic Medicine in Bonn and Kiel. Marked signs of putrefaction or pathological change of the sampled organs were set as exclusion criteria. Within one hour of excision, tissue samples were immersed in RNAlater® solution (Ambion™, Austin, TX, USA) and stored at −80 °C until further processing, when samples were allowed to thaw on ice overnight and approximately 100 mg per sample were subjected to nucleic acid extraction.

Additionally, dried organ tissue samples were prepared by rubbing and pricking into the excised native tissue specimens (except skin) with dry stemmed DNA-free cotton swabs (Sarstedt AG & Co, Nümbrecht, Germany). Swabs were allowed to dry at room temperature for 24 h, then stored at −80 °C and prior to extraction thawed at room temperature and let dry for another 24 h. Dry skin samples were obtained by thoroughly swabbing the forehead of informed and consenting adult volunteers with dry stemmed DNA-free nylon swabs (Copan, Brescia, Italy) that were processed after storage at room temperature for 24 h.

2.1.2. Additional non-target samples for specificity testing

To detect potential cross-reactivity of the selected markers to relevant human tissues not represented in this study, total RNA including small RNAs from adipose, bladder, colon, gallbladder, pancreas, spleen, small intestine and stomach (BioChain Institute, Inc., Newark, CA, USA) was used.

Additionally, to detect potential cross-reactivity to human body fluids, RNA extracts of venous blood, saliva, semen, menstrual blood and vaginal secretion [30] were analyzed with the selected markers. For venous blood, three individual specimens were tested while for the remaining body fluids pooled samples of ten (saliva) and five (semen, menstrual blood and vaginal secretion) donors, respectively, were used.

2.1.3. Aged specimens, mixtures and mock case samples

Two dried swabs per organ tissue type were aged for 28 days prior to extraction in a clean environment with between 18 and 25 °C and without direct exposure to sunlight (see 2.1.1 for sampling procedure).

To further assess the effects of putrefaction on miRNA organ profiling success, native organ tissue samples measuring approximately 1 cm^3 were placed in an outside location immediately after autopsy (without prior storage in RNAlater® solution) for 28 days during June/July (n = 2 per organ tissue type; for weather parameters see Supplementary material 1). Specimens were put into translucent plastic containers with perforated lids, allowing air circulation and exposure to circadian variations in temperature, humidity and insolation while restricting access for birds and insects. Macroscopically visible mold growth was excised prior to total RNA extraction.

Mixtures of different organs of the same individual were prepared by combining portions of two to four tissue samples (previously stored in RNAlater® solution at −80 °C) prior to extraction, either in balanced mixtures or with one tissue type as a minor component (Supplementary Table 4).

In an attempt to simulate human gunshot injuries involving perforated organs, experimental contact shots at basic ballistic models (modified after [31,32]) were conducted to generate backspatter and forward spatter samples. Briefly, the model consisted of 1 l polyethylene bottles, filled with 0.9 l ballistic gelatine (10%, type 'Ballistic III' (Gelita, Ebersbach, Germany), prepared as per manufacturer's instructions). 3 × 3 cm plastic foil bags containing a mixture of 3 ml venous blood from informed and consenting adult volunteers and 300 mg ground organ tissue obtained during a medico-legal autopsy were fixed to the front and back of each model and covered with a layer of approximately 3 mm of silicon. The model was fixated in a self-made contraption (see Supplementary material 2) and contact shots were fired in such a manner that both organ tissue containing bags were perforated by the bullet. We performed three series of shots employing three distinct firearms, so that each organ tissue type, i.e. brain, kidney, liver, lung, heart and skeletal muscle, was shot at by three distinct weapons and each weapon was used to shoot all six organ types. The weapons used were a revolver Ruger Speed-Six with caliber .357 158 grain semi-jacketed soft point ammunition, a semi-automatic pistol Sig Sauer P 225 and a semi-automatic pistol Star, both with 9 × 19 mm 115 grain full metal jacket ammunition. All shots were fired in a forensic ballistics laboratory by an authorized expert and according to German laws and regulations. Prior to each shot, the firearms were thoroughly cleaned with Roti® Nucleic Acid-free (Carl Roth, Karlsruhe, Germany) to avoid contamination. Backspatter from within the gun barrel was sampled using a modified double-swab technique [33] employing stemmed DNA-free cotton swabs with one half moistened with 30 μL of 70% ethanol. The swabs were stored at room temperature for 96 h prior to extraction. Forward spatter was caught on a 38 × 46 cm screen of 0.35 mm filter paper (Carl Roth) that was tautened 40 cm behind the ballistic model and was renewed prior to every shot. Filter papers were stored at room temperature for 120 h before sampling cuttings totaling 9 cm^2 presumably containing adherent organ tissue prior to extraction.

To mimic case work samples of abdominal stabbings, identical kitchen knives with 12 × 1.8 cm blades were used to stab stacks of autopsy samples consisting of the following strata: skin on skeletal muscle, skin on skeletal muscle on kidney, skin on skeletal muscle on liver, skin on skeletal muscle on lung and skin on skeletal muscle on heart. Skin samples were excised from the abdomen and had a thickness of 1 to 2 mm (plus approximately 10 mm of adipose tissue) while the strata of internal organs were approximately 15 mm thick. Experiments were performed using three biological replicates per tissue combination. Smears and tissue material were let dry on the knives at room temperature for 24 h and then collected by swabbing the blade with a stemmed DNA-free cotton swab moistened with 100 μl 70% ethanol. Swabs were stored at room temperature for 24 h prior to extraction.