Forensic Science International: Genetics Supplement Series 7 (2019) 129–131

Contents lists available at ScienceDirect

Forensic Science International: Genetics Supplement Series

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

A comparison of endogenous and exogenous RNA reference marker as relevant for accurate Post-Mortem Interval estimation

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A R T I C L E I N F O

Keywords:
PMI
Reference marker
Gene expression
qPCR

A B S T R A C T

The Post Mortem-Interval (PMI), also known as time since death, is of relevance for forensic routine casework and serves primarily as the basis for the determination of the time that has passed since the time of death. The existing, “classical” approaches are characterised by certain limitations because they are predominantly based on morphological examinations.

The present study deals with the assessment of two molecular messengerRNA-markers in different kinds post-mortem human specimens concerning their ability to use them for the estimation of the time of death. Moreover, a comparison between carefully selected endogenous and one exogenous reference marker for the normalisation of the gene expression data as relevant for accurate PMI estimation is drawn.

The evaluation showed that the used approach is methodologically useful but on consideration of the achieved results it was only possible to determine slightly meaningful tendencies. The tested exogenous reference marker was not suitable for sufficient data normalisation whereas the endogenous reference markers showed a stable expression. However, it was not possible to recognise a specific expression level of the genes within a PMI group when endogenous reference markers were used.

1. Introduction

The Post Mortem-Interval (PMI), also known as time since death, is of prime forensic relevance and serves primarily as the basis for the determination of the time that has passed since the time of death. It used to determine the time of death in order to reconstruct the course of events since the time of the crime can be narrowed down as well as in case of homicides, that claimed more than one victim, a statement about the sequence of the deaths can be made [1]. In addition, a determination of the PMI can contribute to the verification of alibis and thus, to the delimitation of suspects.

However, the existing, “classical” approaches are characterised by certain limitations because they are predominantly based on morphological examinations. Hence, they only work for the early PMI.

Therefore, it was tried to establish a new method for the PMI determination by means of a RNA-based gene expression analysis. Numerous studies in this field showed that messengerRNA (mRNA) in postmortal human specimens is stable enough to serve as biomarker for the estimation of PMI [2]. However, also the selection of candidate reference genes is a crucial factor [3].

In the present study, the gene expression analysis was performed by means of quantitative real-time polymerase chain reaction (qPCR) and subsequent normalisation of the data against an external reference RNA or various mRNA, microRNA (miRNA) and small nucleolarRNA (snoRNA), respectively.

2. Material & methods

2.1. Sample collective

Tissue samples from the brain, heart muscle, spleen, adrenal gland, skeletal muscle and blood of female (N = 11) and male (N = 10) deceased with exactly known PMI (death – autopsy of the dead body) were taken during judicially ordered autopsies (see Table 1).

2.2. PMI marker

In accordance with previous work [4,5] the following PMI marker were chosen for the estimation of the time of death: Hypoxia-inducible factor 1-alpha (*HIF1A*) and Period circadian protein homolog 3 (*PER3*).

2.3. Exogenous reference marker

Based on recommendations from literature [6] the commercially

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https://doi.org/10.1016/j.fsigss.2019.09.050
Received 5 September 2019; Received in revised form 23 September 2019; Accepted 23 September 2019
Available online 30 September 2019
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Table 1
Overview of the sample collective.

	PMI [days]				Age [years]				BMI [kg/m²]			
	Female ♀		Male ♂		Female ♀		Male ♂		Female ♀		Male ♂	
	Min.	Max.	Min.	Max.	Min.	Max.	Min.	Max.	Min.	Max.	Min.	Max.
SPMI (♀ N = 5; ♂ N = 5)	2	4	2	4	68	92	15	73	20,23	41,59	16,81	31,43
MPMI (♀ N = 5; ♂ N = 5)	5	7	5	7	49	85	38	82	11,57	22,40	20,75	29,70
LPMI (♀ N = 1)	10		/	/	91		/	/	22,21		/	/

SPMI = Short PMI (2–4 days), MPMI = Medium PMI (5–7 days), LPMI = Long PMI (10 days).
Min. = Minimum; Max. = Maximum; BMI = Body Mass Index.

available TATAA Universal RNA Spike I (TATAA Biocenter AB, Goteborg, Sweden) was used for the normalisation with an exogenous reference marker.

2.4. Endogenous reference markers

For the selection of candidate endogenous reference markers the online accessible software “Genvestigator” v7.3.1 (Nebion AG, Zurich, Switzerland), a multi-organ microarray data browsing algorithm [7], was used. The search resulted in following choice: *PHF1*, *USF2* (both mRNA for *HIF1A*), *KIAA0586*, *ZNF74* (both mRNA for *PER3*), *RNU24* and *hsa-miR-191-5p* (snoRNA and miRNA for *HIF1A* and *PER3*).

2.5. Workflow

Fig. 1 shows the workflow used for working with the exogenous as well as the endogenous reference markers.

2.6. Data analysis

All obtained quantification cycle (C_q)-values were baseline corrected by means of the “LinRegPCR” analysis program v2017.1 (Heart Failure Research Center, Amsterdam, the Netherlands) and efficiency corrected as well as normalised utilising the “GenEx” software v6 (MultiD Analyses AB, Goteborg, Sweden). The thereby determined ΔC_q-values ($\Delta C_q = C_{q\ (mRNA)} - C_{q\ (reference)}$) were rated as unspecific amplification if > 35 and excluded from further analysis.

3. Results

The RNA concentrations noticeably varied between the tissues and the blood, respectively. However, all samples yielded enough RNA for subsequent analyses.

The evaluation of the achieved results for the exogenous reference marker showed that it was not suitable for sufficient data normalisation. The measured spike gene expression noticeably fluctuated between the different samples but also within the tissue types and the blood, respectively.

For the endogenous reference markers stable expression values were obtained. However, it was not possible to recognise a specific expression level of the genes within a PMI group.

4. Discussion & conclusion

Although the RNA concentrations yielded noticeably different results for the used post-mortem human specimens, all are suitable for mRNA profiling and no difference between the RNA yield and the PMI was observed.

Based on the results for the exogenous reference marker it can be seen that it did not fulfil the precondition for a suitable reference marker. Therefore, it is not recommended to use the TATAA Universal RNA Spike I for data normalisation in gene expression analysis.

The tested endogenous reference markers were suitable for data normalisation. It was proven that these kinds of endogenous reference markers should be chosen as references for gene expression analysis. For further studies it will be tried to not only use PMI marker-specific but also tissue-specific reference markers to thereby possibly achieve

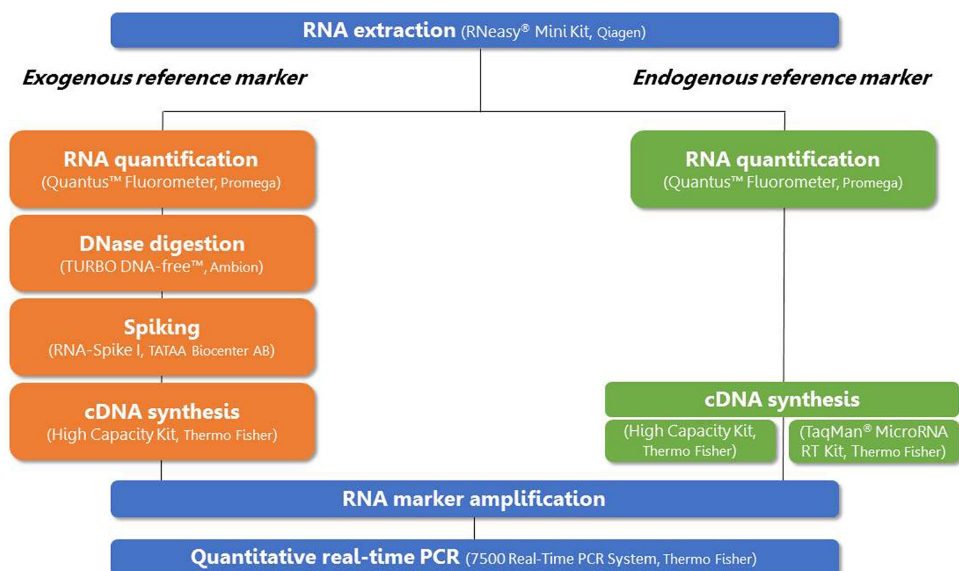

Fig. 1. Overview of the workflow.

PMI-specific expression levels within a PMI group.

All in all, the presented moleculargenetic approach for PMI-determination turned out to be methodologically useful but under consideration of the achieved results as less informative. Nonetheless, the in literature described potential of the tested PMI marker [4,5] is still not disproved but should rather be tested in further studies.

Declaration of Competing Interest

None.

Acknowledgment

We would like to thank the forensic pathologists and human dissectors at the Institute of Legal Medicine Bonn for their help with sample acquisition.

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