

Danio rerio as a model for post-mortem interval estimation based on methylation levels

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Research note

Keywords: Post-mortem interval (PMI), Epigenetics, DNA methylation, Zebrafish, Real time PCR (q-PCR), High Resolution Melting (HRM)

Posted Date: October 31st, 2019

DOI: <https://doi.org/10.21203/rs.2.16607/v1>

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Abstract

Objective The post-mortem interval (PMI) describes the period of time elapsed since death. A precise estimation of PMI has a strong impact in a crime investigation; however, it becomes a complex task due to the influence of intrinsic and extrinsic factors. There are several methods for PMI determination including pathological, biochemical and entomological. Those methodologies have low precision and several practical limitations leading to the emergence of genetic-molecular methods based on degradation of nucleic acids. DNA methylation is a sufficiently stable and time-related epigenetic modification, making it useful for the development of a method to determine PMI. Thus, the methylation levels of a model organism – zebrafish – were analysed since the moment of death, in order to contribute to create a robust model for the PMI estimation.

Results In this study, we evaluated the variation in global methylation levels and at the promoter region of EDARADD gene during 6 post-mortem intervals. An increase of global methylation levels in the initial post-mortem intervals was observed, followed by an abrupt decrease until 48h post-mortem. Followed by the amplification of EDARADD's promoter region by q-PCR and HRM analysis, it was possible to differentiate samples according to relative PMI.

Introduction

A precise estimation of the time elapsed since death - post-mortem interval (PMI) - is one of the major focuses of investigation in forensic science because it allows the corroboration of witness statements, the restriction of the number of suspects and the confirmation of the veracity of their alibis [1]. However, it is a complex problem due to a plethora of factors that affect the process of post-mortem changes. Intrinsic factors include age, sex and physiological and pathological states of the victim. On the other hand, there are extrinsic factors like temperature, humidity and insect activity, which can affect the precise estimation of post-mortem interval. Traditional methods to estimate PMI are generally based on physiological changes such as *algor mortis*, *livor mortis* and *rigor mortis*, but those methods can only provide a rough estimation of PMI [2] [3]. Over the years, several molecular-genetic methods using nucleic acid degradation have been developed for the determination of post-mortem interval. Although those methods are very promising and the parameters analysed depend on the time since death, a new approach appears: the possibility to study the variation of DNA methylation levels to estimate PMI.

Methylation is a well-studied chemical modification of DNA, a molecule with high post-mortem stability proven by several authors [4]. DNA methylation consists in the addition of a methyl group to the fifth carbon of a cytosine (5-mC) preceding a guanine (CpG). The analysis of DNA methylation patterns is a methodology with enormous potential to estimate the PMI, despite not being exploited currently.

In this study, we evaluated the global DNA methylation levels during PMI, as well as the variation of methylation levels at EDARADD gene promoter. For that purpose, a model organism was used—zebrafish

—which permitted a homogeneous sampling, as well as the control of intrinsic and extrinsic factors that could affect the PMI estimation.

Methods

Zebrafish Care

Zebrafish (*D. rerio*) specimens from a culture established at the Department of Biology, University of Aveiro, were maintained in carbon-filtered water at $27.0 \pm 1^\circ\text{C}$ under a 14:10h light/dark photoperiod cycle. Conductivity is kept at $800 \pm 50 \mu\text{S}$, pH at 7.5 ± 0.5 and dissolved oxygen at 95% saturation. Adult fish were fed once daily with GEMMA 500, a commercially available artificial diet.

Sampling and Genomic DNA Extraction

Twelve healthy adult male zebrafishes at 8 months-old were killed by hypothermia induction. To avoid a heterogeneous sampling, all fish were weighed ($274,17 \pm 90,10\text{mg}$) and body size was registered ($3,13 \pm 0,26\text{cm}$).

After death, they were randomly divided into a control group (PMI=0h) and five experimental groups (PMI=1, 4, 24, 48 and 72h) which were composed of two individuals. Specimens were kept at 4°C during the correspondent *post-mortem* time. Subsequently, whole fish samples were homogenised with liquid nitrogen and frozen at -20°C until DNA extraction using modified CTAB method, according to the protocol described by Stefanova et al. (2013) [5]. DNA concentration was measured using Nanodrop1000™ Spectrophotometer (Thermo Scientific).

Global DNA Methylation

Global DNA methylation was measured with the MethylFlash™ Methylated DNA Kit – Colorimetric (Epigentek) following the manufacturer's protocol. The OD intensity of the colorimetric reaction was measured by a Biotek ELx800 plate reader (Winooski, VT, USA). The OD intensity of each sample was normalized to 100 ng of DNA. The absolute quantification of cytosine methylation was calculated in two-steps: first, it was generated a standard curve and then, it was plotted the OD values versus the amount of positive controls at each concentration point, followed by determination of the slope (OD/ng) of the standard curve using linear regression. The cytosine methylation was calculated by using the formula: $[(\text{OD sample} - \text{OD negative control}) / (\text{slope} \times 2)]$ where 2 is a factor to normalize 5-methylcytosine in the positive control to 100%. Cytosine methylation percentage was calculated according to: $[(5\text{-mC Amount (ng)}) / (S)] \times 100$; being 5-mC amount (ng) the value obtained by the formula above and S the amount of DNA per sample. Each control was measured in duplicate and each post-mortem sample in triplicate.

All statistical calculations were performed using Microsoft Excel (2010) and IBM Software Package for the Social Sciences (SPSS, IBM). The t-student’s test was applied in order to find significant differences between pooled zebrafish measurements for each PMI.

Methylation at EDARADD gene promoter

1. Sodium Bisulphite Treatment

DNA methylation analysis was performed with bisulphite conversion, where non-methylated cytosines were converted in uracils keeping 5-methylcytosines intact. DNA bisulphite modification was performed using EZ DNA Methylation-Gold™ Kit (Zymo Research) following the manufacturer's recommendations.

1. Amplification by q-PCR

The amplification by q-PCR was performed for EDARADD gene promoter. In order to analyse EDARADD levels during PMI, a q-PCR array was carried out in 20 µL volume and contained 10µL *Xpert Fast SYBR (uni)* (Grisp).), 0.3 µM of forward and reverse primer (Table 1) and 1µL of the DNA template. Real Time PCR was carried on CFX96™ Real-Time PCR (Bio-Rad), with the following conditions: initial denaturation at 95 °C for 3min, followed by 40 cycles at 95°C for 5s, 30s at the primer’s specific annealing temperature and DNA extension at 72°C for 30s.

Table 1: Information about primers for EDARADD gene promoter.

Gene promoter	Primer	Sequence	Annealing Temperature (°C)	Fragment size (bp)
EDARADD	Forward	GGT AGA TTA AGA GGA AGT TTA TTT TTT TAT	57	231
	Reverse	AAT ACC TCT CCC CAT CTA TTT AAT C		

High-resolution *melting* curve analysis

HRM was conducted immediately after amplification in the following conditions: increase in temperature from 65 °C to 95 °C, with 0.5 °C increment, each step with a 5s hold. The HRM data was analysed using the *Precision Melt Analysis™ Software* (Bio-rad).

Results

Quantification of global DNA methylation levels during PMI

After DNA extraction from the post-mortem samples of the whole fish, the levels of global methylation were measured. For control samples, 8 month-old zebrafish that were not submitted to any PMI, the

methylation levels were around 3%, the same value found in other study. [6]

Figure 1: Variation in DNA Methylation levels during PMI.

Regarding the variation of methylation between 0h and 1h after death, the levels did not undergo statistically significant changes, increasing only 0.35%. As for the variation in methylation between 1h and 4h post-mortem, a statistically significant increase of 1.49% was observed. A statistical data shows this in more detail (see Additional File 1).

Between 4h and 24h after death, the results show a 0.76% decrease in methylation (Figure 1), however, it is not statistical significant. Comparing the variation in the amount of 5-mC between 24 and 48 post-mortem hours, the methylation level decreased about 1.75%, reaching the lowest value recorded throughout the study (see Additional File 1).

Finally, regarding the interval between 48h and 72h post-mortem, the results show a marked increase (3.45%) in the amount of methylated cytosines, reaching the highest value recorded at 72h after death (see Additional File 1).

Variation of methylation levels in EDARADD gene promoter

After bisulphite treatment, the methylated status of the EDARADD gene promoter was evaluated by q-PCR followed by HRM analysis.

Table 2: Data obtained by HRM analysis for EDARADD gene promoter, according to PMI.

PMI (h)	Sample	Cluster	Melting temperature (°C)	Percent confidence (%)
0	ip0	1	72,5	97,9
	ip0'			
	ip0a	2		97,6
	ip0a'			
1	ip1	1	72,5	97,8
	ip1'		72	
	ip1a	5	72,5	98,0
	ip1a'			
4	ip4	6	72	97,9
	ip4'			
	ip4a	2	72,5	97,5
	ip4a'			
24	ip24	1	72,5	98,9
	ip24'			
	ip24a			
	ip24a'	5	72	98,0
48	ip48	3	72	98,2
	ip48'			
	ip48a	4		97,8
	ip48a'			
72	ip72	7	73,5	97,7
	ip72'			
	ip72a		73	98,7
	ip72a'			

As it can be seen in Table 2, samples were grouped in 7 different clusters and, in some cases, independently of the PMI to which they belonged. For example, the samples ip0 and ip0a, even belonging to the same PMI were assembled in cluster 1 and 2, respectively. The samples ip1, ip24 and ip24a were also assembled in cluster 1, which means that there are no significant differences in *melting* curves that distinguish them. On the other hand, it was recorded that the samples corresponding to PMI=72h were assembled in the same cluster, emphasizing their similarity.

Discussion

This study aimed to evaluate the variation of global and target gene promoter methylation levels over the time elapsed since death. The promoter region of EDARADD gene was selected for methylation analyses since it has been documented to be related to the human aging process [7]. The sampling of the study was composed by individuals of the same age in order to observe the variation of methylation levels in this promoter with the PMI. The results indicate an oscillation of methylation levels over the post-mortem interval both in overall methylation and at the promoter region of the EDARADD gene.

HRM analysis allowed to group the samples in different clusters, depending on the PMI. Initially, because it is an extremely sensitive approach, this technique is capable of distinguishing between samples within the same PMI. However, at longer PMIs, the distinction only occurs by the post-mortem interval, meaning that no differences between samples are considered.

Zebrafish was a successful model organism for post-mortem interval estimation, because it allowed a homogeneous sampling and the ability to control all extrinsic factors.

Limitations

In this study it was used 12 specimens. The next step is increase sampling using this PMI and, later includes longer PMI and other type of samples, namely specific tissue samples. Finally this methodology should be tested in other model organisms until is fully improved for application in criminal investigations.

List Of Abbreviations

PMI—post-mortem interval

EDARADD—EDAR-Associated Death Domain

q-PCR—Real time Polymerase Chain Reaction

HRM—High Resolution Melting

Declarations

Ethics approval and consent to participate

Ethical consent was provided by Department of Biology, University of Aveiro.

Availability of data and material

Data will be available upon reasonable request from the corresponding author.

Funding

No funding source available.

Acknowledgements

Authors would like to thank the CESAM-Centre for Environmental and Marine Studies, University of Aveiro for providing the zebrafish facility.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

Author's Contributions

Rita Pina and Luís Souto conceived of the study. Rita Pina developed all the experimental procedures, the analysis of the results and drafted the manuscript. Helena Moreira helps Rita Pina with the analysis of HRM results. All the authors participated in the revision of the paper and approved the final manuscript.

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Figures

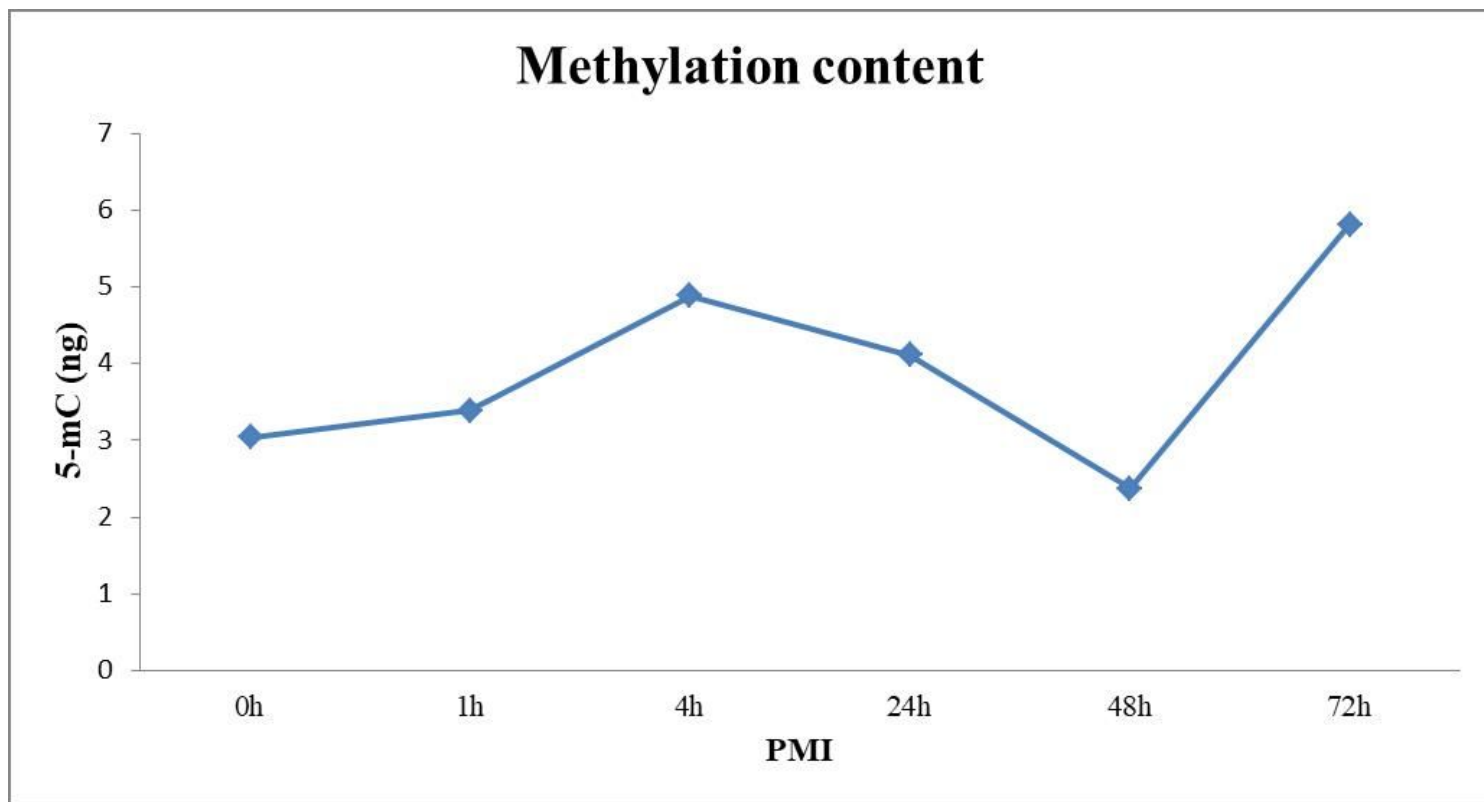


Figure 1

Variation in DNA Methylation levels during PMI

Supplementary Files

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- [AdditionalFiles.docx](#)
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