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Accurate predictions of postmortem interval using linear regression analyses of gene meter expression data

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ABSTRACT

In criminal and civil investigations, postmortem interval is used as evidence to help sort out circumstances at the time of human death. Many biological, chemical, and physical indicators can be used to determine the postmortem interval – but most are not accurate. Here, we sought to validate an experimental design to accurately predict the time of death by analyzing the expression of hundreds of upregulated genes in two model organisms, the zebrafish and mouse. In a previous study, the death of healthy adults was conducted under strictly controlled conditions to minimize the effects of confounding factors such as lifestyle and temperature. A total of 74,179 microarray probes were calibrated using the Gene Meter approach and the transcriptional profiles of 1063 genes that significantly increased in abundance were assembled into a time series spanning from life to 48 or 96 h postmortem. In this study, the experimental design involved splitting the transcription profiles into training and testing datasets, randomly selecting groups of profiles, determining the modeling parameters of the genes to postmortem time using over- and/or perfectly-defined linear regression analyses, and calculating the fit (R^2) and slope of predicted versus actual postmortem times. This design was repeated several thousand to million times to find the top predictive groups of gene transcription profiles. A group of eleven zebrafish genes yielded R^2 of 1 and a slope of 0.99, while a group of seven mouse liver genes yielded a R^2 of 0.98 and a slope of 0.97, and seven mouse brain genes yielded a R^2 of 0.95 and a slope of 0.87. In all cases, groups of gene transcripts yielded better postmortem time predictions than individual gene transcripts. The significance of this study is two-fold: selected groups of gene transcripts provide accurate prediction of postmortem time, and the successfully validated experimental design can now be used to accurately predict postmortem time in cadavers.

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1. Introduction

The postmortem interval (PMI) is the elapsed time between death of an organism and the initiation of an official investigation to determine the cause of death. Its prediction is important to civil investigations such as those involving life insurance fraud because investigators need to determine if the person was alive or not when the policy was in effect [1]. The PMI is also important to criminal investigations, especially suspicious death cases where there are no witnesses, because it can help determine the time relationship between a potential suspect and the victim and eliminate people from a suspect list, which speeds up investigations. Accurate prediction of PMI is considered one of the most important and complex tasks performed by forensic investigators [2].

Several studies have suggested that RNA could be used to estimate PMI [3–7]. While most studies focused on the degradation of mRNA gene markers, some examined gene expression. The RNA degradation studies include: a model to predict PMI based on the degradation of Beta actin (*Actb*), Glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*), Cyclophilin A (*Ppia*) and Signal recognition particle 72 (*Srp72*) genes in mouse muscle tissue samples [3], a model to predict PMI based on degradation of an amplified *Actb* gene and temperature in rat brain samples [4], and a study that predicted PMI based on the degradation of *Gapdh*,*Actb* and 18S rRNA genes in the spleens of rats [5]. The gene expression studies include: a study that found increased expression of myosin light chain 3 (*Myl3*), matrix metalloprotease 9 (*Mmp9*) and vascular endothelial growth factor A (*Vegfa*) genes in human body fluids

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after 12 h postmortem [6], a study that found increased expression of Fas Ligand (*Fasl*) and 'phosphatase and tensin homologue deleted on chromosome 10' (*Pten*) genes with postmortem time in rats [7], and a study that found individual gene transcripts did not increase using PCR-based gene expression arrays of frozen human brain cadaver samples [8]. Common to these studies is the requirement: (i) to amplify cDNA by polymerase chain reaction (PCR) and (ii) to normalize the data with a control in order to facilitate sample comparisons. These requirements introduce methodological biases that could significantly affect interpretation of the data [9–11]. An approach that minimizes or eliminates these biases is highly desirable because it might lead to better PMI predictions.

Since conventional DNA microarray approaches yield noisy data [12], in 2011 we developed the "Gene Meter" approach that precisely determines specific gene abundances in biological samples and minimizes noise in the microarray signal [13,14,16]. The reason this approach is precise is because the behavior of every microarray probe is determined by calibration – which is analogous to calibrating a pH meter with buffers. Without calibration, the precision and accuracy of a meter is not known, nor can one know how well the experimental data fits to the calibration (i.e., R^2). The advantages of the Gene Meter approach over conventional DNA microarray approaches is that the calibration takes into consideration the non-linearity of the microarray signal and calibrated probes do not require normalization to compare biological samples. Moreover, PCR amplification is not required. We recognize that next-generation-sequencing (NGS) approaches could have been used to monitor gene expression in this study. However, the same problems of normalization and reproducibility are pertinent to NGS technology [15,16]. Moreover, a recent publication, by a group from National Institute of Standards and Technology, used the same dilution series approach (as we did) to evaluate and calibrate RNASeq [17]. They found RNASeq comparable to microarrays, in terms of target quantification, but not superior, as it may be perceived by the community. Hence, the Gene Meter approach is currently the most advantageous high throughput methodology to study postmortem gene expression and might have utility for determining the PMI.

The Gene Meter approach has been used to examine thousands of postmortem gene transcription profiles from 44 zebrafish (*Danio rerio*) and 20 house mice (*Mus musculus*) [18]. Many genes were found to significantly increase in abundance relative to live controls. Given that each sampling time was replicated two or three times, we conjectured that the datasets could be used to assess the feasibility for predicting PMIs from gene expression data. Although many approaches are available to determine PMI (see Section 4), an approach that accurately determines the time of death is highly desired and it is the goal of our study to determine if specific gene transcripts or groups of gene transcripts could accurately predict postmortem time. Zebrafish and mice are ideal for testing experimental designs because the precise time of human deaths is often not known, and other variables such as lifestyle, temperature, and health condition are also often not known or sufficiently controlled in human studies. Given that these variables could have confounding effects on the interpretation of gene expression data in human studies, testing experimental designs under controlled conditions using model organisms is ideal. In our study, the timing of death and health of the zebrafish and mice are known, which enables the testing of different experimental designs to provide "proof of principle". Our intent is to use the best design to determine PMI of cadavers in future studies.

The objectives of our study are twofold: (1) to identify specific gene transcripts or groups of gene transcripts that accurately predict the PMI in the zebrafish and mouse, and (2) to design and evaluate a robust experimental approach that could later be implemented to predict PMI in cadavers.

2. Materials and methods

Although the details of zebrafish and mouse processing, the extraction of RNA, and microarray calibrations are presented in a previous study [18], we have provided relevant experimental protocols to aid readers in the interpretation of the results of this study.

2.1. Zebrafish processing

The 44 zebrafish were maintained under standard conditions in flow-through aquaria with a water temperature of 28 °C. Prior to sacrifice, the zebrafish were placed into 1 L of water of the same temperature as the aquaria. At zero time, four fish were extracted and snap frozen in liquid nitrogen. These live controls were then placed at −80 °C. To synchronize the time of death, the remaining 40 fish were put into a small container with a bottom made of mesh and placed into an 8 L container of ice water for 5 min. The small container with the mesh bottom was placed into the flow-through aquarium with a water temperature of 28 °C for the duration of each individual's designated postmortem time. The postmortem sampling times used for the zebrafish were: 0, 15 min, 30 min, 1, 4, 8, 12, 24, 48 and 96 h. At each sampling time, 4 individuals were taken out of the small container in the flow-through aquarium, snap frozen in liquid nitrogen and then stored at −80 °C. One zebrafish sample was not available for use (it was accidentally flushed down the sink) however this was taken into account for calculation of extraction volumes.

2.2. Mouse processing

Twenty C57BL/6JRj male mice of the same age (5 months) and similar weight were used. Prior to sacrifice, the mice had ad libitum access to food and water and were maintained at room temperature. At zero time, the mice were sacrificed by cervical dislocation and each mouse was placed in a unique plastic bag with pores to permit the transfer of gases. The mice were kept at room temperature for the designated postmortem sampling times. The sampling times used were: "zero" time, 30 min, 1, 6, 12, 24 and 48 h. At each sampling time, a brain and two liver samples were obtained from each of three mice, except for the 48 h sampling where only two mice were sampled. The samples were immediately snap frozen in liquid nitrogen and placed at −80 °C.

2.3. RNA processing and labeling

Gene expression samples for each PMI were done in duplicate for zebrafish and in triplicate for mice (except for the 48 h PMI sample that was duplicated). The zebrafish samples were homogenized with a TissueLyzer (Qiagen) with 20 mL of Trizol. The mouse brain and liver samples (~100 mg) were homogenized in 1 mL of Trizol. 1 mL of the homogenate was placed into a centrifuge tube containing 200 μL of chloroform. The tube was vortexed and placed at 25 °C for three min. Following centrifugation for 15 min at 12,000 RPM, the supernatant was placed into a new centrifuge tube containing an equal volume of 70% ethanol. Purification of the RNA was accomplished using the PureLink RNA Mini Kit (Life Technologies, USA). The purified RNA was labeled using the One-Color Microarray-based Gene Expression Analysis (Quick Amp Labeling). The labeled RNA was hybridized to the DNA microarrays using the Tecan HS Pro Hybridization kit (Agilent Technologies). The zebrafish RNA was hybridized to the Zebrafish (v2) Gene Expression Microarray (Design ID 019161) and the

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