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QUANTIFICATION OF RNA DEGRADATION OF BLOOD-SPECIFIC MARKERS TO INDICATE THE AGE OF BLOODSTAINS

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Abstract

Determining the time since deposition of a biological stain can provide essential information to a police investigation; indicating either when a crime occurred, or whether the biological evidence was deposited at the time of a known crime event. Bloodstains are one of the most important biological evidence types to forensic investigators. This research has used reverse transcription quantitative PCR to examine the relative expression ratio (RER) between different types of blood-specific markers, with the aim of developing a method to estimate the age of bloodstains. Targets included three mRNA markers (HBA, PBGD, HBB) and two microRNA markers (miR16, miR451), along with three reference genes (18S rRNA, ACTB mRNA, U6 snRNA). Blood samples from 10 individuals were deposited onto cotton swabs and stored at room temperature to simulate natural ageing. When samples reached a series of desired age points, total RNA was extracted. Analysis of the degradation rate of individual RNAs showed they exhibited unique degradation profiles during the nine-month storage interval, where miRNAs and U6 were shown to be more stable than other markers. The RERs show a non-linear relationship with bloodstain age, and were shown to be useful for bloodstain age estimation.

Introduction

The role of gene expression and the potential applications of ribonucleic acid (RNA) have been explored in forensic science. RNA types such as messenger RNA (mRNA) and microRNA (miRNA) have been subject to increasing interest in the forensic science community.

Identification of biological stains recovered from crime scenes can make a significant contribution to criminal investigations, i.e. whether they originate from blood, saliva, semen, or other body fluids of forensic interest. Recently, it has been shown that identification of cell type-specific mRNAs and miRNAs can provide high specificity for body fluid identification [1, 2].

As RNA molecules are susceptible to degradation, it has been shown that RNA molecules can also be applied to determine the time a biological stain was deposited at crime scene, by analysing their stability and degradation rate [3]. Estimating the age of a biological stain can provide essential information to an investigation; indicating either when a crime occurred or whether the biological evidence was deposited at the time of a known crime event, or before/after, in order to exclude the sample.

In this study, expression and degradation levels of 8 RNAs encompassing mRNA, miRNA and rRNA were analysed in bloodstains over a 9-month period. The aim was to identify the most/least stable RNAs, towards the development of a method to estimate the age of bloodstains.

Materials and Methods:

Sample collection:

Blood samples were collected from 10 volunteers (6 females, 4 males) using disposable Unistick 3 Comfort lancets. 20 µL of blood was deposited onto sterile cotton swabs and stored at room temperature protected from light to simulate natural ageing. Samples were stored until they reached a desired age (0, 3, 6, 15, 30, 90, 180, 270 days).

RNA extraction:

Total RNA was extracted from bloodstained cotton swabs using the TRI® Reagent method (Sigma-Aldrich) [3]. Genomic DNA was digested with the TURBO DNA-free™ Kit (Applied Biosystems) following the manufacturer's protocol. The quantity of the extracted total RNA was determined using a NanoDrop-1000 Spectrophotometer (Thermo Scientific).

Reverse transcription quantitative PCR (RT-qPCR):

cDNA was synthesised from extracted RNA using the High-Capacity cDNA Reverse Transcription Kit for mRNA/rRNA markers, and TaqMan® microRNA Reverse Transcription Kit for miRNA markers (Applied Biosystems) following the manufacturer's instructions.

Blood-specific markers potentially suitable for bloodstain identification were selected from the forensic science literature [1, 2]; mRNA: HBA, HBB, PBGD; miRNA: miR16 and miR451. ACTB, 18S and snoU6 were selected as reference genes.

Real-time PCR was carried out using the TaqMan® Universal PCR Master Mix II Kit, with no AmpErase® UNG (Applied Biosystems) following the manufacturer's protocol with pre-designed primers.

Statistical analysis:

Quantitative PCR data was analysed using MxPro (Agilent Technologies) and GenEx statistical software (version 5.4.4). Microsoft Excel 2011 was used to present basic data and line graphs and Minitab® 17 was used for statistical analysis.

Results and discussion:

Degradation rate:

The degradation rate and stability of RNA markers including blood-specific markers and reference genes were assessed to determine the best marker for age estimation.

Figure 1 illustrates that RNA markers degrade at different rates in aged stains with miRNA markers exhibiting the highest stability, as expected due to their small size (18 to 24 nucleotides) [4].

Both HBA and HBB showed rapid degradation in the first six days. However, HBA continued to degrade in a linear fashion across the 270 days, while HBB remained at the same level and started to degrade again at 270 days. In

contrast, PBGD showed linear degradation up to 15 days, then no C_q values after that, showing it was degraded to below the sensitivity of the assay used for its detection.

The ΔC_q values for miR16 and miR451 were around zero in the first 180 days, indicating high stability. However, after 15 days miR16 started to show slight degradation, and miR451 started to degrade after 270 days.

With regards to reference genes, only U6 showed high stability and its ΔC_q values remained around zero across time points.

Relative Expression Ratio (RER):

The RER was calculated using the C_q values of less stable markers to that of the most stable marker, and the relationship between bloodstain age and RER values examined. An Anderson-Darling normality test showed that data were not normally distributed (p = 0.022), so nonparametric statistical analysis was used.

Spearman's correlation indicates a positive correlation between bloodstain age and the RERs of HBA/HBB (r=0.99, p<0.0001), HBA/miR451 (r=0.97, p< 0.0001), HBA/miR16 (r=0.78, p=0.023) and both miRNA markers to U6 (r=0.98, p<0.0001). Additionally, HBA/U6 also showed a positive correlation (r=0.99, p< 0.0001). PBGD was excluded from statistical analysis as it did not show C_q values after 15 days.

Furthermore, regression analysis was used to estimate the relationship between the RERs of blood-specific markers and age of bloodstains. The RERs of HBA/HBB and miR16/U6 gave a high value of R^2 with narrow confidence intervals when applying a second-order polynomial (Figure 2), therefore could potentially be used to estimate the age of bloodstains.

Conclusion:

The outcomes of this research provide evidence that different RNA molecules degrade at different rates in bloodstains, with miRNA markers exhibiting

strong stability, likely due to their small size. The RERs of blood-specific markers represent a potentially powerful method to estimate the age of bloodstains. This research is continuing by applying the same method to different body fluids (saliva and semen).

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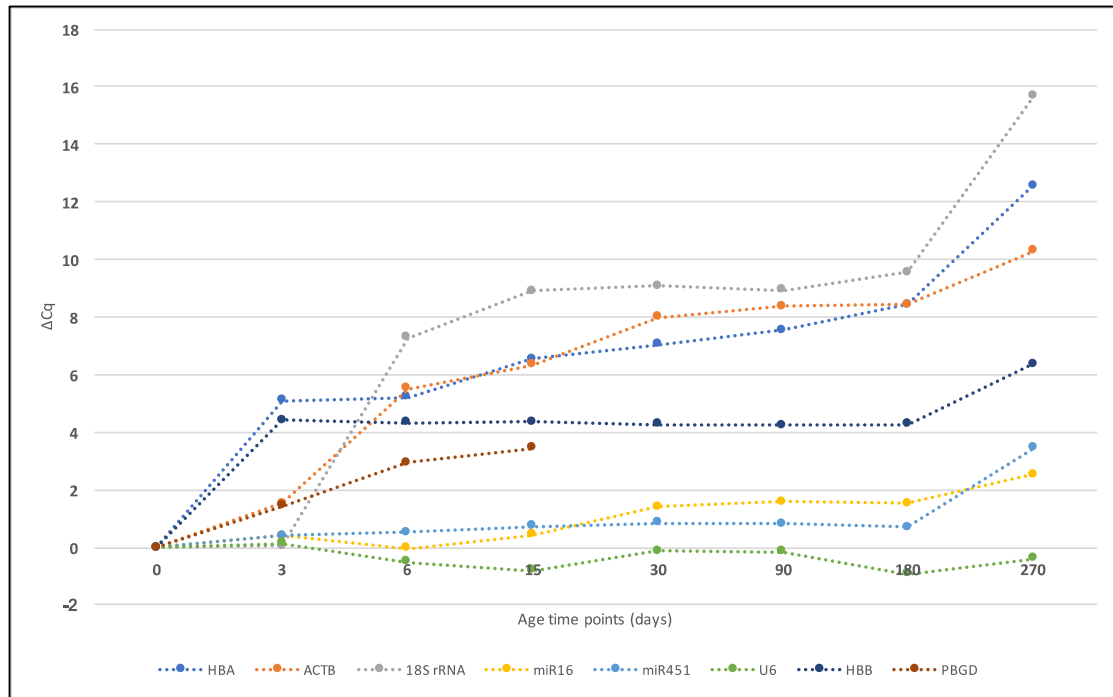[4]Kim VN. Small RNAs: Classification, Biogenesis, and Function. Molecules and Cells. 2005;19(1):1-15.

Figure 1: ΔC_q ($C_{q\ time\ x} - C_{q\ time\ 0}$) for HBA, HBB, PBGD, ACTB, 18S, miR16, miR451, and U6 in total RNA extracted from blood samples stored at room temperature for 270 days. Data represents the mean of n = 10. Error bars were omitted for clarity.
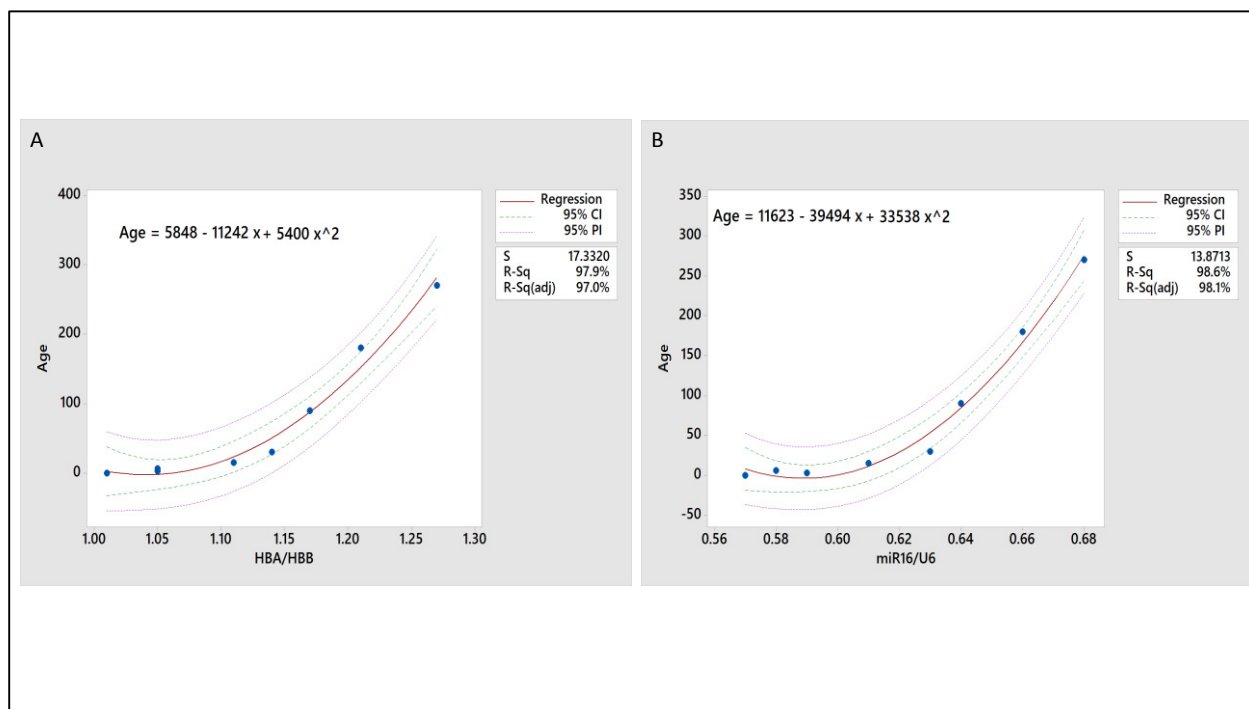
Figure 2: Regression analysis of the relationship between bloodstain age (up to 270 days) and RER.
A second-order polynomial curve was applied to RERs: (A) HBA/HBB and (B) miR16/U6. Data represents mean of n = 10.