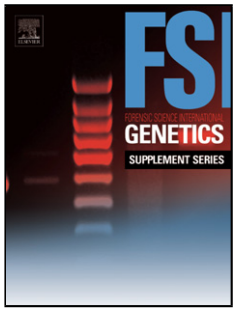

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Evaluating the use of hypoxia sensitive markers for body fluid stain age prediction

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In order to augment DNA profiling and body fluid identification techniques, efforts are being made to increase the amount of information available from a crime scene stain, which includes efforts to identify externally visible characteristics through phenotypic analysis. A key question surrounding crime scene stains is the length of time between deposition of the stain and its subsequent recovery, in that is the stain recovered related to the incident in question or from a previously deposited stain number of weeks earlier? The inability to answer this fundamental question has a detrimental effect upon the successful completion of a criminal investigation. Once a body fluid leaves the body, the oxygen concentration in the environment changes, therefore it may be that this change could cause a change in expression of hypoxia-sensitive biomarkers. Here, a range of blood, saliva and semen samples were collected at 0 days, 7 days, 14 days, 21 days and 28 days of degrading at room temperature, before undergoing total RNA extraction and cDNA synthesis. All samples then underwent quantitative PCR targeting Vascular Endothelial Growth Factor A (VEGFA) and Hypoxia-Inducible Factor 1 Alpha (HIF1A), with B-Actin (ACTB) as a reference gene. A range of linear and quadratic correlation values was obtained from the qPCR data and used to develop a predictive model with a mean absolute deviation (MAD) of 5.3, 2.0, and 4.3 days for blood, saliva, and semen respectively. Blind testing indicated that a stain age prediction model based upon VEGFA with ACTB as a reference gene could be used on stains up four weeks old with a margin of error ranging from 2 days through to 5 days. Whilst a large potential time frame exists using this model, this represents a major step towards the target of having an accurate stain age prediction model.

Introduction: Considerable effort is being made to enhance the amount of genetic information obtained from crime scene stains, thus augmenting the standard DNA profiling processes. For example, the characterisation of mRNA, miRNA, and DNA methylation markers to enhance body fluid identification capability [1-4]. One area of interest that is proving challenging is the ability to predict the age of the body fluid stain, i.e. the time since deposition. The ability to predict the age of any biological sample would be of great benefit to the forensic investigation. It would provide a temporal linkage between the contributor of the stain and the deposition of the stain or it could be used to exclude evidence that does not correspond to the time a crime was committed. For example, was the blood deposited less than 24 hours ago when the alleged incident occurred, or did it happen 2 months ago when the involved party had an accident and cut his finger? A linkage between ageing of a biological sample or the post-mortem interval (PMI) and the degradation of RNA was investigated by some research groups [4-6]. Most of these studies targeted different markers and different techniques to obtain a correlation between target markers and time or age. Recent advances in molecular biology suggest that hypoxia-inducible factor 1 (HIF1), a transcription factor that functions as a global regulator of hypoxic gene expression, plays a major role in the response of hypoxia together with other factors including erythropoietin (EPO) and vascular endothelial growth factor (VEGF) [8]. The expression of these factors involved in oxygen homeostasis increases the protein and mRNA levels in organisms or cells suffering from hypoxia or ischemia. Therefore, it may be possible to predict the age of biological samples deposited in a crime scene targeting oxygen-sensitive markers, as the exposure to the change in oxygen concentration may cause an over- or under- proliferation of proteins in the short term. Thus this study aimed to evaluate the degradation patterns of a small range of hypoxia-sensitive mRNA markers to potentially predict the age of blood, saliva, and semen stains. Whilst previous studies have explored this issue over a period of months, this

study explores whether such changes can be reliably characterised over a period of 4 weeks, thus representing a shorter time frame and therefore may be more practical to the criminal investigation.

Methods and materials: 8 individuals provided blood and saliva samples for each of 5-time points (from fresh to 4 weeks), and 6 individual provided semen samples for each of 5-time points. Saliva was collected via expectoration, blood was collected using the finger-prick method and deposited on to sterile filter paper, and semen was collected through deposition into a falcon tube. Saliva and semen were then sampled using sterile swabs. All samples were then stored at room temperature in the dark (to minimize variations) for 4 weeks. At relevant times, sections of blood, saliva, and semen were removed and underwent total RNA extraction using the RNeasy Mini Kit (Qiagen, UK) with on-column DNA digestion as per manufacturer's instruction with minor modifications. Synthesis of cDNA was carried out using Multiscribe First-Strand Synthesis System for quantitative-PCR (Invitrogen, UK) using random hexamers as per manufacturer's instructions. cDNA products were characterised using the relative quantitation (RQ) method by ABI 7500 Fast Real-Time PCR Machine (Life Technologies, UK) using FAST Master Mix mini kit with low Rox with SYBR green (Primer Design, UK). Following the amplification, a high-resolution melting curve analysis (HRMA) was carried out to verify the product. Unlabelled primers were used (MWG Eurofins) targeting HIF1A, VEGFA, and ACTB. The data was normalised against a chosen reference gene and calibrated against the Day 0/Fresh samples.

Results

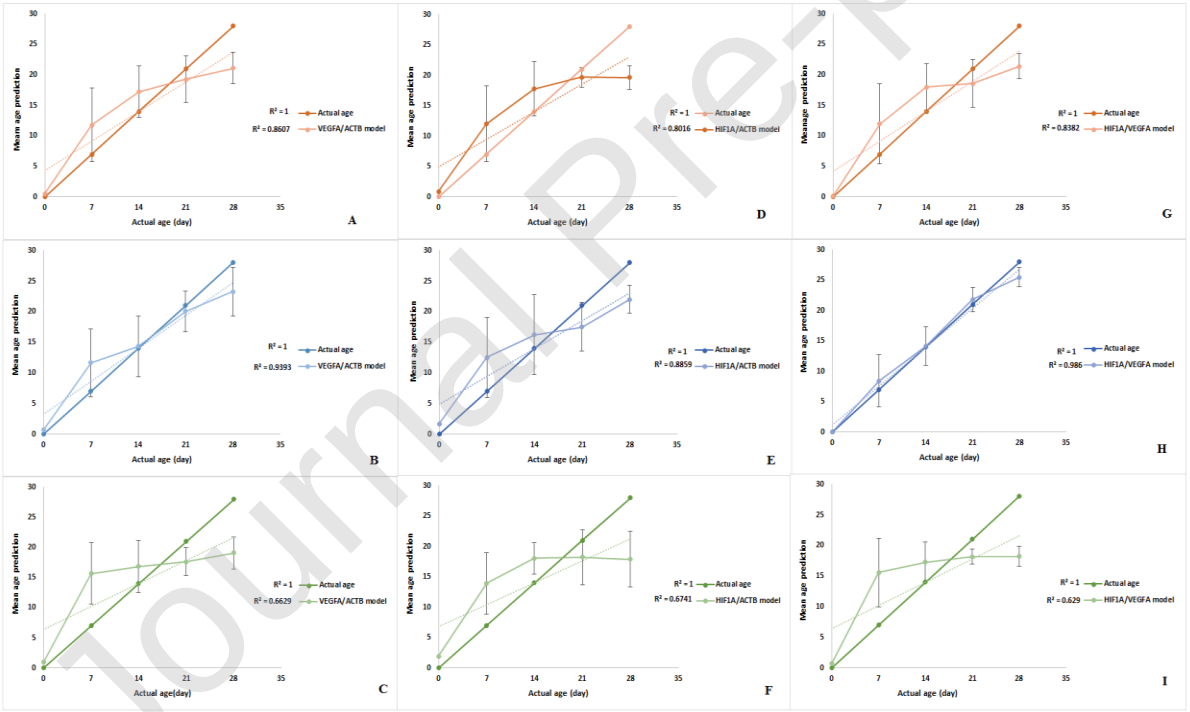

Figure 1. Mean age predictions were calculated and plotted against actual age. A, B and C show VEGFA/ACTB (where ACTB is the reference gene) models in blood, saliva, and semen, respectively and D, E, and F show HIF1A/ACTB models in blood, saliva and semen, with G, H, and I showing HIF1A/VEGFA model. Two parameters were used to evaluate which model was best, R^2 value and the standard deviation. These show that the HIF1A/VEGFA (where VEGFA is the reference gene) model was best of stain age prediction in saliva. Overall, all models were able to accurately predict some time points, namely differentiating between 0 and 21 days. All error bars represent one standard deviation

Discussion: Blood, saliva, and semen samples were taken and characterised, to assess their decomposition profile over a month. It was clear that a linear trend with a correlation value of 0.94 and 0.92 could be observed in VEGFA and HIF1A respectively, when ACTB is used as a reference gene, and a correlation value of 0.99 when HIF1A with VEGFA as the reference gene, thus indicating a linear decrease in marker expression over a month period for saliva samples. Linear correlations could be observed in the data from the blood samples taken at the same intervals, however, the correlation was higher (0.93, 0.87and 0.84, respectively) and also linear correlation was observed in semen (0.66, 0.77, and 0.73, respectively). It is thought that saliva shows a higher linear correlation as it is a less dynamic fluid than blood and saliva with the latter having more intracellular processes in progress and thus introducing a wider range of variable, thereby making any decomposition processes less linear. Prediction models were developed for blood, saliva, and semen, with the lowest mean absolute deviation (MAD) of 2.1, 4.2, and 5.00 days which detected with HIF1A/VEGFA model in saliva, blood, and semen, respectively. Once the predictive models for blood, saliva, and semen were developed, blind testing was carried out. The data were analysed in three ways, targeting VEGFA and HIF1A with ACTB as the reference gene, and then targeting HIF1A with VEGFA as the reference gene. Also, multiple regression models were tested in blood, saliva, and semen. The predicted stain age was calculated using the models developed and, compared with the actual age, deviation, R^2 value, and MAD recorded. In blind blood and saliva samples, the strongest R^2 value was observed with multiple regression model targeting VEGFA with ACTB as the reference gene, and HIF1A with ACTB. This was an expected result because this model has the strongest R^2 for all models obtained in blood. In blind semen samples the VEGFA/ACTB showed the highest R^2, despite a stronger linear correlation being observed with multiple regression model. Consequently, it has been demonstrated that hypoxia-sensitive biomarkers could be used for predicting in stain age up to 1 month, at least in saliva stains and, to a lesser extent blood and semen stains. There is a substantial margin of error in that any calculated day could be within a time frame ranging from ~2 days through to ~5 days. However, whilst this time frame is rather substantial, it is an improvement upon existing capability, whereby the difference between fresh stains and 1-month-old stains cannot be determined.

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