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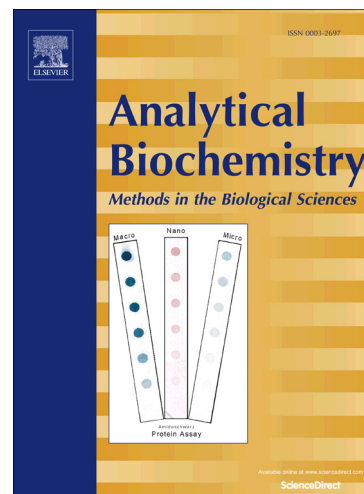
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Differentiating between monozygotic twins through DNA methylation specific high resolution melt curve analysis

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SHORT TITLE = DNA Methylation and HRMA of MZ Twins

Although STR profiling is extremely powerful in identifying individuals from crime scene stains, it is unable to differentiate between monozygotic (MZ) twins. Efforts to address this include mutation analysis through whole genome sequencing and through DNA methylation studies. Methylation of DNA is affected by environmental factors; thus, as MZ twins age, their DNA methylation patterns change. This can be characterised by bi-sulfite treatment followed by pyrosequencing. However, this can be time consuming and expensive, thus unlikely to be widely used by investigators. If the sequences are different, then, in theory, the melting temperature should be different. Thus the aim of this study is to assess whether high resolution melt curve analysis can be used to differentiate between MZ twins. Five sets of MZ twins provided buccal swabs which underwent extraction, quantification, bi-sulfite treatment, PCR amplification and high resolution melting curve analysis targeting two markers, Alu-E2F3 and Alu-SP. Significant differences were observed between all MZ twins, targeting Alu-E2F3, and in four out of five MZ twins, targeting Alu-SP (p<0.05). Thus it has been demonstrated that bi-sulfite treatment followed by high resolution melting curve analysis could be used to differentiate between MZ twins.

Introduction

Whilst standard DNA profiling targeting short tandem repeats is successful in identifying individuals from stains, there have been a number of incidences where the suspects were monozygotic (MZ) twins. In such cases, it has not been possible to determine from which of the twins the stain could have originated.

Efforts in this area have included whole genome sequencing, which looks at potential single nucleotide polymorphisms, through mutation analysis [1]. Such specialised techniques may not be readily available in general forensic laboratories. One particular

area showing promise is DNA methylation, an epigenetic feature that alters in response to environmental exposure [2-4]. The principle behind DNA methylation analysis on samples from twins is that, as the MZ twins mature, they will be exposed to different environmental stimuli; for example, if one twin were to take up smoking, then this person may present a different DNA methylation pattern. This could be characterised following bi-sulfite treatment and sequencing to identify the differences [5, 6].

This study explores the use of high resolution melting curve analysis [6, 7] to identify differences in DNA methylation patterns between MZ twins. The principle behind this is that, as the DNA methylation patterns change through exposure to different stimuli, the use of the bi-sulfite conversion step will change the sequences. Different sequences can lead to different melting temperatures. Consequently, the aim of this study is to evaluate whether high resolution melting curve analysis will allow for the differentiation between MZ twins.

Methods and materials
Sample collection and extraction
Five sets of MZ twins provided buccal swabs, after informed consent was obtained, providing ten samples in total. An additional buccal swab was obtained from a full sibling of one pair of twins. DNA extraction was conducted using the buccal swab protocol of the QIAamp DNA Mini Kit (Qiagen, UK).

DNA quantification
All extracted samples then underwent qPCR DNA quantification using an Investigator Quantiplex Kit (Qiagen, UK) on the RotorGene Q Real-time PCR Machine (Qiagen, UK). This allows for the normalisation of the DNA prior to bisulfite treatment.

Bi-sulfite treatment

Extracted DNA then underwent bisulfite treatment using the EpiTech Bisulfite Kit (Qiagen, UK) as per the manufacturer's recommendations. This step converts all non-methylated cytosines into uracil, leaving any 5-methylcytosines unchanged. Thus differences in methylation patterns result in differences in sequences following bi-sulfite treatment.

Amplification

Both treated and untreated DNA extracts then underwent amplification targeting the *Alu*-E2F3 (2 CpGs) promotor and fragments of the *Alu*-SP (17 CpGs) regions using the Applied Biosystems® 7500 Fast Real-Time PCR Machine and SYBR Green chemistry utilising the following primers: *Alu*-SP Forwards 5'-tttggtgattaggaaggtgggta-3', Reverse 5'-aaactaatctcaaactccctacctc-3', *Alu*-E2F3 Forwards 5'-ggtaataattttaaaatttgggggt-3' and Reverse 5'-attaaaaaaaccaatcaacccataa-3' [8]. The Fraga *et al* study [8] provides further information regarding the positions of DNA methylation in both regions.

High resolution melting curve analysis (HRMA)

HRMA was conducted immediately after amplification on the Fast 7500 Real-Time PCR Machine (Life Technologies, UK), with an initial phase of 95°C for 15 seconds, then at 60°C for 60 seconds, before a 1% increase to 95°C. Data collection took place during the 1% increase. The data was analysed using SDS 7500 Software Version 2.0.6.

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Results

Following bi-sulfite treatment and subsequent amplification of *Alu*-SP, as can be seen in Figure 1, one set of MZ twins had different melting temperatures. This is the largest difference observed and was obtained from 53-year-old MZ male twins.

Note to Editor – Insert Figure 1

Figure 1 – A representative melt curve of one set of twins (53-year-old males), exhibiting a difference in melting temperature, targeting Alu-SP. Each group of peaks represents one individual.

As can be seen in Figure 2, the extent of the differences in the melting temperatures between different sets of MZ twins varies considerably. However, there are significant differences in the melting temperatures of products amplified by targeting *Alu*-E2F3 across all MZ twins (p<0.05), with two sets of MZ twins showing a particularly higher significant difference (p<0.001).

Note to Editor – Insert Figure 2

Figure 2 –A column chart indicating the differences in melting temperature between five different pairs of MZ twins, targeting Alu-E2F3. Pair 6 refers to the same individual to ensure consistency with the techniques. Paired sample t-tests were carried out and those denoted as * indicates a p-value of >0.05 and those denoted as ** indicate a p-value of >0.001. An absence of the asterisk indicates no significant difference. The age range of the twins at the time of sample collection are as follows 1) – 20-25yo, 2) – 40-45yo, 3) – 20-25yo, 4) – 40-45 yo, 5) – 53yo, and 6) – 20-25yo.

Figure 3 shows the same set of samples, but when targeting a different set of markers, namely *Alu*-SP. In this case, only four of the five sets of MZ twins showed a significant difference in melting temperature with the *Alu*-SP amplicon.

Note to Editor – Insert Figure 3

Figure 3 – A column chart indicating the differences in melting temperature between five different pairs of MZ twins, targeting Alu-SP. Pair 6 refers to the same individual to ensure consistency regarding the techniques. Paired sample t-tests were carried out and those denoted as * indicate a p-value of >0.05 and those denoted as ** indicate a p-value of >0.001. An absence of the asterisk indicates no significant difference. See Figure 2 legend for ages.

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Discussion

The aim of this study was to evaluate the effectiveness of high resolution melting curve analysis for differentiating between MZ twins. This was achieved by isolating DNA from buccal swabs, performing bi-sulfite treatment and then carrying out HRMA on the *Alu*-SP and *Alu*-E2F3 amplicons.

Both markers allowed for the differentiation between MZ twins on the basis of different melting temperatures; albeit with *Alu*-E2F3 performing better than *Alu*-SP. One particular set of twins showed a remarkably large difference in the melting temperature of both amplicons. Interestingly, this particular set of MZ twins were 53 years old at the time of sampling and they were the eldest of the five sets of twins. The remainder of the twins were in their 20s or 40s and no trend could be gleaned from these twins. This is of particular interest because, as the MZ twins age and become exposed to different environments, their DNA methylation patterns should diverge the longer they are in different environments.

Conversely, the *Alu*-E2F3 and *Alu*-SP in relatively young MZ twins may not have sufficiently different melting temperatures to be of discriminating value. In addition, any MZ twins who have been in a similar environment may also not have sufficiently different melting temperatures.

Thus it is clear that there are limitations behind the use of these techniques for forensic purposes, in that it is possible that the DNA methylation pattern of MZ twins may not be sufficiently different for use in a court of law. However, the cost of HRMA compared with mutation analysis is considerably cheaper and requires less sophisticated instrumentation. Consequently, it can be used as a preliminary test should the test be inconclusive, and then it can proceed to the more expensive mutation analysis.

There are further limitations with the use of HRMA for this purpose in that a large sample volume is generally required for DNA methylation analysis, a volume that may not be available, particularly following standard DNA profiling. In addition, it is suggested that there is discordance between body fluids in that one individual's DNA methylation pattern in the blood could differ from the DNA methylation in the saliva. This is problematic as recent high profile cases have involved semen samples. Since DNA methylation patterns are not thought to be preserved across body fluids, it may be necessary for both of the MZ twins to provide semen samples for comparison rather than the usual reference buccal swab.
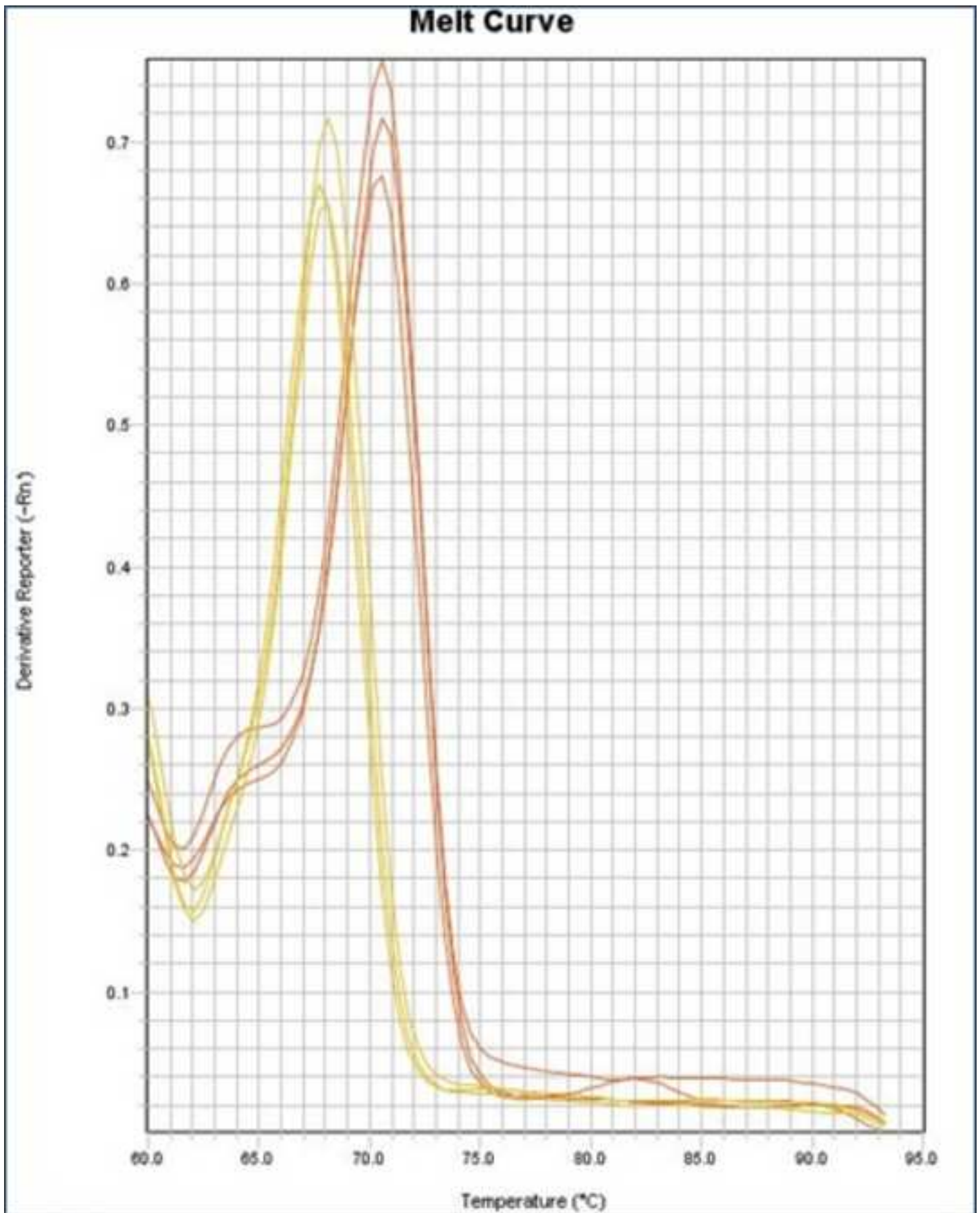
Whilst it is clear that methylation patterns are age-dependant, this does present an operational problem in that there is limited scope for cold-cases. The methylation pattern of an individual could be significantly different from that of a stain that has been stored for a substantial period of time. Further work will be required in order to provide an idea of an acceptable period of time in which HRMA of MZ twins could be carried out.

Whilst there is considerably more work to be carried out before this could be used in casework (such as the identification of further markers to improve statistical calculations in forensic investigations), it has been demonstrated that HRMA of DNA methylation markers *Alu*-E2F3 and *Alu*-SP could be used to differentiate between MZ twins.

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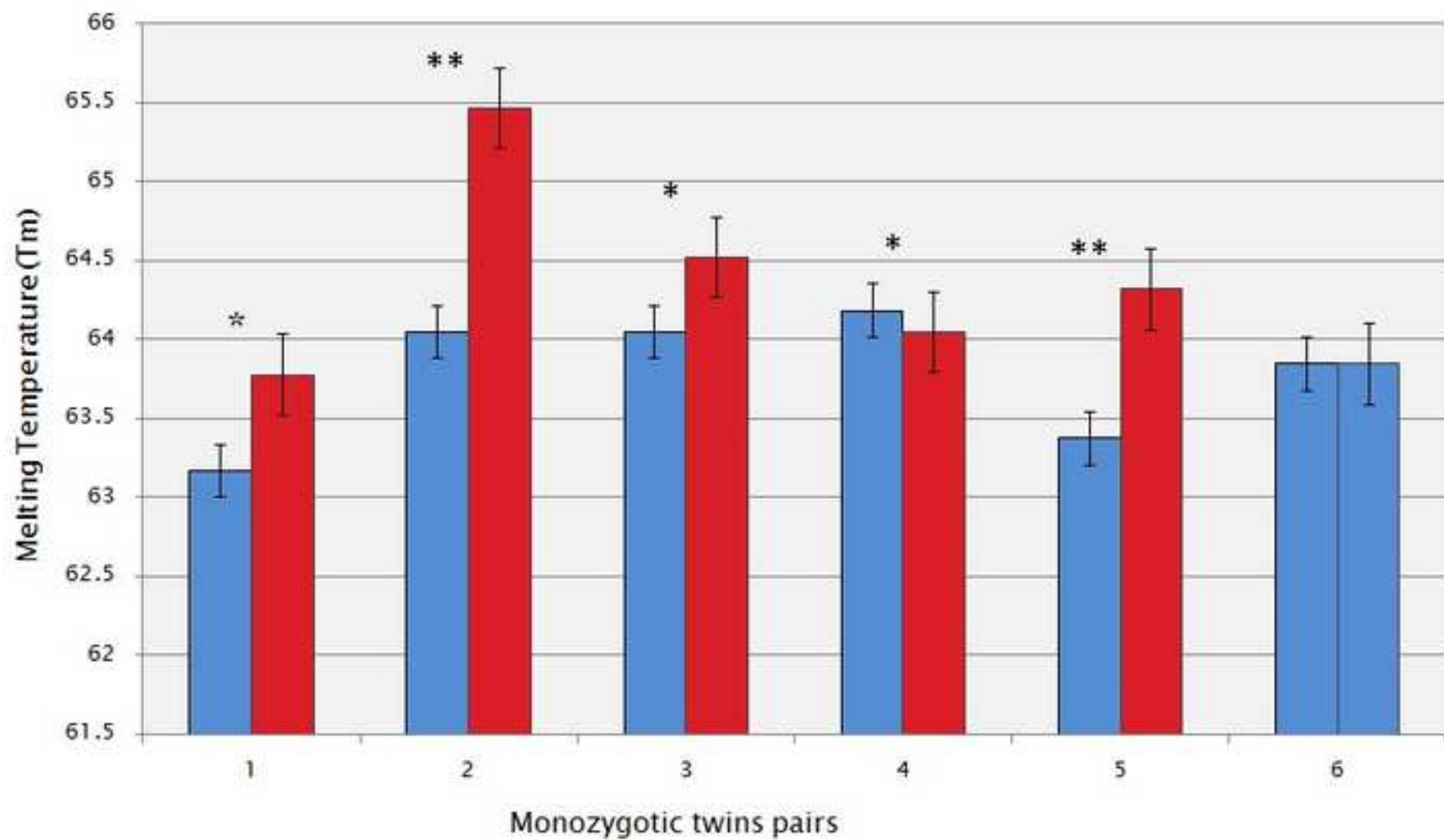
Figure 1

Melt Curve

Figure 2

Monozygotic twins pairs

Figure 3