EVALUATION OF ALPHA-GLOBIN MRNA AS A MOLECULAR MARKER FOR IDENTIFICATION OF HUMAN BLOODSTAINS

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Abstract
Identification of questioned stain collected from crime scene as whether or not human blood is meaningful for DNA analysis. Anti-human serum and human specific DNA sequence had been successfully used for human bloodstain identification. However, none of these had information on the age estimation of bloodstain. Therefore, this research aims to evaluate alpha-globin mRNA as a molecular marker for both human blood identification and age estimation of bloodstain. The oligonucleotide primers of human alpha-globin mRNA were designed using Primer3. The designed primers were then blast with alpha-globin mRNA from four animal species (cow, pig, dog and chicken) using Primer-Blast. No sequence homology was found which confirmed that amplification of non-specific sequence would not be occurred. PCR reactions were then performed using synthesized human and animal cDNA as DNA template. Interestingly, a 168 bp amplified fragment was observed only in human blood sample. This result suggested that human blood identification was successfully performed by using alpha-globin mRNA as a molecular marker. However, for the age estimation of bloodstain, further investigation is needed.

Keywords: Human alpha-globin mRNA, Human blood identification, Forensic science

Introduction

Identification and age estimation of human bloodstains is valuable for investigator in order to 1) reduce number of samples used for DNA analysis 2) exclude suspect from the investigation and/or 3) disprove statement of suspects in which they insist that their bloodstains presented before the crime has occurred. The analyses of bloodstains age were mostly based on the changes of hemoglobin to its derivatives which could be observed by the color change and the solubility of bloodstains (Anderson et al. 2005). In addition, the activities of enzymes and proteins found in bloodstains were also used to detect the age of bloodstain (Rajamannar 1977). However, none of these methods could discriminate human blood from animals and their results also depended on the quantity of samples. Therefore, if any of methods can 1) identify human bloodstains 2) estimate the age of bloodstain and 3) analyze sample in a small quantity, in a single step, it will be very useful.

Although, studies had shown that DNA is a good source for species identification and the results can be obtained even small amount of sample is present (Crouse and Schumm 1995; Nakaki et al. 2007). But, DNA lacks information on age estimation due to its stability. Therefore, mRNAs have been used to estimate the age of bloodstains because it degrades with time. Previous study had shown that the age of bloodstain could be determined by the reduction of β-actin mRNA number using real-time RT-PCR (Anderson et al. 2005). Hence, this study aims to use human alpha-globin mRNA as a target for identification and age

estimation of human bloodstain. But before that, experiment on human blood identification from alpha-globin mRNA needs to be primarily conducted. Here, such experiment will be discussed.

Methodology

Sample

Human blood samples (1 man and 1 woman) were obtained from blood bank, Faculty of Medicine, Khon Kaen University. Animal blood samples: *Bos taurus* (cow), *Sus scrofa* (pig), *Canis lupus familiaris* (dog) and *Gallus gallus* (chicken) were taken from Ubon ratchathani College of Agriculture and Technology. All blood samples were collected in sample tubes with addition of 50 µl 200 mM EDTA.

RNA extraction

RNA extraction from 400 µl human and animal blood samples were performed using GF-1 Blood total RNA kit (Vivantis, Malaysia) according to manufacturer's instruction. Briefly, 400 µl blood samples was mixed with 300 µl Buffer BR and then 180 µl RNase-free water, 20 µl Proteinase K was subsequently added. The solution was mixed and incubated at 65^{o}C for 10 min. The mixture was centrifuged. The supernatant was transferred to Homogenization column and then centrifuged. The flow through liquid was kept and then 0.5% volume of 80% ethanol was added. The liquid was mixed and loaded onto RNA Binding column. The column was centrifuged and then washed with 500 µl Wash buffer. DNase I digestion Mix was subsequently added to the column and incubated at room temperature for 15 min before being added by 500 µl Inhibitor Removal buffer. The liquid was removed from the column by centrifugation and the column was then washed with 500 µl Wash buffer twice. Total RNA was then eluted from the column by adding 60 µl of RNase-free water. The eluted RNA was then quantified by using UV-VIS spectrophotometer (Shimadzu, Japan).

cDNA synthesis

RevoScript^{TM} Reverse Transcriptase PreMix (Oligo dT$_{15}$primer) kit was used for cDNA synthesis. Briefly, 20 ng of RNA was added into the RevoScript^{TM} RT PreMix tubes. RNase/DNase-free water was then added to make a total volume up to 20 µl. The tubes were incubated at 50°C for 60 min to allow the cDNA synthesis. The reaction was then ceased by incubation at 95°C for 5 min to inactivate the RTase enzyme. The cDNA solutions were then stored at -20^{o}C until use.

Primer design

The nucleotide sequences of human and animal (cow, pig, dog and chicken) alpha-globin mRNAs were obtained from GenBank database (http://www.ncbi.nlm.nih.gov/genbank). The human alpha-globin mRNA sequence was used as a template DNA for primer design by Primer3 program. The forward and reverse primers were then compared with animal alpha-globin mRNA using Primer-blast program. The alpha-globin mRNA sequences were retrieved from GenBank database. The accession numbers of cow, pig, dog and chicken alpha-globin mRNAs were NM_001077422, XM_003481084, NM_001270885 and NM_001004376, respectively. Primers showing no complementary DNA sequence with those of animals will be chosen for PCR amplification.

PCR amplification

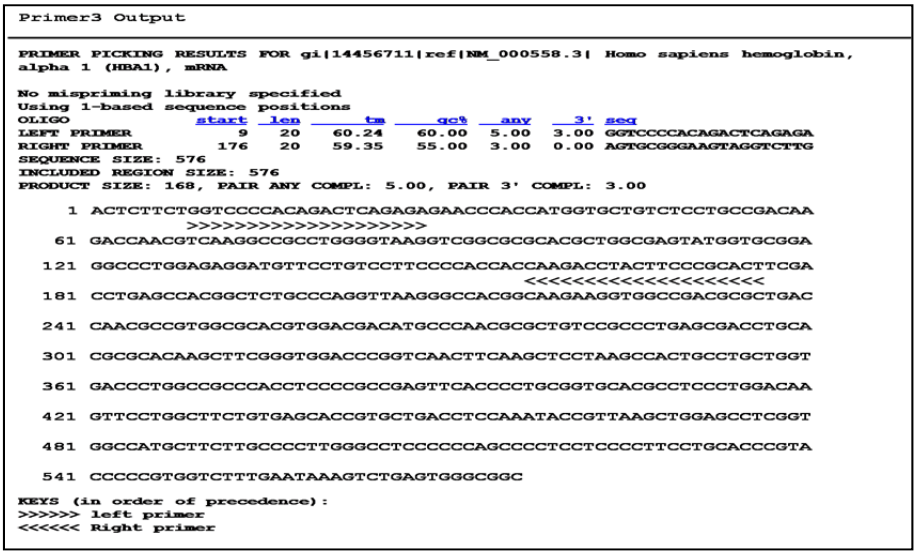
The total volume of PCR reaction was 20 µl containing 5 ng cDNA templates, 0.5 µM of each primer, 2xPCR Master Mix and deionized water. Amplification was performed in a

Thermal Cycler with the following cycling condition: initial denaturation step at 94°C 3 min followed by 35 cycles of 94°C 30 sec, 65°C 45 sec, 72°C 45 sec and a final extension at 72°C 3 min. The PCR product was subsequently analyzed on 1.5% agarose gel.

Results and Discussion

Primer design

The nucleotide sequence of human alpha-globin mRNA (Accession Number NM_000558) obtained from GenBank was used as a template DNA for primer design by Primer3 program. The result is shown in Fig.1. From the result, the size of amplified DNA fragment by these primers is 168 bp. These primers were then compared to alpha-globin mRNA of four animal species to investigate any of unspecific binding sites which may have occurred during PCR amplification. No homology sequence was found (data not shown). Therefore, these primers were then used for PCR analysis. The length of PCR product and the nucleotide sequences of Human alpha-globin primers were shown in Table 1.



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Primer3 Output

PRIMER PICKING RESULTS FOR gi|14456711|ref|NM_000558.3| Homo sapiens hemoglobin,
alpha 1 (HBA1), mRNA

No mispriming library specified
Using 1-based sequence positions
OLIGO            start  len      tm      gc%    any    3'  seq
LEFT PRIMER          9   20   60.24    60.00   5.00   3.00 GGTCCCCACAGACTCAGAGA
RIGHT PRIMER       176   20   59.35    55.00   3.00   0.00 AGTGCGGGAAGTAGGTCTTG
SEQUENCE SIZE: 576
INCLUDED REGION SIZE: 576
PRODUCT SIZE: 168, PAIR ANY COMPL: 5.00, PAIR 3' COMPL: 3.00

     1 ACTCTTCTGGTCCCCACAGACTCAGAGAGAACCCACCATGGTGCTGTCTCCTGCCGACAA
                >>>>>>>>>>>>>>>>>>>>
    61 GACCAACGTCAAGGCCGCCTGGGGTAAGGTCGGCGCGCACGCTGGCGAGTATGGTGCGGA
   121 GGCCCTGGAGAGGATGTTCCTGTCCTTCCCCACCACCAAGACCTACTTCCCGCACTTCGA
                                                <<<<<<<<<<<<<<<<<<<
   181 CCTGAGCCACGGCTCTGCCCAGGTTAAGGGCCACGGCAAGAAGGTGGCCGACGCGCTGAC
   241 CAACGCCGTGGCGCACGTGGACGACATGCCCAACGCGCTGTCCGCCCTGAGCGACCTGCA
   301 CGCGCACAAGCTTCGGGTGGACCCGGTCAACTTCAAGCTCCTAAGCCACTGCCTGCTGGT
   361 GACCCTGGCCGCCCACCTCCCCGCCGAGTTCACCCCTGCGGTGCACGCCTCCCTGGACAA
   421 GTTCCTGGCTTCTGTGAGCACCGTGCTGACCTCCAAATACCGTTAAGCTGGAGCCTCGGT
   481 GGCCATGCTTCTTGCCCCTTGGGCCTCCCCCCAGCCCCTCCTCCCCTTCCTGCACCCGTA
   541 CCCCCGTGGTCTTTGAATAAAGTCTGAGTGGGCGGC

KEYS (in order of precedence):
>>>>>> left primer
<<<<<< Right primer
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Figure 1 The sites of forward and reversed primers based on the sequence of human alpha-globin mRNA (Accession Number NM_000558).

Table 1 The nucleotide sequences of designed primers with the expected size of PCR product.

Human alpha-globin primer	PCR product (bp)
Forward 5′-GGTCCCCACAGACTCAGAGA-3′	168
Reverse 5′-AGTGCGGGAAGTAGGTCTTG-3′	

PCR assay of designed primer

The designed primers were analyzed by PCR technique using synthesized cDNA from human and animal blood as DNA template. A DNA fragment of 168 bp was amplified in the PCR reaction which contained human cDNA template. Interestingly, such PCR product was not observed with those of animals (Fig.2). These results approved that the human blood could be identified by PCR amplification using these designed primers. Similar study on human blood identification by using designed primers from other gene was reported (One et al. 2001).

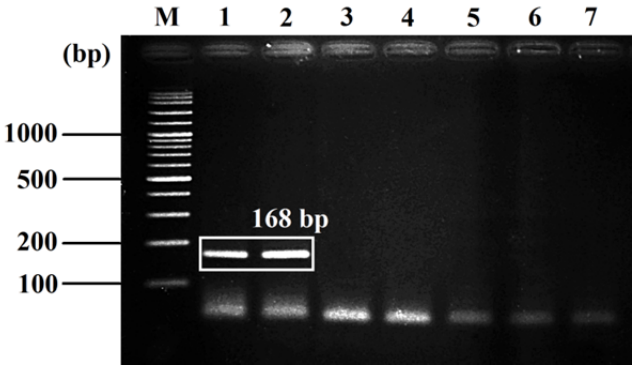

Figure 2 Amplification of human alpha-globin mRNA from cDNA samples. Lane 1: man, Lane 2: woman, Lane 3: cow, Lane 4: pig, Lane 5: dog, Lane 6: chicken, Lane 7: Control (non cDNA template), Lane M: 100 bp DNA ladder.

Conclusion

Human blood can be identified by reverse-transcription PCR using a pair of primers specific to human alpha-globin mRNA. A PCR product with the size of 168 bp indicates human blood samples. Primers used in this study will be further analyzed for age estimation of blood samples by real-time PCR.

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