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Perspectives

Identification of Molecular Biomarkers for Estimation of Postmortem Interval Using Blood Samples - A Pilot Study

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Abstract

Accurate estimation of the post-mortem interval (PMI) is an important research topic in forensic science. Unfortunately, all current methods to determine the time of death are unreliable in some way. The aim of the present study was to investigate the usefulness of blood samples and molecular biomarkers for the determination of PMI of a corpse. For this, diverse molecular markers such as human 18S rRNA and several mRNAs of housekeeping genes including ACTB, B2M, PPIA, GUSB, HPRT1, PGK1, RPLPO, TBP, TFRC in whole blood samples were analyzed in a time-dependent manner at two temperature conditions, at 25°C and 4°C. The expression and integrity of diverse molecular biomarkers were investigated using microfluidic electrophoresis chip and RT-qPCR TaqMan assays. The results indicate that GAPDH, B2M, and HPRT1 mRNA levels at 25°C and GAPDH at 4°C were significantly time-dependently decreased over 30 days. This pilot study may provide informative molecular biomarkers to predict more accurately PMI compared to traditional methods.

Keywords: Postmortem interval; RNA markers; Real-time PCR; Blood; Housekeeping genes;18S rRNA.

Introduction

Determination of the postmortem interval (PMI) is a major focus of investigation in forensic science. A panel of physical methods, such as the measurement of body temperature or the electrical stimulation of facial muscles, allows initial determination of PMI with considerable accuracy [1]. However, accurate PMI estimation still remains difficult due to many intrinsic factors such as age, sex, and physiological and pathological states of the deceased, as well as extrinsic factors such as temperature, humidity, and insect activity [2]. All methods presently used to estimate the time of death are affected by some degree of inaccuracy. These methods only provide an approximation since several variables including environmental temperature, body structure, cause of death, location of the body, or putative drug consumption can influence the rate of post mortem changes. In recent years, analysis of the biochemical changes in certain markers, such as glucose and electrolytes, for PMI estimation has made significant progress [3,4]. However, detection of postmortem changes in protein, RNA, and DNA markers has not yet been systematically studied. Several studies have tried to determine changes in molecular markers to provide more useful information regarding PMI [5-7]. In general, mRNA is thought to be much less stable than DNA and protein. However, there are several reports that suggest the contrary. RNA

degradation in the body after death might be a useful marker for the precise estimation of PMI [8].

The aim of this study was to characterize the expression and degradation levels of RNA markers, including human 18S rRNA and several mRNAs of housekeeping genes including β-actin (ACTB), β2-microglobulin (B2M), peptidylprolyl isomerase A (PPIA), β-glucuronidase (GUSB), hypoxanthine-guanine phosphoribosyl transferase (HPRT1), phosphoglycerate kinase 1 (PGK1), 60S acidic ribosomal protein P0 (RPLPO), TATA-binding protein (TBP), and transferrin receptor protein 1(TFRC), in whole blood samples after collection at different time points and temperature conditions (at 4°C and 25°C). The gathered information was then used for estimation of an accurate PMI. Real-time reverse transcription polymerase chain reaction (RT-PCR) TaqMan assay was used to detect changes in RNA markers.

Materials and Methods

Blood samples

This study was performed at Yonsei University in Wonju, Republic of Korea, from December 2013 to April 2014. Whole blood samples were collected using EDTA blood collection tubes. All participants provided written informed consent. The Institutional Ethics Committee of Yonsei University Wonju Campus approved the study (approval number 1041849-201406-BM-025-02). A total of six samples of EDTA whole blood from study participants were incubated for nine different time points (none, 6 hr, 1, 3, 5, 10, 15, 20, and 30 days) at two temperature conditions, 25°C and 4°C. After incubation, blood samples were harvested and stabilized in RNAgard Blood tubes (Biometrica Inc., San Diego, CA, USA) and stored at -20°C before total RNA preparation.

Total RNA extraction

Next, 2.5 mL of stabilized blood samples were transferred to 50 mL conical tubes, to which 3 mL of Precipitation Buffer was added. The mixture was then incubated at room temperature on a shaking incubator for 30 minutes and centrifuged at room temperature for 30 minutes at 9,000 *x g*. The supernatant was then decanted, and 350 µL of Resuspension Buffer was added to the pellet and resuspended. To this solution, 250 µL of 100% ethanol was added and centrifuged for 1 minute at 8,000 *x g*. Next, 400 µL of Wash Buffer 1 was added and centrifuged at 8,000 *x g* for 1 minute, followed by addition of 100 µL of DNase I Mix and incubation at room temperature for 20 minutes. The solution was then treated with 400 µL of Wash Buffer 1 and centrifuged at 8,000 *x g* for 1 minute. To the pellet, 350 µL of Wash Buffer 2 was added, centrifuged at 8,000 *x g* for 1 minute (repeat twice), and then centrifuged at 11,000 *x g* for 2 minutes to dry the pellet. Next, 50 µL of RNase-free water was added, incubated for 1 minute at room temperature and centrifuged for 1 minute at 8,000 *x g* to elute RNA. The contents and purity of RNA were measured at 260 nm and 280 nm, using a Nanodrop spectrophotometer, Infinite® 200 Pro NanoQuant (Tecan, Mannedorf, Switzland).

RNA quality analysis

RNA integrity and degradation levels were assessed using an automated chip-based microfluidic capillary electrophoretic separation system, 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA), and the Agilent RNA 6000 Nano Kit (Agilent Technologies) according to the manufacturer's instructions. Results were analyzed using Agilent 2100 Expert software v.B.02.07.SI532 (Agilent Technologies).

Gene Expression Analysis

The RT-qPCR TaqMan® assay was carried out on an ABI 7500 Fast system (Applied Biosystems, Foster City, CA, USA) for thermal cycling and fluorescence detection, according to manufacturer instructions. PCR primers and corresponding TaqMan® probes were designed (IDT, Coralville, IA, USA) for 11 different human 18S rRNA and housekeeping gene mRNA of ACTB, B2M, PPIA, GUSB, HPRTI, PGK1, RPLPO, TBP, GAPDH and TFRC (Table 1). For real-time thermal cycling, 500 ng/ uL of cDNA and THUNDERBIDR® Probe qPCR Mix (Toyobo, Osaka, Japan) was used. After initial denaturation at 95°C for 3 min, the PCR for DNA detection was run for 40 cycles (15 sec at 95°C, 1 min at 55°C) using an ABI 7500 fast system (ABI, Carlsbad, California, USA). The threshold cycle (*Ct*) value for each reaction was automatically calculated and recorded using the 7500 software v2.0.6.

Statistical analysis

Statistical analysis was performed using Microsoft® Office Excel v. 2010 (Microsoft, Redmond, Washington, USA).

Results

Time-dependent changes in RNA integrity

RNA integrity number (RIN) was measured using an automated microfluidic chip system, and RIN values ranged from 1 to 10, where 1 represents totally degraded RNA and 10 represents completely intact RNA [9]. A shift toward shorter fragment sizes is observed with progressing degradation. It was obvious that band patterns of 18S and 28S rRNA were clear until three days after blood collection at both temperatures, at 4°C and 25°C. However, the band patterns of 18S and 28S rRNA gradually faded after ten days, and RIN values were unmeasurable (Figure 1 and Table 2).

Time-dependent changes in RNA markers according to RT-qPCR

In order to determine the usefulness of RNA markers to estimate PMI, time-dependent changes in 11 RNA markers in

Target genes	Accession no.	Name of primer and probe	Sequences of primer and probe (5' - 3')
18S rRNA	HQ387008.1	18S-F-1	TGC TCA ATC TCG GGT GGC TGA A
		18S-R-2	GGA CAT CTA AGG GCA TCA CAG
		18S-P-3	FAM - GAG ACT CTG GCA TGC TAA CTA G–BHQ1
ACTB	NM_001101.3	ACTB -F-1	TCA CCA TGG TGA GCT GGC GG
		ACTB -R-2	CCT TGC ACA TGC CGG AG
		ACTB -P-3	FAM – ACA GAG CCT CGC CTT G–BHQ1
B2M	NM_004048.2	B2M-F-1	CCT GCC GTG TGA ACC ATG TGA CT
		B2M-R-2	ACC TCC ATG ATG CTG CTT AC
		B2M-P-3	FAM – GGA CTG GTC TTT CTA TCT CTT GT–BHQ1
PPIA	AK290085.1	PPIA-F-1	AAT TCA CGC AGA AGG AAC CAG ACA GT
		PPIA-R-2	CAA GAC TGA GAT GCA CAA GTG
		PPIA-P-3	FAM – GTG GCG GAT TTG ATC ATT TGG–BHQ1
GUSB	NM_001293105.1	GUSB-F-1	TGC AGG GTT TCA CCA GGA TCC AC
		GUSB-R-2	GTT TTT GAT CCA GAC CCA GAT G
		GUSB-P-3	FAM – GCC CAT TAT TCA GAG CGA GTA–BHQ1
HPRTI	NM_000194.2	HPRT1-F-1	AGC CTA AGA TGA GAG TTC AAG TTG AGT TTG G
		HPRT1-R-2	GCG ATG TCA ATA GGA CTC CAG
		HPRT1-P-3	FAM – TTG TTG TAG GAT ATG CCC TTG A–BHQ1
PGK1	NM_000291.3	PGK1-F-1	ACT CTC ATA ACG ACC CGC TTC CCT
		PGK1-R-2	GAC AGC AGC CTT AAT CCT CTG
		PGK1-P-3	FAM – CTA ACA AGC TGA CGC TGG A–BHQ1
RPLP0	NM_001002.3	RPLPO-F-1	CCC TGT CTT CCC TGG GCA TCA C
		RPLPO-R-2	TGT CTG CTC CCA CAA TGA AAC
		RPLPO-P-3	FAM – TCG TCT TTA AAC CCT GCG CGT G–BHQ1
TBP	NM_001172085.1	TBP-F-1	CAC AGG AGC CAA GAG TGA AGAA CAG T
		TBP-R-2	CAA GAA CTT AGC TGG AAA ACC C
		TBP-P-3	FAM – GAT AAG AGA GCC ACG AAC CA–BHQ1
TFRC	AK291723.1	TFRC-F-1	ACT TCA AGG TTT CTG CCA GCC CA
		TFRC-R-2	CCC AGT TGC TGT CCT GAT ATA G
		TFRC-P-3	FAM – TCT GGA TAA AGC GGT TCT TGG–BHQ1
GAPDH	NM_001256799.2	GAPDH-F-1	CCA TCT TCC AGG AGC GAG ATC C
		GAPDH-R-2	ATG GTG GTG AAG ACG CCA GTG
		GAPDH-P-3	FAM - TCC ACG ACG TAC TCA GCG CCA GCA –BHQ1

Table 1. Oligonucleotide primers and TaqMan probes used in this study to detect 18s rRNA and housekeeping gene mRNA levels.

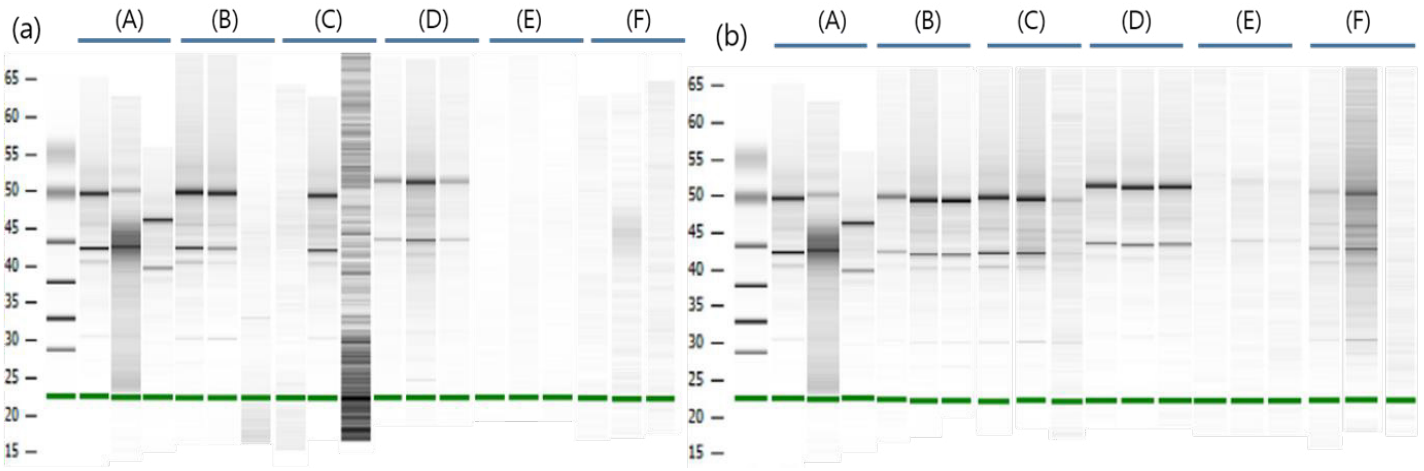

Figure 1. A total RNA sample was degraded for different times, and the resulting samples were analyzed on an Agilent 2100 Bioanalyzer enabling the quality analysis of RNA using only 1 ul of sample via the Total RNA Nano assay. A shift toward shorter fragment sizes is observed with progressing degradation. (A) o hr, (B) 6 hr, (C) 1 day, (D) 3 days, (E) 10 days, and (F) 15 days. (a)at 25^0C (b) at 4^0C.

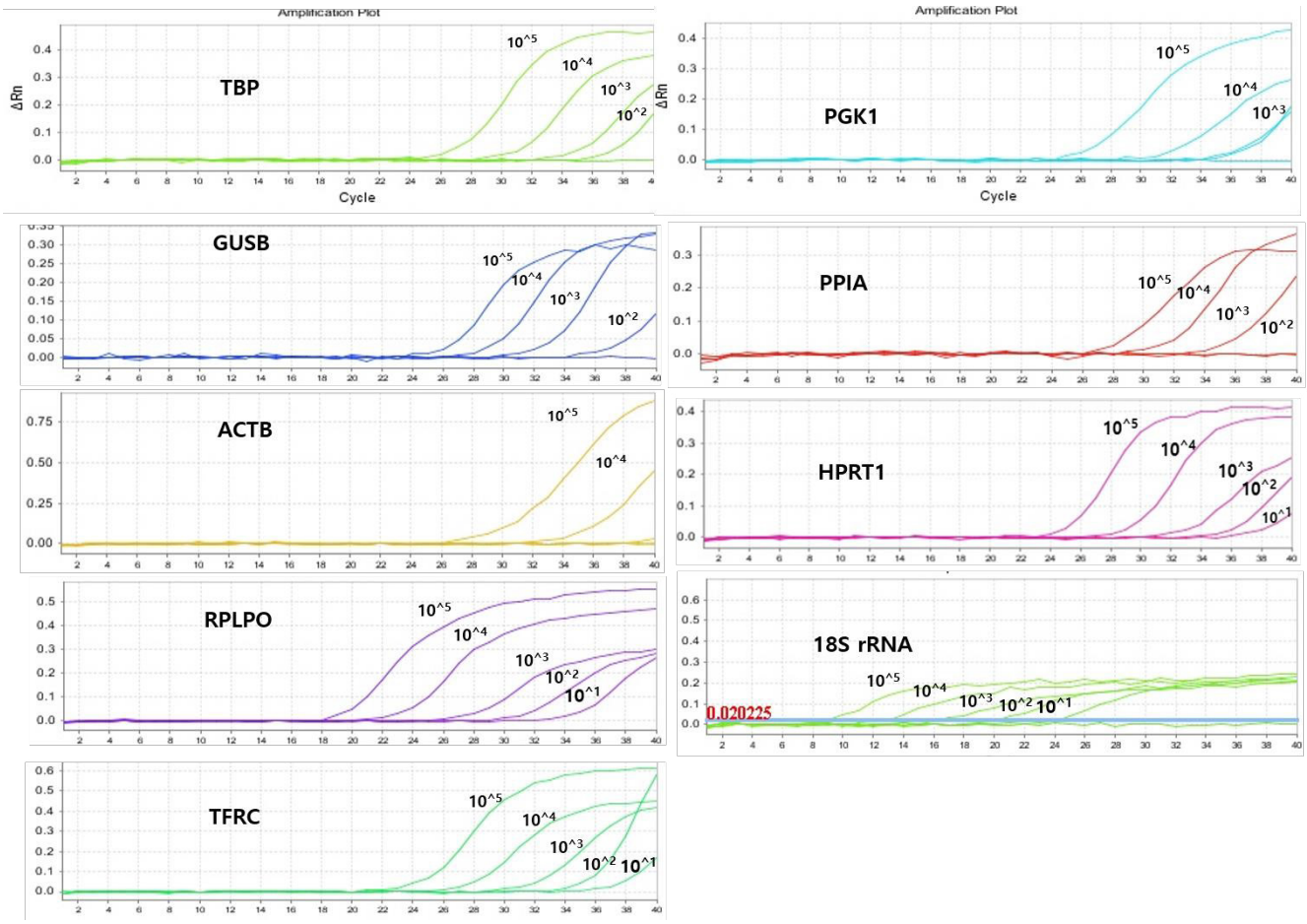

Figure 2. Detection limits of the three target probes in ten-fold serial diluted blood samples.

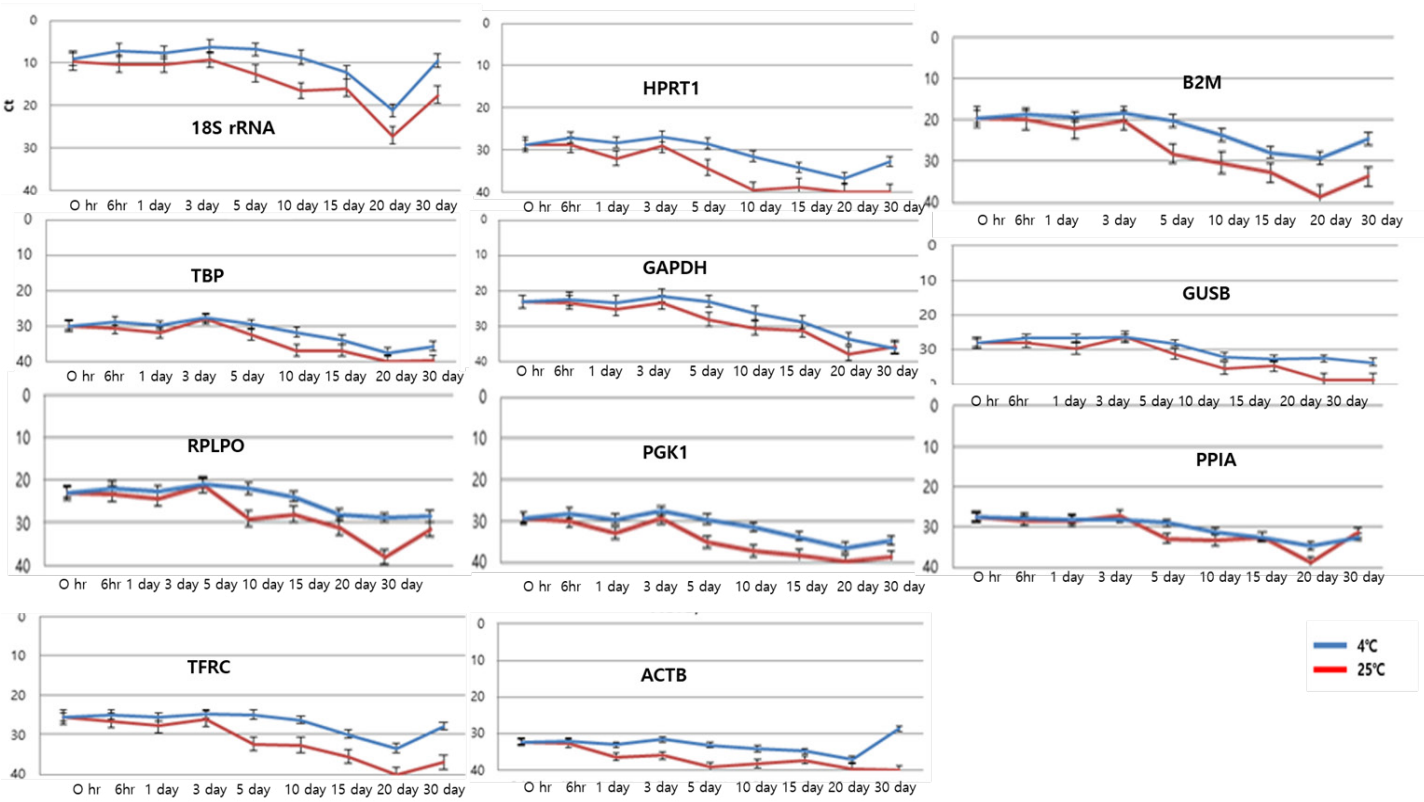

Figure 3. Relationshhip between postmorterm time (0 hr-30 days) and Ct value for estimation of postmortem interval using RT-qPCR.

	Temperature	Time point					
		0 hr	6 hr	1 day	3 days	10 days	15 days
RNA conc. (ng/ul)	25℃	109	82	89	49	2	2
	4℃	109	48	81	45	2	2
RIN rate	25℃	7.2	6.0	8.5	8	N	N
	4℃	7.2	8.6	5.5	9.3	4.8	2.0

* RIN score is on a scale of 1-10 (1=lowest; 10=highest)

Table 2. RNA integrity number (RIN) of for each sample as an indication of RNA integrity, the 18S/28S ratio, and an estimation of concentration.

blood samples were analyzed using RT-qPCR targeting ACTB, B2M, GAPDH, PGK1, GUSB, HPRT1, PPIA, RPLPO, TBP, and TFRC mRNAs and 18S rRNA.

As shown in Figure 2, all RNA markers were degraded slower at 4°C than at 25°C. The mRNA levels of GADPH, B2M, and HPRT1 at 25°C and GAPDH at 4°C were maintained at a relatively constant level for three days. After five days, the mRNA levels of those markers gradually decreased. 18S rRNA and mRNA levels of ACTB, PPIA, GUSB, PGK1, RPLPO, TBP, and TFRC were not remarkably changed at either temperature.

Discussion

This study investigated RNA markers for estimating PMI from blood samples. Blood samples are easily obtained from living persons and thus can be used for methodical evaluation. However, blood samples pose several problems when using RT-qPCR because the aspects involved in estimating PMI are much more complicated in the scene of incident than in experimental conditions.

In the present study, to overcome the limitations of previous PMI estimation approaches, the integrity of 18S and 28S rRNA and mRNA levels of ten housekeeping genes were analyzed. Analysis methods included microfluidic electrophoresis chip and RT-qPCR TaqMan assays over nine different time points (0, 6 hr, 1, 3, 5, 10, 15, 20, 30 days) and two temperature conditions, at 4°C and 25°C.

Microfluidic electrophoresis results from this study show that PCR bands of 18S and 28S rRNA were maintained for 15 days at 4°C, and 18s rRNA and mRNA levels of all mRNA markers were detected using real-time RT-PCR through 30 days at 4°C and 25°C. In contrast to DNA, RNA is generally believed to be rapidly degraded after death and *in vitro* due to ubiquitous ribonuclease activity. Many studies, however, reported rather high RNA stability in certain organs such as brain tissues [10-14]. Results from the present study show that all RNA markers were gradually decreased in a time-dependent manner; however, RNA levels were able to be detected through 30 days after blood collection. Thus, these results were concordant with previous studies regarding RNA in brain tissues [15].

This study confirmed that levels of certain RNA markers such as 18S rRNA and HPRT1, B2M, and GAPDH mRNAs at 25°C and GAPDH mRNA at 4°C are significantly decreased in a time-dependent manner through 30 days after blood collection, indicating the useful of these markers to estimate PMI even if a dead body is left for several days at variable temperatures. These RNA markers may be affected by released intracellular substances such as enzymes and proteins from hemolysis of red blood cells in whole blood.

In conclusion, we hypothesized that the RNA degradation level in various blood specimens could be measured quantitatively and used as an indicator of PMI for up to 30 days post mortem. We expect that further study using PMI estimation will add valuable information.

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