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The Effect of Elapsed Time on the Quantity of mRNA in Skin: A Study to Evaluate the Potential Forensic Use of mRNA to Determine the Postmortem Interval

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Ribonucleic acid (RNA) has potential use in forensic science for the determination of postmortem interval. We report the first study on serial sampling of messenger RNA (mRNA) from surgical specimens to determine if there is a correlation between mRNA quantity and elapsed time. Skin tissues were collected from modified radical mastectomy specimens. After a defined period of time, bisected skin sections were cut and frozen in liquid nitrogen. Serial collection of the specimens was conducted, and frozen sections were obtained from all samples. Quantitative real-time reverse transcription-polymerase chain reaction was performed using the extracted RNA to measure the transcriptional activity of 2 selected housekeeping genes. The selected loci were mRNA sequences that exhibited time-dependent quantitative changes in a previous study. We collected 44 samples from 9 different patients, with 3-10 samples collected per patient. The amount of mRNA transcripts present in the serial samples showed a weak time-dependent correlation trend only in some cases. Further studies to evaluate different target mRNA sequences are necessary, as is exploration of additional methods to evaluate mRNA transcript degradation.

Key words : messenger RNA, postmortem interval, reverse transcription-polymerase chain reaction

Introduction

Molecular technology using deoxyribonucleic acid (DNA) is widely used in the field of forensic science. However, ribonucleic acid (RNA) is less widely used because of the rapid decay rate of RNA. Nevertheless, it has been suggested that RNA may have potential applications in the field of forensic science.[1] For example, the fragility of RNA could be exploited to help determine the postmortem interval (PMI) if there is a correlation between the quantity of residual RNA and elapsed time. Bauer et al[2, 3] suggested a method of quantifying messenger RNA (mRNA) as an indicator of PMI. On the other hand, Heinrich et al[4] failed to detect a correlation between PMI and RNA degradation.

There is no firmly established method to evaluate the integrity, stability or quantity of RNA in degenerated specimens. Previous studies which tried to show the relationship of RNA and PMI have used percentage of poly A in total RNA,[5] quotient of areas drawn by capillary electrophoresis in two genes,[2, 3] copy numbers or cycle threshold (Ct) value of a certain gene by real-time polymerase chain reaction (PCR),[4, 6-11] or ratio of Ct value in two genes by real-

time PCR.[12, 13] The materials were also varied, such as blood from healthy volunteers,[2, 3, 13] organs/tissues from autopsy/biopsy,[4, 5, 7-12, 14-17] or animal model.[6] All the previous studies were conducted using samples collected at just one point in time from one patient or deceased individual. None of them were done with skin tissue.

In this study, we performed serial sampling of mRNA from surgical individual specimens in order to evaluate the correlation between mRNA quantity and elapsed time.

Material and Methods

Material collection and sampling

Modified radical mastectomy specimens were selected from surgical specimens from 31 May 2010 to 9 April 2010. The recorded times of reception were designated as the starting times of each specimen, and a portion of skin that was grossly unremarkable and had no influence on diagnosis was bisected using a clean blade. The bisected skin was then kept at a gross examination room which maintained its temperature of 20−22℃. After a certain length of time had passed, a portion of bisected skin was cut into several 0.5−1.0 cm^2 pieces and these pieces were frozen in liquid nitrogen. Serial collections were made from each specimen and all collected samples were stored at −70℃ until experimental analyses. This protocol was approved by the Institutional Review Board of Samsung Medical Centre (IRB File No. 2010-04-058-001).

Ten to fifteen frozen sections with a thickness of 20 ㎛ were obtained from each sample. The material was thawed and then RNA was extracted using an RNeasy RNA purification kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. These kits do not contain DNase, and additional DNase treatments were not performed.

The ABI High-Capacity cDNA Archive Kit (Applied Biosystems, Carlsbad, CA, USA) and random hexamers were used for cDNA synthesis. Quantitative PCR was performed using an ABI Prism 7900 HT (Applied Biosystems). Four loci from two housekeeping genes, *ACTN1*, *ACTN2*, *FASN1*, and *FASN2* (called *FASN4* in a previous study[2]) were selected for the present study. The selected loci were mRNA sequences that had showed time-dependent quantitative changes in two previous studies[2, 3]. The functions and locations are given in Table 1 and Figure 1. Primer Express 3.0 software (Applied Biosystems) was used to design primers and probes (Table 2) in order to discriminate between RNA species. A panel of gene-specific primer pairs was also used for further expression profiling using the Taqman® probe method. All amplifications were performed three times. The experimental Ct value for each RNA segment was obtained and calibrated against those of the other segments. The ⊿⊿Ct method was used to compare the amount of product present in later samples to that present in the earliest sample, even though the earliest time point was not necessarily the zero time point.

Results

Forty-four samples were collected from nine cases, with 3−10 samples collected per case. The median of earliest elapsed times was 8.3 hours (range: 0 to 18 hours). The median of maximum elapsed times was 40.0 hours (range: 24.5 to 92.5 hours). Degeneration of extracted RNA from specimens was confirmed by electrophoretic analysis showing no visible band for 18S and 28S (Fig. 2A). However, all RNA specimens

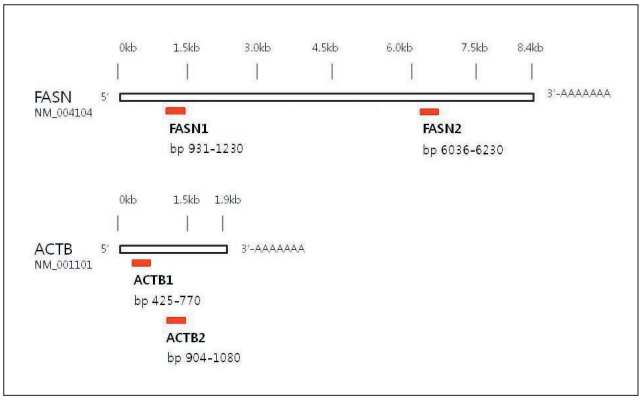

Fig. 1. The schematic shows the locations and size of target sequences on each mRNA.

contained enough RNA to be measured with Ct values ranging 19.1 to 38.0, with the exception of FASN1 in one specimen (Case 7, Table 3, Figure 2B). The mean Ct values of FASN1, FASN2, ACTB1 and ACTB2 were 33.3, 27.8, 22.9 and 27.3. The difference between the means of the Ct values of FASN1 and those of FASN2 was statistically significant ($p < 0.01$). The standard variation of Ct value of FASN2 (1.17) and ACTB1 (2.38) were relatively smaller than their counterparts among cases and elapsed times.

The relationship between the Ct values of each specimen with their elapsed time showed no remarkable change or trend over time with minimal gradient in every gene (Fig. 3). The relative quantification of FASN1 was calculated using the Ct value of FASN2 as a calibrator, because the combination (quotient FASN1/FASN2) showed time-related decrease in a previous study.[2] The values of

Table1. Specification of the Genes Tested in the Present Study

Symbol	Name	Function	Localization
ACTB	Actin, beta	Cytoskeletal structural protein	7p22
FASN	Fatty acid synthase	Enzyme for long-chain saturated fatty acids synthesis	17q25

Table 2. Primer and Probe Sequences

Sequence Name	Primer Sequence	Probe Sequence	Size
FASN1*	5′-CTCATCCGCTCGTTGTACCAG-3′ 5′-GCTGGGATCTCAGGGTTGG-3′	FAM‡-CCGGAGTGGCCCCT-NFQ	300bp
FASN2	5′-GGTCTTGAGAGATGGCTTGCTG-3′ 5′-TTGGCAAAGCCGTAGTTGCT-3′	FAM-TTCCAGGACGTCTGCA-NFQ	195bp
ACTB1†	5′-CAGATCATGTTTGAGACCTTCAACA-3′ 5′-GTGGCCATCTCTTGCTCGAA-3′	FAM-TCCTCACCGAGCGCG-NFQ	346bp
ACTB2	5′-ATCCACGAAACTACCTTCAACTCC-3′ 5′-AAGGAGCAATGATCTTGATCTTCAT-3′	FAM-TGGCATTGCCGACAGG-NFQ	177bp

*FASN, Fatty acid synthase ; †ACTB, Actin, beta ; ‡FAM, Fluorescein amidite

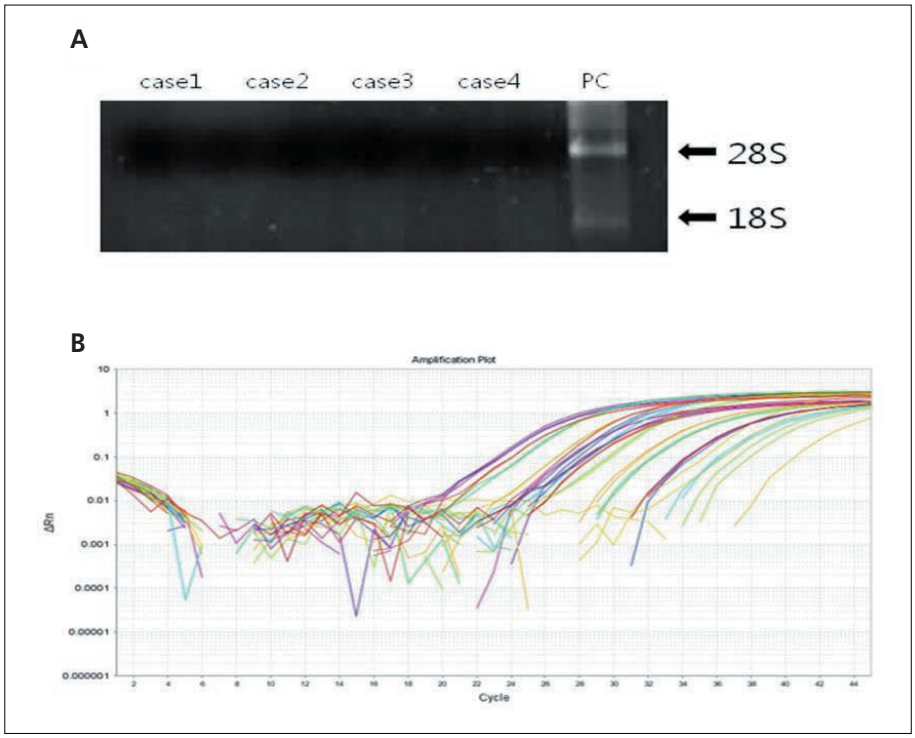

Fig. 2. (A) This image shows a representative presentation of electrophoresis of extracted RNA compared to positive control (PC) with visible band for 18S (lower arrow) and 28S (upper arrow). (B) This plot shows the amplification of sequences in real-time PCR.

Table 3. Ct Values of each RNA Segment and Relative Quantification by the $\Delta\Delta C_t$ Method

Case	Serial Number	Elapsed Time (h)	ΔCt				2^-ΔΔCt (Target/Reference)		
			FASN1	FASN2	ACTB1	ACTB2	FASN1 /FASN2	FASN1 /ACTB1	ACTB2/ ACRB1
1	1	18.0	33.31	25.57	20.96	26.50	1.00	1.00	1.00
	2	21.0	33.42	26.46	21.48	27.01	1.72	1.33	1.00
	3	39.0	33.12	27.25	21.83	27.27	3.66	2.09	1.07
	4	42.0	35.38	28.06	22.44	27.91	1.33	0.67	1.06
	5	45.0	34.21	28.04	22.46	27.95	2.98	1.52	1.03
	6	48.5	35.75	28.48	22.95	28.73	1.38	0.73	0.85
	7	63.0	35.83	28.37	23.16	28.83	1.21	0.80	0.91
	8	66.0	36.79	27.91	22.46	27.92	0.45	0.25	1.06
	9	68.0	34.07	27.85	22.31	28.27	2.86	1.50	0.74
2	1	13.0	38.00	27.58	21.75	27.52	1.00	1.00	1.00
	2	16.0	34.60	27.47	22.20	27.08	9.76	14.40	1.86
	3	19.0	33.34	27.79	21.90	26.71	28.94	27.81	1.94
	4	22.0	33.27	27.94	21.98	26.39	34.00	31.05	2.56
	5	37.0	36.08	28.64	23.21	27.90	7.85	10.39	2.12
	6	40.0	31.63	27.77	22.79	27.15	93.72	169.29	2.65
3	1	14.5	31.63	27.99	23.47	28.30	1.00	1.00	1.00
	2	17.5	33.35	27.49	23.01	27.46	0.22	0.22	1.30
	3	20.3	33.74	28.07	23.12	27.27	0.25	0.18	1.61
	4	24.0	33.19	27.57	22.52	26.53	0.25	0.18	1.77
	5	41.5	35.38	28.03	22.92	27.15	0.08	0.05	1.52
4	1	16.5	33.38	28.68	23.51	28.00	1.00	1.00	1.00
	2	19.5	34.19	30.49	25.33	30.02	2.01	2.01	0.87
	3	21.5	37.97	30.00	24.50	29.00	0.10	0.08	0.99
	4	24.5	37.01	29.36	23.96	28.23	0.13	0.11	1.17
5	1	17.5	34.06	27.38	21.74	25.53	1.00	1.00	1.00
	2	19.0	34.62	27.11	21.50	25.34	0.56	0.57	0.97
	3	21.0	33.02	27.03	21.49	25.87	1.61	1.72	0.66
	4	24.0	32.38	27.20	21.55	25.87	2.82	2.80	0.69
	5	46.0	34.28	27.46	21.74	25.98	0.91	0.86	0.73
6	1	0.0	33.91	26.71	20.86	24.48	1.00	1.00	1.00
	2	2.0	33.02	26.95	21.18	25.02	2.19	2.31	0.86
	3	5.0	29.89	26.13	19.11	22.47	10.81	4.83	1.20
	4	27.0	30.95	27.60	21.60	35.03	14.41	13.06	0.00
7	1	3.5	Ud	28.06	34.97	36.81	Ud	Ud	1.00
	2	25.5	29.87	26.83	24.79	24.58	Ud	Ud	4.12
	3	28.5	28.86	25.38	22.16	22.40	Ud	Ud	3.02
8	1	1.5	28.73	24.87	20.89	22.61	1.00	1.00	1.00
	2	4.5	31.08	27.40	21.74	29.61	1.13	0.35	0.01
	3	25.5	31.84	28.68	22.04	33.86	1.62	0.26	0.00
	4	28.5	32.28	29.77	27.83	29.73	2.54	10.47	0.88
9	1	1.0	31.23	28.68	25.40	25.89	1.00	1.00	1.00
	2	25.0	31.23	28.95	23.07	24.91	1.22	0.20	0.39
	3	88.5	31.57	29.21	23.61	25.83	1.14	0.23	0.30
	4	92.5	31.61	29.74	24.75	26.18	1.61	0.49	0.52
Average			33.33	27.82	22.91	27.34			
Standard deviation			2.21	1.17	2.38	2.81			

*FASN, Fatty acid synthase ; †ACTB, Actin, beta ; ‡Ud, Undetermined

relative quantification are shown in Table 3. As shown representatively in Figure 4, this calculation revealed inconsistent, irregular distribution between cases.

FASN1 and ACTB1 showed the biggest difference between the Ct values ($p < 0.01$). The relative quantifications of FASN1 were done by $\Delta\Delta$Ct method using the Ct value of ACTB1 of the earliest specimen in the same case as a calibrator. As shown representatively in Figure 5, there was a weak decreasing trend in some cases.

In short, the quantity of mRNA transcript present in the samples showed a weak time-dependent decreasing tendency in some cases but not in general.

Discussion

Several aspects of our study were different from those of previous studies conducted to evaluate the usefulness of RNA as a means of determining the PMI. This is the first study with skin tissue from surgical specimens. Skin was selected as the experimental target because of its easy accessibility on the body. Easy accessibility would be an advantage if this technique is revealed to be reliable in practice. Our patient population and state of the specimens were well controlled and fairly homogenous. All patients were female and all specimens were breast skin. All specimens were free from disease, so no physiological or pathological conditions influenced RNA decomposition. Furthermore, we obtained at least three successive samples from each specimen to investigate changes in mRNA levels over time. Previous studies were conducted using samples collected at just one time point from one patient or deceased individual, so the result showed statistical tendency, not serial change of each independent case.

The initial sampling time, sampling interval, and number of specimens collected per case were not strictly controlled. Moreover, it may be considered unusual to use the ΔCt of the earliest sample as the

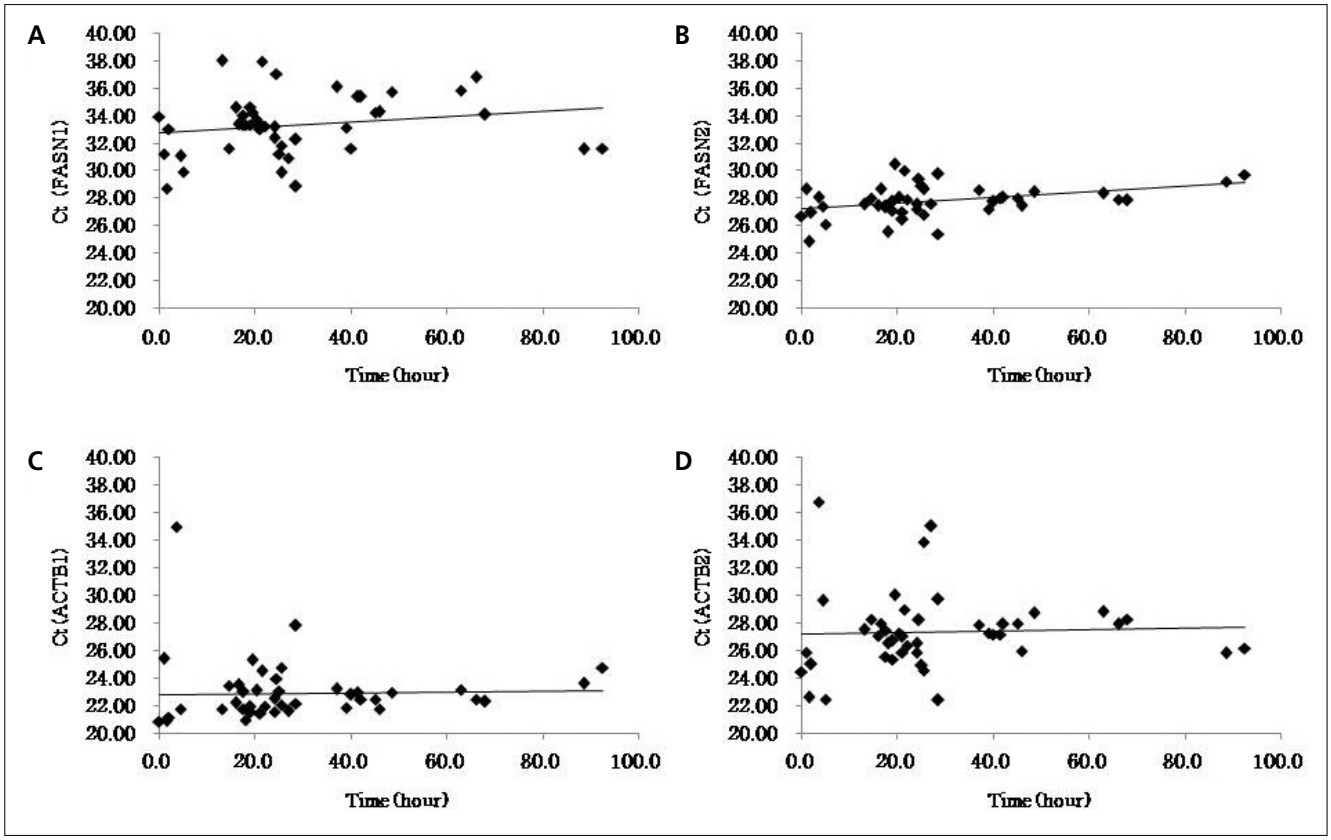

Fig. 3. These graphs show the relationship between the Ct values with elapsed time.

calibrator, even though the earliest time is unlikely to be the zero time point. However, this reflects the sampling conditions for forensic specimens in practice. In most real criminal resources, the initial ⊿Ct for the zero time point also cannot be determined.

Quantitative real-time PCR is a well established method for measuring transcript levels, but several systematic errors in the application of methods, including inappropriate choice of reference genes for normalizing, may compromise the interpretation of results. In the context of PMI determination, the choice of reference genes is more problematic. If the combination of one gene with time-dependent degeneration and another gene with great stability were found, the measurement of the ratio of the two genes would be the best reliable method to determine PMI. In this study, we selected target mRNA sequences for amplification that had shown time-dependent quantitative changes in previous studies.[2, 3] FASN-mRNA was chosen as target sequence because of its size of >8 kb and its ubiquitous expression as the so-called house-keeping gene.[18] In comparison, ACTB was selected as a reference gene because of its short length and ubiquitous expression.

However, in contrast to previous reports, the relative transcript abundance of pairs of sequences from the target genes did not show any strong time-dependent correlations. Also, the Ct value of each gene did not show time-associated change. This discrepancy between studies may be due to different experimental techniques. First, the former studies targeting FASN and ACTB used a semi-quantitative method (capillary electrophoresis), not quantitative real-time PCR. No compatible study using real-time PCR is reported yet. Second, we used the random-hexamer primer rather than the oligo-dT primer. Bauer et al[2, 3] applied oligo-dT primer because the 5′-end of the mRNA will be underrepresented in the cDNA population to a degree corresponding to the extent of degradation in the RNA preparation. Typically, the Ct value for the primer pair

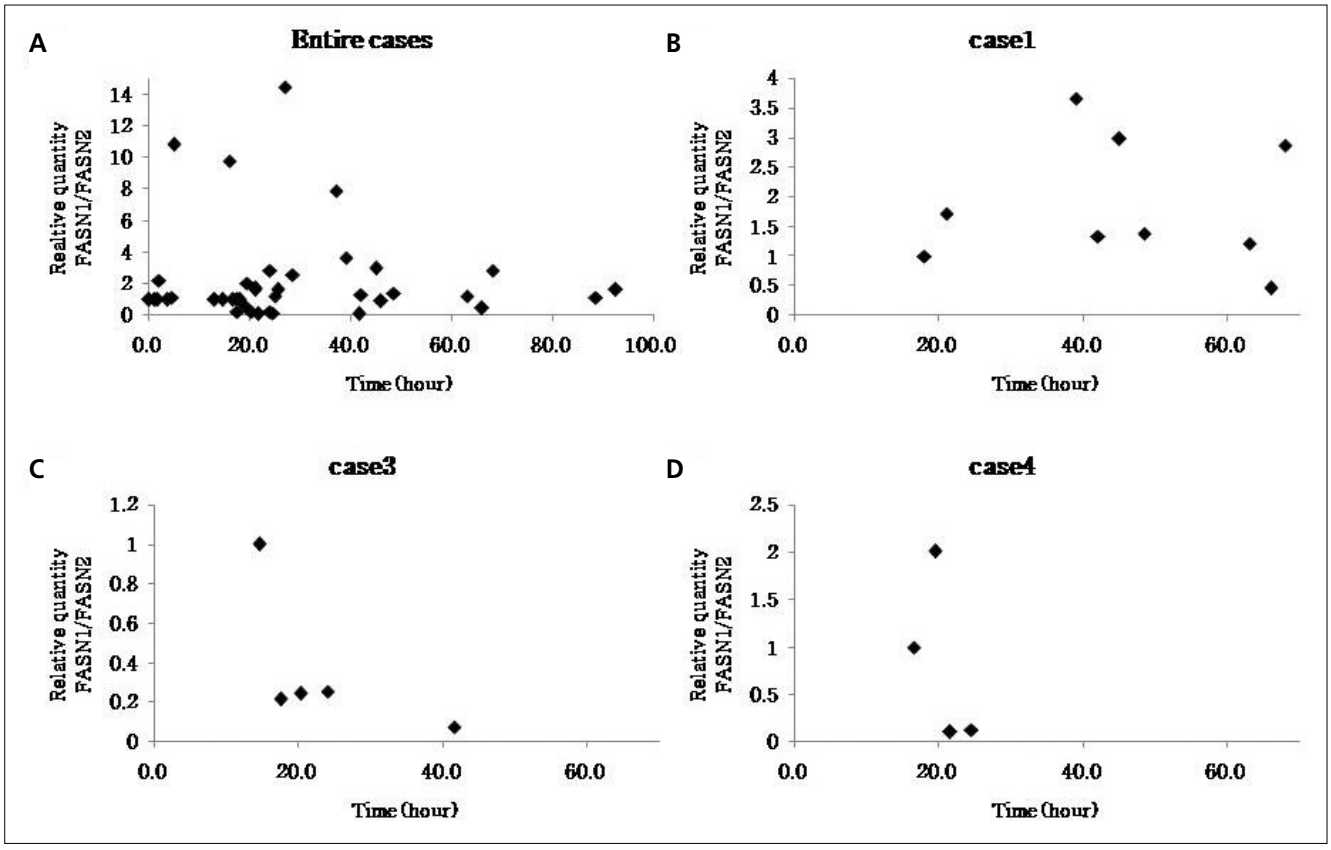

Fig. 4. These graphs show the relative quantifications of FASN1 along elapsed time (calibrator: FASN2).

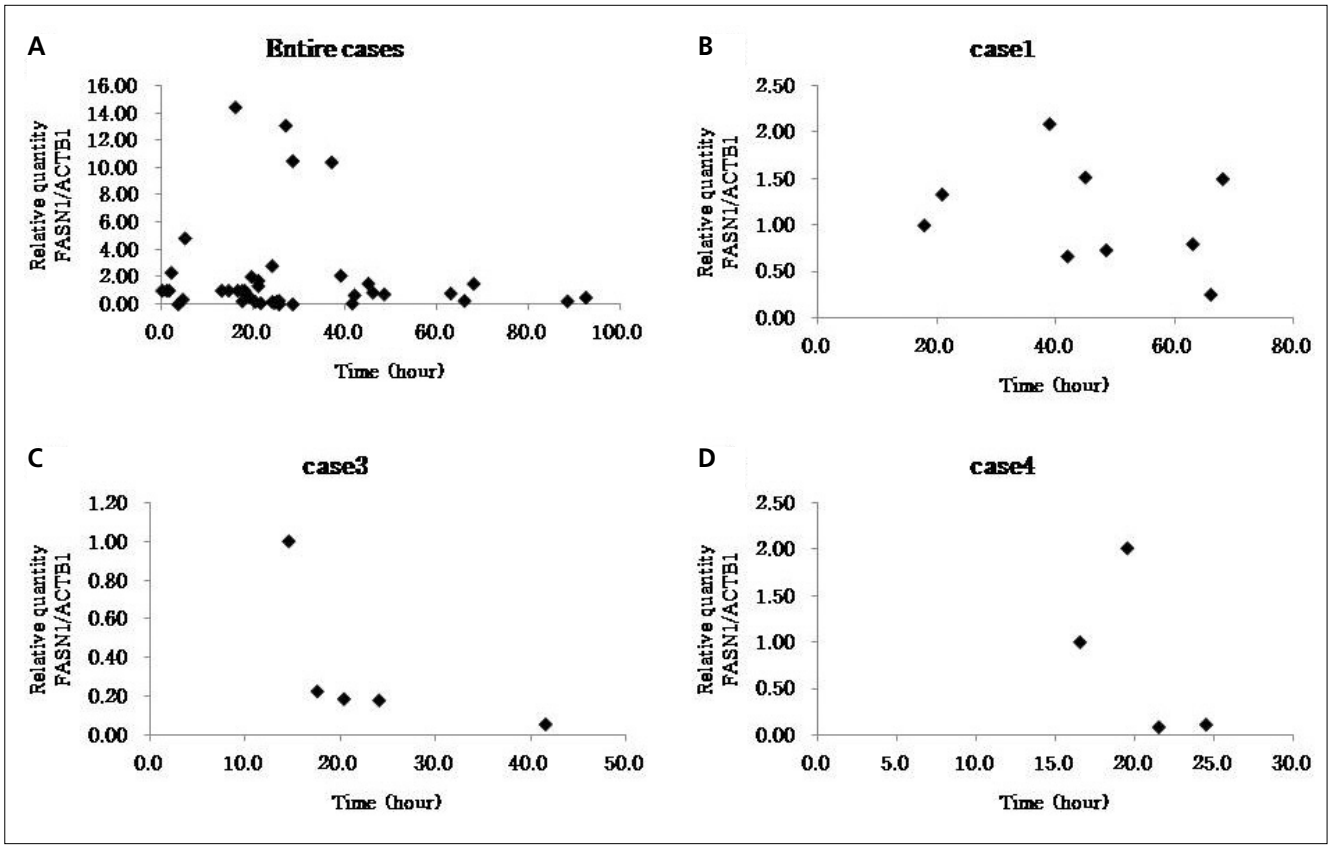

Fig. 5. These graphs show relative quantifications of FASN1 along elapsed time (calibrator: ACTB1).

at the 5′-end of a cDNA will be higher than the Ct value of the primer pair at the 3′-end when the oligo-dT primer is used.[19] In this study, the random-hexamer primers were used to maximize the effect of the efficiency of reverse transcription. As the first step to set up the protocol for mRNA degradation study with skin tissue, confirming that a measurable amount of mRNA existed was a priority. Reverse transcription was conducted successfully for all samples, including those left at room temperature for 92.5 hours. This is consistent with previous studies that showed that mRNA can be extracted and detected after more than several days postmortem,[4, 7, 14, 20] and as long as several months from well-preserved blood stains.[3] In short, random-hexamer primer might be a good choice to acquire sufficient quantities of mRNA for analysis, but not to evaluate the degree of degradation. Based on the result of these studies so far, the next step of experiment could be one using the same sample preparation technique with more regular interval, same loci, oligo-dT primer, and real-time PCR. Additional DNase treatment could be tried for excluding unnecessary cDNA contamination. Taking the first sample from each specimen as early as possible might be helpful for more desirable normalization, too.

Many chemical-based methods have been studied for use in determining the PMI,[21] as have some DNA-based ideas.[22–24] To date, none of the chemical methods for determining the time of death have been found to be practically applicable.[21] It is therefore perhaps not that surprising that our RNA-based method did not yield useful results.

But there are still lots of issues to research in order to determine whether mRNA can be used for determining PMI. From the results of previous genome-wide analyses of mRNA degradation rates, there are large differences between the stabilities of individual mRNAs, and that mRNA stability is strongly related to the molecular function of the

encoded protein.[25] Improved understanding of the dynamics of mRNA degradation will aid the selection of better markers for the determination of PMI. It is also possible that the age, the gender and the pathologic condition of the individual and/or the manner of death may affect mRNA degradation and PMI predictability. Therefore, further investigation to confirm the role of mRNA in determining PMI should be performed including various conditions of specimens or patients, different target mRNA sequences, and diverse reliable quantitation methods.

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