Jikeikai Med J 2004 ; 51 : 123-8

Forensic Age Estimation by Detection of Deletion Level in Human Mitochondrial DNA

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ABSTRACT

The presence of 4,977-base pair (bp) mitochondrial DNA deletions was examined in specimens of human skeletal muscle (pectoralis major) and cardiac muscle obtained at forensic autopsy from 53 persons aged 1 month to 91 years. Causes of death included neither sudden cardiac death nor any mitochondrial diseases. A simple method using the polymerase chain reaction and electrophoresis to detect the 4,977-bp deletion level was developed. The level of this deletion was estimated by observing visible bands in 2% agarose gel following 28 cycles or 40 cycles of both of the polymerase chain reaction and graded as −, 1+, and 2+. The use of 1 ng of template DNA (total DNA) was sufficient to detect the 4,977-bp deletion. The 4,977-bp deletion level tended to increase with age, and the age distribution did not differ between the two tissues. In 15 of the 53 subjects (28.3%) deletion levels differed between skeletal and cardiac muscle, but neither muscle type had a significantly higher deletion level. These findings suggest that the level of the 4,977-bp mitochondrial DNA deletion is age-dependent and does not differ between skeletal muscle and cardiac muscle. This simple method can be used to estimate age in forensic applications.

(Jikeikai Med J 2004 ; 51 : 123-8)

Key words : forensic age estimation, 4,977-base pair deletion, mitochondrial DNA, muscle tissue, polymerase chain reaction

INTRODUCTION

Several types of deletion that arise from strand slippage during replication in human mitochondrial DNA (mtDNA) are associated with aging[1-3]. The 4,977-base pair (bp) deletion of mtDNA is common, and its relation with aging has been reported for cardiac muscle, skeletal muscle[4-7], the brain[8,9], lung[10], skin[11], and blood[12,13]. Of these tissues, muscle shows the most marked deletion change because muscle cell is postmitotic.

When age is unknown in forensic autopsy cases, it is usually estimated on the basis of morphological findings of bones, teeth, or other tissues. It is interesting to carry out molecular biological analysis as well as these analyses. The purpose of this study was to obtain data on the 4,977-bp mtDNA deletion in muscle and to apply the deletion to forensic age estimation. Recently, Wurmb-Schwark et al. attempted to apply the 4,977-bp deletion to forensic age estimation using 100 ng of total DNA extracted from skeletal muscle for the real-time polymerase chain reaction (PCR)[14]. In this study, the level of the 4,977-bp deletion was evaluated in skeletal muscle (pectoralis major) and cardiac muscle obtained from 53 forensic autopsy cases. The relationship between deletion level and aging was examined with conventional PCR with 1 ng of template DNA (total DNA),

Received for publication, August 31, 2004

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and the difference in the deletion level between skeletal and cardiac muscle in each subject was examined.

MATERIALS AND METHODS

1. *Tissues and DNA samples*

Cardiac and skeletal muscle (pectoralis major) tissues were obtained from 53 forensic autopsy cases at the Department of Forensic Medicine, The Jikei University School of Medicine. The ages of the subjects ranged from 1 month to 91 years (mean age, 41.7±27.4 years), and the cause of deaths are shown in Table 1. Subjects dying of sudden cardiac death or mitochondrial disease were excluded. After samples were rinsed 3 times in 35 ml of Tris-ethylenediaminetetraacetic acid solution with a vortex mixer, DNA was extracted from 50 to 100 mg of each tissue with the proteinase K and phenol/chloroform method. The extracted DNA was quantified with a spectrophotometer according to optical absorbance at 260 nm.

Table 1. Distribution of causes of death among 53 forensic autopsy cases

	Cause of death	Number of cases
Natural death	Infectious disease	10
	Others	12
Unnatural death	Acute asphyxia	7
	Head injury	9
	Traumatic shock	10
	Poisoning	2
	Fire death	3

2. *PCR*

Twenty microliters of PCR mixture containing 1 ng of template DNA, 4 μl of 5×Green GoTaq Reaction Buffer (Promega, Madison, WI, USA), 0.2 mM of deoxyribonucleotide triphosphate, 0.5 μM of each primer, and 0.6 units of GoTaq DNA Polymerase (Promega) was used. Amplification was performed with a thermal cycler (PC-700, Astec, Tokyo, Japan) with the following thermal conditions: initial denaturation at 94°C for 1 minute followed by 20, 28, or 40 cycles at 94°C for 1 minute, 50°C for 1 minute, and then 72°C for 1.5 minutes. Two microliters of the PCR products was resolved in 2% agarose gel and photographed after staining in ethidium bromide.

The primer pair used for the 4,977-bp deletion was L828/H1349, and that used for the positive control was L1317/H1349. The sequences of these primers are shown in Table 2. In the presence of the 4,977-bp deletion, the 241-bp fragment was amplified using the L828/H1349 primer pair. The PCR product of the positive control was the 325-bp fragment.

3. *Estimation of the 4,977-bp deletion level*

Owing to a quality check of the template DNA, 20 thermal cycles of PCR for the positive control were carried out and 28 thermal cycles of DNA amplification were carried out for the 4,977-bp deletion. If the 241-bp fragment was detected, the extent of deletion was implied to be high and the deletion level was graded as del.2+. When no visible band was detected after 40 thermal cycles, the deletion level was graded as del.−. Additionally, when a visible band was not detected after 28 thermal cycles but a 241-bp fragment was detected after 40 thermal cycles, the

Table 2. Primers and PCR products

Primer	Sequence 5′→3′	Nucleotide position*	Length of PCR product
L828	CCCCTCTAGAGCCCACTGTAAAGC	8,282-8,305	241 bp**
H1349	CCTGTGAGGAAAGGTATTCCTGCT	13,476-13,499	
L1317	AGGCGCTATCACCACTCTGTTCG	13,175-13,198	325 bp

* Nucleotide numbering of mtDNA is according to Anderson et al.[15].
** Predicted length of the PCR product in the presence of the 4,977-bp deletion.

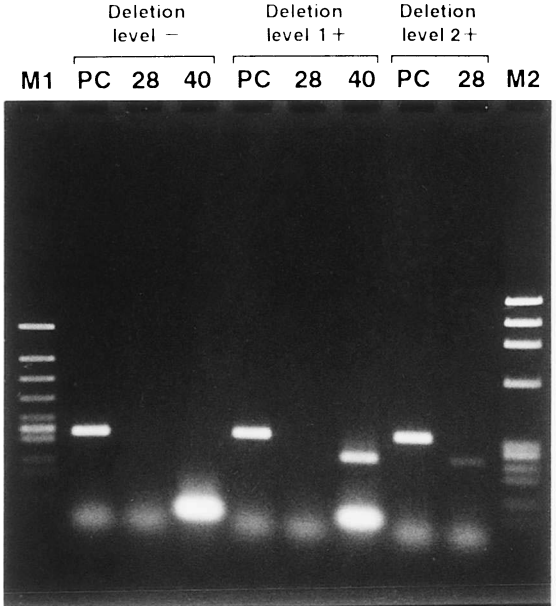

Fig. 1. Detection of 4,977-bp mtDNA deletions using PCR and 2% agarose gel electrophoresis. Lanes: PC, PCR with 20 thermal cycles using the primer pair L1317/H1349 for positive control; 28, PCR with 28 thermal cycles using the primer pair L828/H1349 for the deletion level 1+; 40, PCR with 40 thermal cycles using the primer pair L828/H1349 for the deletion level 2+; M1, size marker ϕX174/Hinc II digest; M2, size marker ϕX174/Hae III digest.

deletion level was graded as del.1+ (Fig. 1).

4. *Statistical analysis*

The data were statistically analyzed with Welch's t-test and the Mann-Whitney's U-test. Differences with a p value less than 0.05 were considered significant.

5. *Ethics*

This study was approved by the Ethics Committee for Biomedical Research, The Jikei University School of Medicine, and samples were anonymized in an unlinkable fashion. This study was conducted in accordance with the Ethics Guidelines for Human Genome/Gene Analysis Research (Guideline of Japanese Ministry of Education, Culture, Sports, Science and Technology; Ministry of Health, Labor and Welfare; Ministry of Economy, Trade and Industry, of March 29, 2001), Part IV: Handling Of Human Specimens, Section 11. Use Of Existing Specimens, (4) Subrule 4: Subrule Concerning The Use Of Group C Human Specimens Provided Prior To The Enforcement Of The Present Guidelines, 1).

Results

PCR with the L828/H1349 primer pair was performed as a positive control, and the 325-bp fragment was detected in all samples. The mean age of subjects in the del.− group was significantly less than that in the del.1+ group, which was significantly less than that in the del.2+ group (Fig. 2). The level of the 4,977-bp deletion increased with age. There was no significant difference in the age distribution between skeletal muscle and cardiac muscle for each deletion-level group. Deletion levels in skeletal muscle were del.− for all subjects younger than 16 years and del.2+ for all subjects older than 83 years. Furthermore, the oldest subject in the del.− group was aged 69 years, whereas the youngest subject in the del.2+ group was aged 38 years. Deletion levels in cardiac muscle were del.− for all subjects younger than 20 years and del.2+ for all subjects older than 71 years of age. The oldest subject in the del.− group was aged 69 years, whereas the youngest subject in the del.2+ group was aged 29 years.

Of the 53 subjects, 15 (28.3%) showed deletion levels that differed between skeletal muscle and cardiac muscle. In individual subjects, the respective deletion levels were either del.− and del.1+ or del.1+ and del.2+; however, deletion levels of both del.− and del.2+ were not observed in an individual subject. For 8 subjects ranging in age from 24 to 83 years (mean age, 50.6±19.6 years), the deletion level was higher in cardiac muscle than in skeletal muscle (Fig. 3). For 7 subjects ranging in age from 16 to 71 years (mean age, 33.9±19.9 years) the deletion level was higher in skeletal muscle than in cardiac muscle. Neither of these two deletion patterns was predominant, and there were no significant differences in mean age. A total of 4 different deletion patterns were observed (Fig. 4), but none was significantly more common.

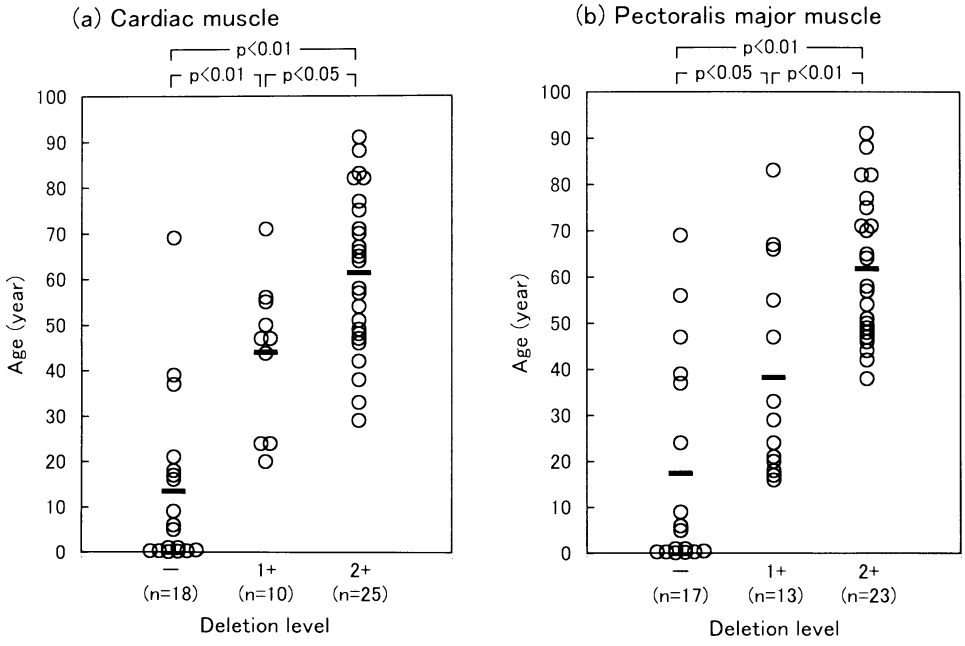

Fig. 2. The age distribution of each 4,977-bp mtDNA deletion level for (a) cardiac muscle and (b) pectoralis major muscle. Dash represents the mean age.

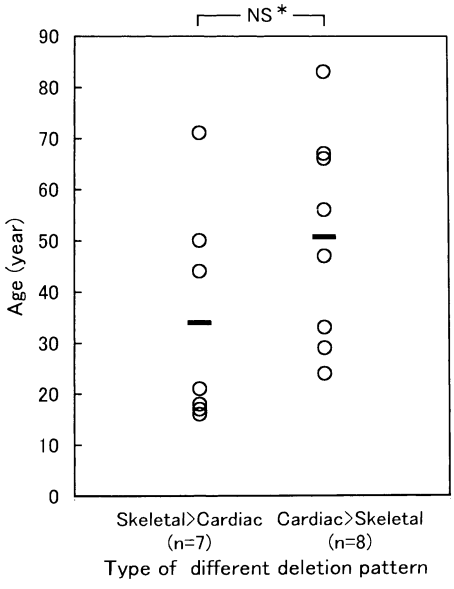

Fig. 3. The age distribution for 15 cases with different deletion patterns. Skeletal>Cardiac, the 4,977-bp mtDNA deletion level was higher in pectoralis major muscle than in cardiac muscle; Cardiac>Skeletal, the deletion level was higher in cardiac muscle than in pectoralis major muscle. *not significant ($p>0.05$)

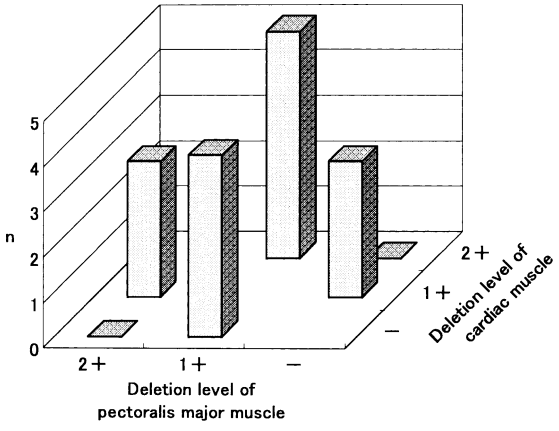

Fig. 4. Distribution of 4 different deletion patterns among 15 cases.

DISCUSSION

A relation between the 4,977-bp mtDNA deletion in muscle tissue and aging has previously been reported[6,7,14]. The 4,977-bp deletion in both skeletal muscle and cardiac muscle was previously evaluated in small series of subjects[4,5]. In the present study, the presence of the 4,977-bp deletion was investigated in skeletal and cardiac muscle from 53 subjects aged 1 month to 91 years.

The presence of the 4,977-bp deletion was also age-dependent in the present study. In previous reports, however, the age at which the 4,977-bp dele-

tion was observed differed greatly. For example, the minimum age at which the 4,977-bp deletion was detected was 24 years for skeletal muscle and 40 years for cardiac muscle in one study[5] and 50 years for skeletal muscle in another study[6]. In the present study, the minimum age of subjects with the 4,977-bp deletion was 16 years for skeletal muscle and 20 years for cardiac muscle.

The disagreement between earlier studies and the present study might be attributed to differences in the number and age distribution of subjects. Properly evaluating the relationship between the 4,977-bp deletion and age is extremely difficult with a small number of subjects or subjects with an extremely skewed age distribution. Another possible source of disagreement is the varying quantities of template DNA used in different studies, which include 1 ng[6], 10 ng[1-3], 100 ng[7,14], and 1,000 ng[6]. Furthermore, the various techniques used, including PCR and electrophoresis[1,3,6], kinetic PCR[2,4,5,7], and real-time PCR[14], have differing levels of detection sensitivity.

In the present study, 1 ng of total DNA was used as a template, and 28 cycles or 40 cycles or both of PCR products were visualized with ethidium bromide after electrophoresis through a 2% agarose gel. The 4,977-bp deletion was classified as del.−, del.1+, or del.2+. The age range which 70% of number of subjects were included in is as follows in each deletion level. When the deletion level was del.−, the age distribution was less than 9 years in skeletal muscle and less than 17 years in cardiac muscle. When the deletion level was del.2+, the age distribution was older than 49 years in both skeletal muscle and cardiac muscle. When the deletion level was del.1+, the age distribution was from 17 to 67 years in skeletal muscle and from 24 to 56 years in cardiac muscle. A classification with this method would have only three levels but is sufficiently useful. Various methods of estimating age in forensic applications, such as conventional morphological analysis and real-time PCR, often have ranges of 10 years or more[14]. General estimates of age are based on a large amount of information. The use of 1 ng of template DNA is suitable for forensic applications because it is often only possible to obtain a small amount of DNA from forensic specimens. The 4,977-bp deletion level can be evaluated using a minimum general PCR apparatus and basic processing techniques.

A quality check of the template DNA was carried out with 20 cycles of PCR using a shifted primer (L1317) as the internal control. Checking the quality of the template DNA extracted from degraded forensic samples is useful. Moreover, these PCR products were confirmed as being of similar quantity by observation of band densities in agarose gel after electrophoresis. Producing equal quantities of 20-cycle PCR products before reaching a plateau means that the quantity of mtDNA in total DNA is approximately equivalent. The PCR product of the internal control was confirmed for all 53 subjects of this study.

Fifteen of 53 (28.3%) subjects had 4,977-bp deletion levels that differed between skeletal muscle and cardiac muscle. When the 4,977-bp deletion levels differed in an individual subject, they differed by only a single stage. Large differences, such as del.− and del.+2, were not observed (Fig. 4). Of the 15 subjects, 8 had higher 4,977-bp deletion levels in cardiac muscle and 7 had higher deletion levels in skeletal muscle. In contrast, earlier studies[4,5] found higher deletion levels in skeletal muscle.

In conclusion, the 4,977-bp deletion levels in cardiac and skeletal muscle are associated with aging, and no difference in the age distribution occurs in either tissue. A simple procedure involving PCR and 2% agarose gel electrophoresis and 1 ng of template DNA was developed to detect the 4,977-bp deletion level. These basic data and this simple method can be used to estimate age in forensic applications.

Acknowledgements: The author would like to thank Professor Akihiro Takatsu and staff of the Department of Forensic Medicine at The Jikei University School of Medicine for collecting specimens, providing critical suggestions for this study, and reviewing this manuscript.

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