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Exploration of reference genes for the development of a diagnostic kit on vascular aging in human saliva

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Identifying reliable biomarkers in saliva can be a promising approach to developing a rapid diagnostic kit for detecting vascular aging. This study investigated the most suitable reference gene for polymerase chain reaction (PCR) in saliva that is not affected by vascular aging variables. Whole saliva samples were collected to assess the expression of reference genes: actin beta (ACTB), 18S ribosomal RNA (18S rRNA), beta-2-microglobulin, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The most abundantly expressed gene was 18S rRNA, and the least expressed gene was GAPDH. Four genes were ranked according to their relative stability, as determined by mathematical algorithms, indicating that ACTB and 18S rRNA were stably expressed as reference genes. 18S rRNA was identified as the most promising reference gene for detecting systemic diseases using saliva from patients with vascular aging in these limited experimental conditions.

Keywords: Vascular aging, Reverse transcription-quantitative polymerase chain reaction (RT-qPCR), Reference gene, Human whole saliva

INTRODUCTION

Various body fluids, such as blood, saliva, and urine, are affected by systemic diseases. Detecting disease-specific biomarkers from these fluids is likely to accelerate the development of rapid diagnostic kits or instruments. Among those, saliva includes glandular saliva secreted by the three major salivary glands (parotid, submandibular, and sublingual) and minor salivary glands, as well as gingival crevicular fluid, exfoliated mucous membrane epithelium, and oral bacteria and their metabolic products. Salivary flow significantly decreases with age[1)], and the components of saliva have been found to change[2)]. In addition to age-related changes in volume and composition, saliva has been shown to reflect systemic conditions and may be used for clinical diagnosis. One advantage of saliva over blood is that it is easier to collect and noninvasive for the patient, reducing anxiety and discomfort during collection. For the examiner, a technique for collection was not required, and there was no coagulation after collection; therefore, handling is easy until analysis.

Various reports have been published on disease-specific biomarkers in the saliva for chronic heart failure[3)], cardiovascular disease[4-7)], Parkinson's disease[8)], Alzheimer's disease[9)], oral cancer[10)]. In addition, commercialized testing tools using saliva have been provided: a kit that can diagnose influenza and severe acute respiratory syndrome coronavirus by immunochromatography (Espline® SARS-CoV-2N, Kobayashi Pharmaceutical, Osaka, Japan) and a biosensor that can detect salivary amylase activity as a stress marker (salivary amylase monitor, Nipro, Osaka, Japan). Although these were mainly analyses of salivary proteins, the presence of salivary mRNA and its possible amplification by polymerase chain reaction (PCR) has already been demonstrated[11)]. The PCR testing of saliva has recently become popular because of the outbreak of novel coronavirus infections. There is a comparative quantification method that quantifies the relative expression levels of a target and reference gene; however, the accuracy of reverse transcription-quantitative PCR (RT-qPCR) is affected by the expression stability of the reference gene[12)]. Since gene expression has been reported to vary depending on the target tissue[13)], the selection of appropriate reference genes for data normalization is important. According to the Guidelines on the Minimum Information for Publication of Quantitative Real-Time PCR Experiments, it is necessary to indicate that the expression of the reference gene is invariant under experimental conditions[14)]. We consider that the usage of analytical tools (geNorm, BestKeeper, and NormFinder) to determine the best reference gene was in line with the recommendations of the guidelines.

We previously attempted to identify the salivary biomarkers of vascular aging[15)]. In that study, actin beta (ACTB), which is frequently used in RT-qPCR, was utilized as a reference gene. An optimal salivary reference gene does not fluctuate with factors that affect vascular aging, such as blood pressure, body mass index (BMI), sex, brachial-ankle pulse wave velocity (baPWV), and age, would be a good candidate

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for this study. However, no studies have evaluated the variable behavior of reference genes in the saliva owing to vascular age-related factors. Our goal is to provide a simple diagnostic kit for detecting vascular aging using saliva; however, validating the reference gene is a priority for discovering a convincing factor related to the aging. This study aimed to establish the most suitable reference gene for RT-qPCR in saliva that is not affected by vascular aging variables.

MATERIALS AND METHODS

Participants

The participants were 10 employees of Osaka Dental University Hospital (7 males and 3 females, mean age 41.20±13.67), and written informed consent was obtained from all participants. This study was approved by the Medical Ethics Committee of Osaka Dental University (Approval No. 111150) and conducted in compliance with the Declaration of Helsinki. It was conducted at Osaka Dental University Hospital from February 2021 to June 2023.

Measurement of vascular aging variables

Blood pressure plethysmography was performed by a clinical laboratory technician at the Department of Internal Medicine, Osaka Dental University Hospital. baPWV, ankle-brachial index (ABI), an indicator of vascular stenosis, brachial systolic pressure, brachial diastolic pressure, and heart rate (HR) were measured using FORM-5 (Fukuda Denshi, Tokyo, Japan). Carotid ultrasonography was performed using a ProSound α10 (Hitachi Aloka Medical, Tokyo, Japan).

Saliva collection and storage

Unstimulated whole saliva was collected at 5–6 p.m. None of the participants was allowed to eat or drink 1 h prior to collection. A 50-mL centrifuge tube (AS ONE, Osaka, Japan) was frozen before collection, and saliva was placed directly into the tube. Saliva was aliquot into RNase/DNase-free tubes, mixed with RNA protect saliva reagent (Qiagen, Hilden, Germany) and vortexed. All samples were anonymized and immediately stored at −80ºC.

Confirmation of reference gene expression levels in saliva

Total RNA was extracted using the RNA Protect Saliva Mini Kit (Qiagen), following the manufacturer's instructions. cDNA synthesis was performed using the SuperScript IV VILO Master Mix (Thermo Fisher Scientific, Waltham, MA, USA). RT-qPCR was performed on Step One Plus Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) with the conditions as follows: 50ºC for 2 min, 95ºC for 20 s, followed by 45 cycles consisting of 95ºC for 1 s and 60ºC for 20 s. ACTB, 18S ribosomal RNA (18S rRNA), beta-2-microglobulin (B2M), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were used as candidate reference genes (Table 1). The $2^{-\Delta\Delta Ct}$ method was used to compare the expression of each reference genes.

Comparison of reference gene expression levels for each vascular aging variable

The participants were classified based on the measurement of the following vascular aging variables: blood pressure, BMI, sex, baPWV, and age. The threshold cycle (Ct) values were used to determine gene expression.

Stability analysis for reference gene selection

The selection of appropriate reference genes directly influences the results of RT-qPCR target gene analyses. Various statistical programs have been developed to investigate the stability of different reference genes. The most stable reference genes were evaluated using the geNorm, BestKeeper, and NormFinder algorithms and RefFinder, an online software tool that integrates them. geNorm and BestKeeper were used, with each algorithm embedded in the statistical software, Easy R[16]. geNorm is based on the principle that the expression ratio of two ideal reference genes is identical in all samples regardless of the experimental conditions[17]. The pairwise variation of all reference genes was determined, and a gene stability measure (M-value) was calculated. The gene with the lowest M-value was evaluated as the stable gene, and two candidate genes with high stability were selected. BestKeeper uses two indices[18]. One evaluation index was the standard deviation (SD) of the Ct values for each reference gene. The other index was the Pearson correlation coefficient between the Ct value and index. Genes with SD values<1 and correlation coefficients>1 were selected as stable reference genes. NormFinder is an algorithm that evaluates variations in expression by establishing a model to evaluate variations within and between reference genes[19]. By calculating the stability of gene expression, genes with lower stability values were evaluated as stable reference genes. RefFinder

Table1 List of Genes for RT-qPCR

Gene symbol	Gene name	Taqman primer-probe assay ID	Accession number
ACTB	Actin beta	Hs01060665_g1	NM_001101.5
18S rRNA	18S ribosomal RNA	Hs9999901_s1	NR_003286.4
B2M	Beta-2-microglobulin	Hs00187842_m1	NM_004048.4
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	Hs02786624_g1	NM_001256799.3

is a web-based algorithm that integrates these three algorithms to compare and rank candidate reference genes. It assigns appropriate weights to individual genes and calculates the geometric mean of the weights for the overall ranking[20].

Statistical analysis

Statistical analysis was conducted using Bell Curve for Excel (Social Survey Research Information, Tokyo, Japan). The Mann–Whitney *U* test was used to determine statistical significance. All data are expressed as medians.

RESULTS

Evaluation of the vascular aging variables

The data of measurements performed on 10 participants are listed (Table 2); ABI, HR, and carotid plaque were within the standard values in all participants and excluded from the comparison data.

Evaluation of the Ct values in the reference genes

To search for valid reference genes in saliva samples, the RNA expression of four reference genes (ACTB, 18S rRNA, B2M, and GAPDH) was analyzed by RT-qPCR using TaqMan gene expression assays (Fig. 1). The highest expressed gene was 18S rRNA (Ct-value: 19.18±2.94), and the lowest expressed gene was

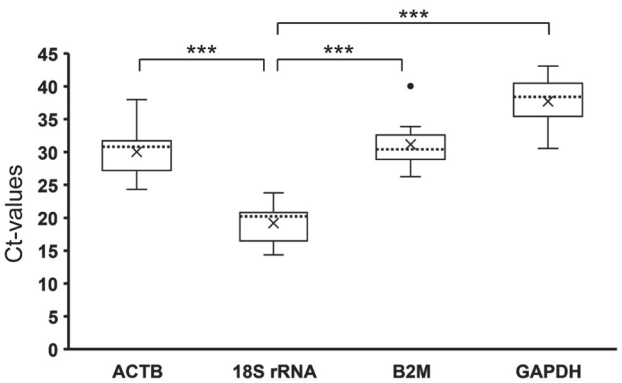

Fig. 1 Expression levels of four reference genes. Dotted lines indicate medians, crosses indicate means, and dot indicates outliers (***p<0.001).

Table2 Participants' body composition data

Parameters	Categories	Number (n=10)	Average (Min–Max)
Age (yrs)	<40 >40	6 4	41.2±13.7 (26–62)
BSBP (mmHg)	<140 >140	7 3	131.1±17.1 (103–153)
BDBP (mmHg)	<90 >90	8 2	80±12.5 (59–95)
baPWV (cm/s)	under 1SD over 1SD	4 6	1,359.2±221.9 (1,048–1,658)
ABI	≤0.9 1.3≤ >0.9 1.3>	0 10	1.1±0.07 (1.04–1.25)
BMI (kg/m^2)	under 25 over 25	4 6	26.58±4.4 (19.9–33.5)
Weight (kg)	<70 >70	5 5	74.2±18.1 (51.6–102.8)
Height (cm)	<165 >165	4 6	166.2±10.3 (149–184)
HR (bpm)	<60 100< >60 100>	0 10	72.2±10.3 (58–91)
Sex	male female	7 3	—
npf	with Plaque without Plaque	0 10	—

BSBP: Brachial systolic blood pressure; BDBP: Brachial diastolic blood pressure; baPWV: Brachial-ankle pulse wave velocity; ABI: Ankle-brachial index; BMI: Body mass index; HR: Heart rate; npf: Number of participants with plaque in carotid over that of the total participants

GAPDH (37.72±4.08). There was a significant difference (p<0.001) between the Ct values of 18S rRNA and those of the other reference genes. In this study, 18S rRNA was found to be the most highly expressed gene in the saliva samples.

Comparison of reference gene expression levels for each vascular aging variable

The expression levels of ACTB, 18S rRNA, and B2M were compared with the variables involved in vascular aging. GAPDH was excluded because its mean Ct value exceeded 35 (Fig. 2). Five variables related to vascular aging were assessed: blood pressure, BMI, sex, baPWV, and age. Blood pressure was determined according to the Japanese Society of Hypertension Guidelines for the Management of Hypertension 2019 (*i.e.*, participants with systolic blood pressure of 140 mmHg or higher or diastolic blood pressure of 90 mmHg or higher defined as the hypertensive group and others as the normotensive group). BMI was defined as 25 or more for the obese group and less than 25 for the normal group according to the Guideline for the Management of Obesity Disease 2022. To define vascular aging, outliers that exceed 1SD of the normal distribution of PWV based on the data were used[21]. If the baPWV exceeded +1SD for the participant's age at the time of measurement, the participant was classified into the high-baPWV group; otherwise, the participant was classified into the low-baPWV group. Age was classified into two groups based on the mean participant age of 41.20 (*i.e.*, 40 years or older and less than 40 years). For all reference genes, there were no statistically significant differences in expression levels among the classified participants.

Algorithmic stability analysis of reference genes

geNorm, BestKeeper, and NormFinder were used to assess the stability of the reference genes (Fig. 3). The M-value calculated using the geNorm algorithm indicated gene expression stability. An M-value below the threshold of 1.5 indicates stable expression[17]. The geNorm results showed that ACTB and 18S rRNA were stably expressed. BestKeeper uses two evaluation indices: the SD of the Ct value and the Pearson correlation coefficient. Genes with SD<1 and high correlation coefficient are selected as stable reference genes, and in the present results, all SD values were 1 or higher. The gene with the lowest SD and highest correlation coefficient was 18S rRNA. NormFinder evaluated the reference gene with the lowest stability value and identified ACTB as the most stable. RefFinder integrated the above three algorithms to compare and rank reference genes, and ACTB was the most stable reference gene (geomean of ranking values: 1.32).

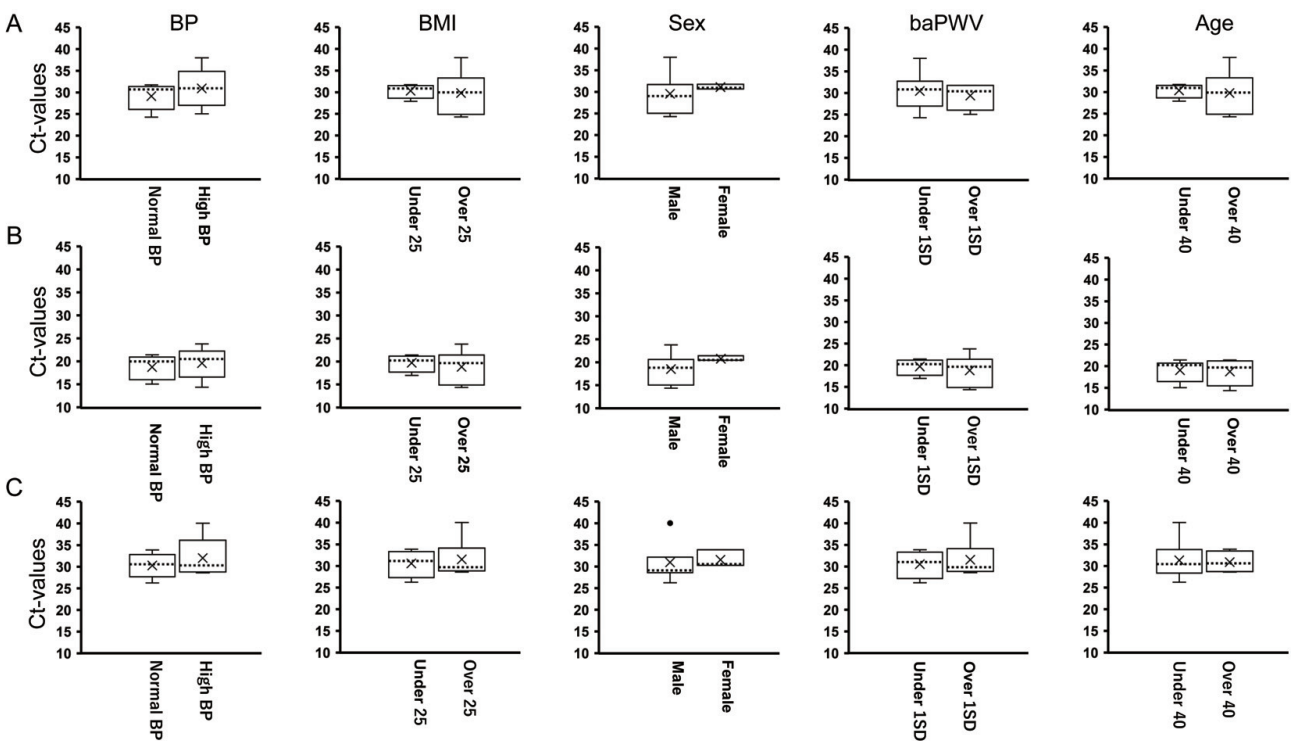

Fig. 2 Comparison of reference gene expression levels for each vascular aging variable.
(A) ACTB, (B) 18S rRNA, (C) B2M. Dotted lines indicate medians, crosses indicate means, and dot indicates outliers.
BP: blood pressure; BMI: body mass index; baPWV: brachial-ankle pulse wave velocity

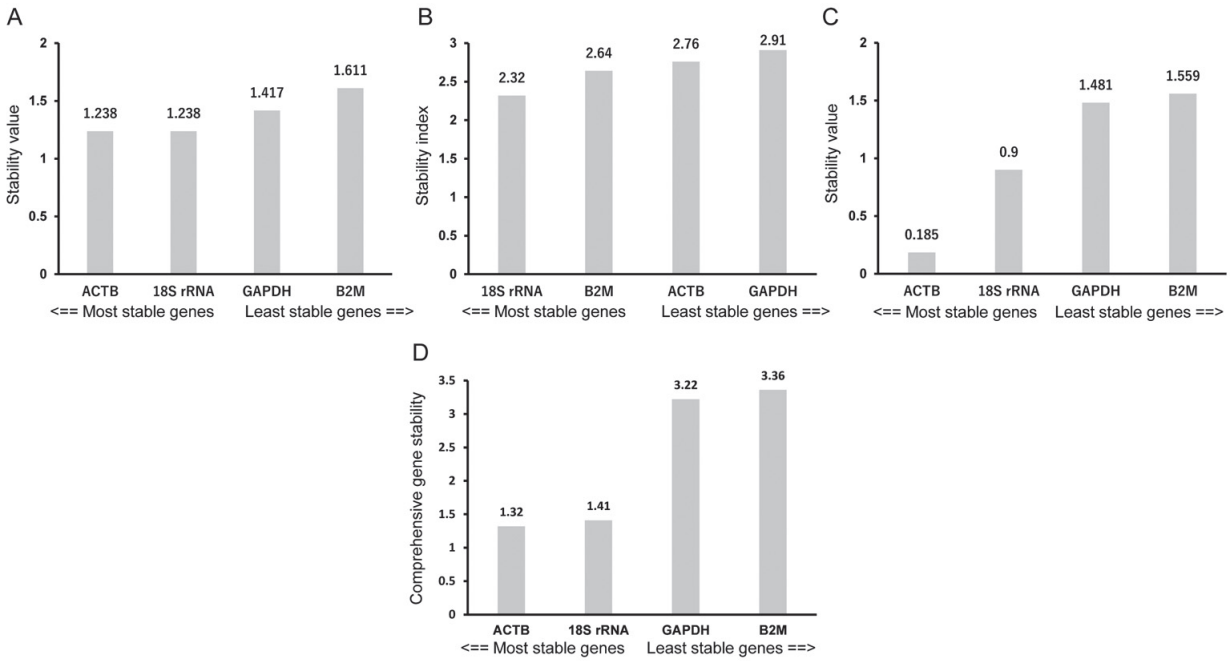

Fig. 3 Algorithmic stability analysis of four reference genes.
(A) geNorm, (B) BestKeeper, (C) NormFinder, (D) RefFinder

DISCUSSION

In this study, we compared the expression levels of four candidate reference genes and the alteration of their expression levels due to vascular aging variables to establish a reference gene unaffected by factors related to vascular aging by RT-qPCR using whole saliva samples. The results showed that 18S rRNA had the highest expression and was unaffected by vascular aging variables. In addition, algorithms using geNorm, BestKeeper, NormFinder, and RefFinder indicated that ACTB and 18S rRNA are reference genes with stable expression. These results suggest that ACTB and 18S rRNA are useful reference genes for RT-qPCR using saliva samples from participants with vascular aging and that the expression of reference genes may not be affected by blood pressure, BMI, sex, baPWV, or age.

In the present study, there was a significant difference ($p<0.001$) between the Ct values of 18S rRNA and those of the other reference genes. Significant differences in the Ct values of 18S rRNA and GAPDH in saliva samples have been previously reported[22] and are consistent with the results of the present study. Ostheim *et al*.[23] also reported that the expression of ACTB, B2M, and GAPDH is not associated with demographic characteristics, such as sex and age.

Saliva contains information used in the diagnosis of oral and systemic diseases, such as DNA, mRNA, miRNA, and proteins, which have been reported to be useful in the diagnosis of autoimmune diseases, cardiovascular diseases, and stress[24]. Saliva samples are gaining attention as alternatives to blood samples because of their simplicity and low invasiveness. Our ultimate goal was to develop a saliva-based diagnosis of vascular aging. To the best of our knowledge, this is the first study to find a stable reference gene that is unaffected by variables related to vascular aging.

The four genes used in this study were adopted as reference genes because they are universally present in eukaryotes and exhibit stable expression levels. ACTB is a cytoskeletal gene involved in cell motility and cell division; GAPDH is an enzyme involved in glycolysis, DNA replication, and apoptosis; B2M is a component of the major histocompatibility class I complex human leukocyte antigen and is involved in immune responses; 18S rRNA is a component of the ribosomal small subunit and is involved in protein synthesis. Variations in the expression of these reference genes have been previously reported, depending on the tissue, cell, inflammatory response, and sample processing conditions[25-28]. Therefore, when confirming gene expression using RT-qPCR, it is necessary to identify useful genes from multiple candidate reference genes. In this study, ACTB and 18S rRNA were suggested to be useful reference genes for saliva samples from participants with vascular aging. The transcription of DNA to RNA was performed using RNA polymerase. rRNA is transcribed by polymerase I, whereas mRNA is transcribed by polymerase II; therefore, both RNAs expression mechanisms may vary. In addition, because rRNA is more abundant in cells than mRNA, when rRNA is used as an internal standard for PCR, its expression level must be confirmed to ensure that it is not affected by experimental conditions. Furthermore, the oligo(dT) primer is specific for the poly(A) tail and therefore, cannot be applied to rRNA. The reason why

different reference genes were identified as the most stable genes in Fig. 3 may be due to different algorithms. Therefore, we verified the stability of the genes using multiple algorithms. The comprehensive overall ranking of expression stability was ACTB across all algorithms, followed by 18S rRNA. ACTB can be used as a reference gene without any restrictions on the type of reverse transcriptase compared to 18S rRNA, however, the Ct value may be higher and less reliable. On the other hand, a comparison of expression levels showed that 18S rRNA was more highly expressed than ACTB, with a significant difference in the Ct value ($p<0.001$). ACTB had a Ct value>35 in some participants, whereas 18S rRNA had a Ct value<30 in all participants. Therefore, 18S rRNA is recommended as a stable reference gene for the saliva of participants with vascular aging in these limited experimental conditions.

The vascular aging of participants, as indicated by baPWV and carotid plaque, was observed in an early stage. The mean age of the participants was 41.20±13.67 years, which did not include the generally defined older adult population aged over 65 years. Given that the expression of reference genes may be influenced by aging, it is necessary to confirm that the expression of these genes remains stable even in saliva collected from individuals older than 65 years.

Although this study was conducted using unstimulated saliva, many of the participants with vascular aging for the actual experiments were senior patients, and saliva collection might be difficult because of age-related decreases in saliva volume[1]. In such difficult cases, stimulated saliva should be collected. Since the composition of unstimulated and stimulated saliva is known to change[29,30], the expression of these genes might also change. Therefore, the changes in gene expression caused by the differences in collection methods must be verified. Basic Policy on Economic and Fiscal Management and Reform 2022[31], which was decided by the Cabinet in June, incorporated the concrete consideration of universal oral health checks. This system allows people of all generations to receive dental checkups throughout their lives. We aim to develop a diagnostic kit for vascular aging in human saliva, which may support this system, and aim to contribute to people's general health through a dental office.

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