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Notes

Direct Detection of Single Nucleotide Polymorphism (SNP) by the TaqMan PCR Assay Using Dried Saliva on Water-soluble Paper and Hair-roots, without DNA Extraction

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We have developed a new method for directly using unprocessed biological specimens as templates for the TaqMan assay. DNA extraction and purification had been believed to be required for the assay, but our new method could avoid hindering fluorescence detection, even if the templates were used directly. Saliva was needed to be put on water-soluble paper and dried, and hairs were cut to be about 10 mm long. This method could reduce both the time and effort involved, and also the risk of contamination. It should prove to be very valuable for genetic diagnoses in various fields.

Keywords Single nucleotide polymorphisms, TaqMan assay, saliva, hair

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Introduction

Single nucleotide polymorphisms (SNPs) have been associated with a predisposition to common diseases and individual variations in drug responses.[1,2] Various assays have been developed to analyze SNPs, such as direct DNA sequencing after polymerase chain reaction (PCR) amplification,[3] allele-specific primer-PCR (ASP-PCR),[4] PCR-restriction fragment length polymorphisms (PCR-RFLP),[5] and TaqMan PCR.[6] It is necessary to employ sample-preparation processes, including the extraction and purification of DNA, for all of these methods. General sample-preparation processes are labor-intensive and time-consuming, and it is costly to use simplified kits for large-scale studies. Recently, we developed simple SNP genotyping methods in which sample-preparation processes were eliminated. We succeeded to use terminal hair-root samples for an ASP-PCR genotyping method,[7] and dried blood samples spotted on general filter papers for a PCR-RFLP method.[8] We have now made the transition to the TaqMan assay as a more useful method for practical use, because it is the most preferred technology for large-scale association studies among the above methods. Since the TaqMan assay detects the fluorescence intensity of probes by laser scanning, it is thought that solids, such as filter paper and hairs, hinder the detection by blocking the laser scanning. We used water-soluble paper instead of general filter paper to avoid blocking the laser scanning. The new method described here is the first to be reported using unprocessed biological specimens directly as templates for the TaqMan assay. The general sample-preparation processes, including the use of proteases or denaturating agents, were totally eliminated by this method. This will be very useful for conducting large-scale association studies in various fields.

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Experimental

Subjects and samples

Forty-four healthy, non-pregnant female students at Mukogawa Women's University participated as volunteers in this study. The Medical Ethics Committee of Mukogawa Women's University approved the study protocol. Informed consent was obtained from all the subjects. The subjects were asked to rub their inner cheek surfaces by their teeth, and to collect saliva in small plastic tubes. Then, *ca.* 50 μL of saliva was put onto pieces of 30 mm^2 water-soluble paper (60MDP, Mishima Dissolve Paper, Nippon Paper Papylia Co., Ltd., Shizuoka, Japan), which were dried for one hour at room temperature. Hair-root samples were provided from 4 out of 44 students.

Genotyping

SNPs of the *ADH1B* (Arg47His; rs1229984) and *ALDH2* (Glu487Lys; rs671) genes were genotyped by the TaqMan assay on an ABI 7300 Real Time PCR System (Applied Biosystems). The mixture was 20 μL, and consisted of 10 μL of a Thunderbird Probe qPCR Mix (QPS-101, TOYOBO, Osaka, Japan), 0.4 μL of a 50×ROX reference dye (TOYOBO), 1 μL of a 20×ADH1B TaqMan Probe & ADH1B Primer Mix (C_2688467_20, TaqMan® Drug Metabolism Genotyping Assays, ABI) or a 20×ALDH2 TaqMan Probe & ALDH2 Primer Mix (C_11703892_10, ABI), 2 μL of KOD FX Neo Buffer (KFX-201, TOYOBO), and 6.6 μL of distilled water. The thermal cycling process was performed according to the Applied Biosystems PCR conditions: 2 min at 50°C, 10 min at 95°C, 40 cycles of denaturation at 95°C for 15 s, and annealing and

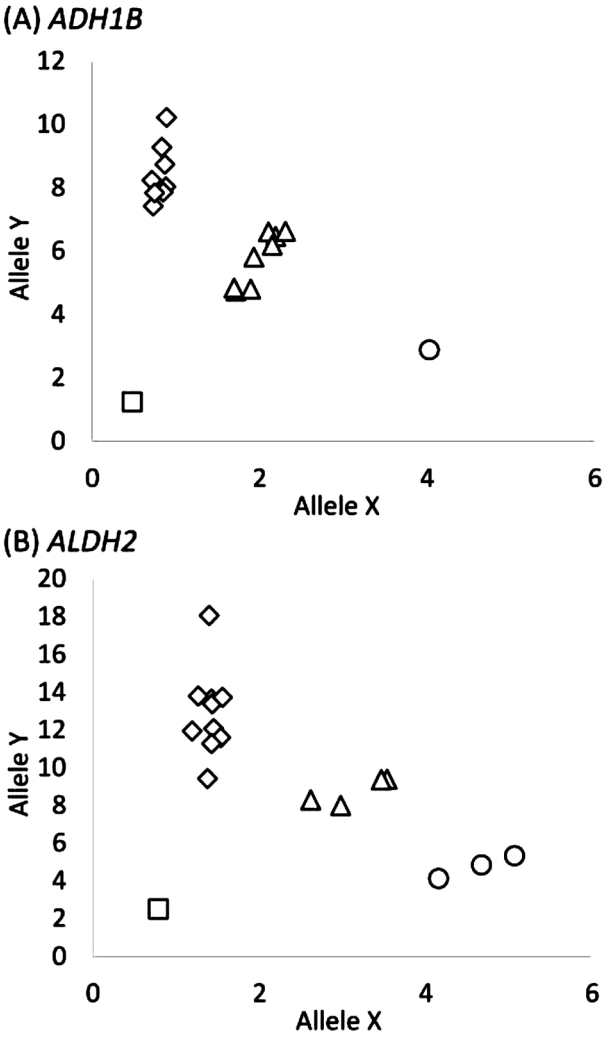

Fig. 1 Results of *ADH1B* and *ALDH2* genotypes among 17 out of 44 samples. (A) Results of *ADH1B*. The round shape indicated *ADH1B*1/*1*; the diamond shape indicated *ADH1B*2/*2*; the triangle shape indicated *ADH1B*1/*2*; the square shape indicated negative control. (B) Results of *ALDH2*. The round shape indicated *ALDH2*2/*2*; the diamond shape indicated *ALDH2*1/*1*; the triangle shape indicated *ALDH2*1/*2*; the square shape indicated negative control. The position of a given symbol was defined by the fluorescence reading obtained for the two fluorogenic probes. The X-axis represented the ratio of the fluorescence intensity for the allele-specific probe labeled with the VIC and ROX passive reference dye; the Y-axis represents the ratio of the fluorescence intensity for the allele-specific probe labeled with the FAM and ROX passive reference dye.

extension at 60°C for 1 min. The results were analyzed by ABI Prism 7300 SDS software. The dried saliva and hair-roots were used as templates. The dried saliva attached to a piece of water-soluble paper was cut into a 1.2-mm diameter disk and put into the reaction mixture directly. Four hair-root samples of one subject were cut into different lengths (15, 10, 6 and 4 mm from the hair-root end) and put into the reaction mixtures directly. Three hair-root samples were provided from subjects whose *ADH1B* genotypes were *1/*1, *1/*2 and *2/*2. They were analyzed, and their results were compared to those of saliva samples of the same subjects.

Table 1 Genotype distribution of *ADH1B* and *ALDH2* in student volunteers

Group	*ADH1B*	*ALDH2*	*n*
1	*1/*1	*1/*1	0
2	*1/*2	*1/*1	10
3	*2/*2	*1/*1	15
4	*1/*1	*1/*2	1
5	*1/*2	*1/*2	6
6	*2/*2	*1/*2	7
7	*1/*1	*2/*2	0
8	*1/*2	*2/*2	1
9	*2/*2	*2/*2	4
Total			44

Results and Discussion

Saliva samples on water-soluble paper were successfully genotyped by the TaqMan assay. The results of *ADH1B* and *ALDH2* genotypes of 17 out of 44 samples are shown in Fig. 1. The samples were divided into three different clusters. In the detection of *ADH1B*, the samples at position X-4.0/Y-3.0 along the horizontal axis were genotyped as homozygotes of allele *1. The cluster at position X-0.8/Y-9.0 along the vertical axis was genotyped as homozygotes of allele *2. The cluster at position X-2.0/Y-6.0 along the diagonal line was genotyped as heterozygotes of both the *1 and *2 alleles (Fig. 1A). Genotyping of *ALDH2* was performed in the same way (Fig. 1B). It was easy to identify three genotypes, since each group was clearly separated. We could not obtain accurate results when we used general filter paper (Qualitative Filter Paper No.1, ADVANTEC, Japan) instead of water-soluble paper (data not shown). When general filter paper was used, three genotypes were found to be very close, and it was hard to judge each genotype. The filter papers produced some noise in the optical system, and consequently the software regarded them as being fluorescence signals. Filter paper might scatter the laser beam, or some fluorescing substances might be eluted. Using filter paper eventually led to erroneous decision-making. On the other hand, the water-soluble paper did not affect the fluorescence detection. It was not detected as unwanted signals, because it is quite thin and started melting when it was put into the reaction mixtures. It was also revealed that the saliva attached to the water-soluble paper could hold enough DNA to be genotyped. The crude DNA from the saliva could be amplified, because we added the buffer of a DNA polymerase kit (KOD FX Neo) in order to obtain a high amplification efficiency.[7,8] The genotype distribution is given in Table 1. The same results were obtained by the PCR-RFLP method (data not shown).

We also confirmed whether hair-roots could be used for the TaqMan assay. Clear results were obtained using hair-roots, but their lengths affected the detection (Fig. 2). The 10 and 6 mm hairs were detected with stronger signals than other samples. The former two hairs were attached to the walls of the tubes completely, from the end of the hair-roots to the edge. The 15 mm hair was too long and the 4 mm hair was too short to be set vertically in the tubes, and they were not attached to the tube walls. In 15 and 4 mm hair mixtures, the water surfaces were not horizontal because of the hairs' locations and the surface tension. We revealed that it is necessary to make the hairs become attached to the wall of tubes so as to avoid any hindering the laser scanning. The length from 6 to 10 mm was

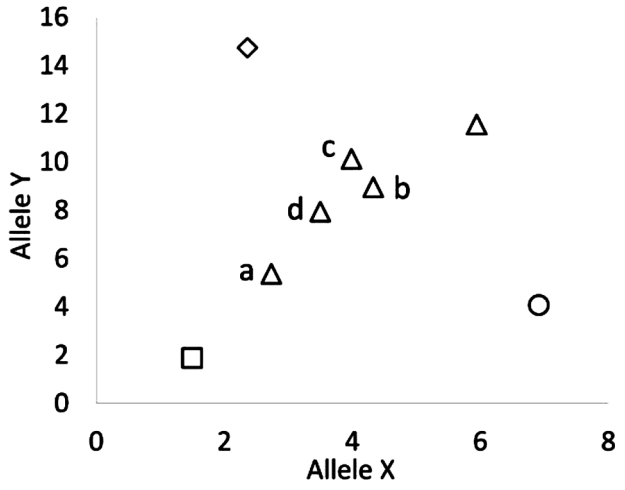

Fig. 2 Results of the *ADH1B* genotype of hair-root samples. The hair lengths from the roots were as follows: a) 15 mm, b) 10 mm, c) 6 mm, d) 4 mm. They all were from one subject who had the *1/*2 type of *ADH1B*. The unmarked round, diamond and triangle marks indicate positive controls of allele *1/*1, *2/*2 and *1/*2, respectively. The negative control is indicated by a square shape. The X-axis and the Y-axis are represented in the same way as in Fig. 1.

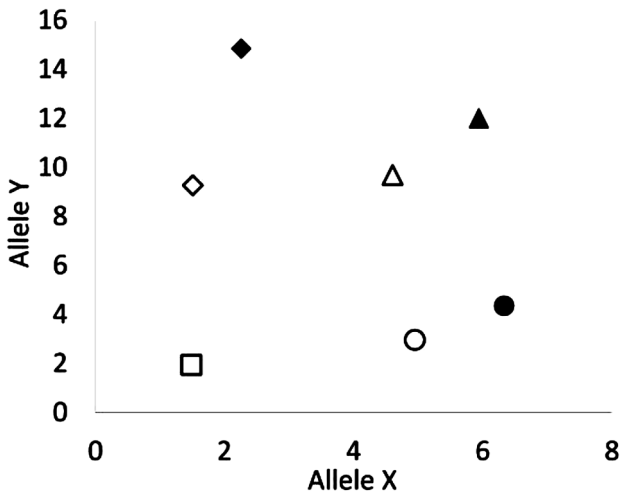

Fig. 3 Results of the *ADH1B* genotype of saliva samples and hair-root samples. The open marks are hair-root samples and the closed marks are saliva samples. The symbols and the axes have the same meanings as in Fig. 1.

suitable to be attached to the wall of reaction tubes and the hairs of these lengths demonstrated clear results.

The results of the hair-roots were compared with those of saliva samples. Both types of specimens were detected correctly by this method, but the saliva samples obtained stronger signals than the hair-roots (Fig. 3). The saliva samples could be stored at room temperature, and they were verified to maintain sufficient DNA to be genotyped for one year. It was much easier to handle them than other liquid biological specimens because they could be stored at room temperature, and stored compactly; further, they were easy to ship or carry. The risk of contamination was considerably decreased since the samples were solids. Furthermore, it is valuable to use saliva as a template in studies that have healthy subjects, since the sampling of saliva is noninvasive.

We developed this direct TaqMan PCR method that can use unprocessed saliva and hair-root samples as templates. DNA extraction was not required to use saliva samples attached to water-soluble paper and hair-root samples were cut to be about 10 mm long. This assay is the first to be reported as a true one-pot SNP genotyping method, because all of the reagents and the unprocessed saliva or hair-root samples were added simultaneously, and no additional steps were required before or after PCR. This method can reduce both the time and effort, as well as the risk of contamination. This high-throughput SNP genotyping method is suitable for practical use in clinical sites as well as in academic laboratories.

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