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Short Communication

SNP typing of aldehyde dehydrogenase2 gene with Cycleave ICAN

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ABSTRACT

A simple and non-expensive platform is critical to realize on-site SNP typing. In this study we typed an SNP existing at the 487th residue of human aldehyde dehydrogenase2 [wild: Glu (GAA); mutant: Lys (AAA)] using our unique isothermal DNA amplification method, ICAN and cycling probes. Both genotypes were identified by the naked eye using a non-expensive UV transilluminator as well as with real-time PCR apparatus or a fluorescence detector. Since ICAN does not need thermal cycling, a cost- and space-limiting factor when fabricating apparatus, the combination of ICAN and cycling probes will be able to realize affordable on-site SNP typing in the near future.

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1. Source/description

Aldehyde dehydrogenase2 (ALDH2) is an enzyme which acts on acetaldehyde, a metabolic intermediate of alcohol, to convert to acetate, and is also a bioactivator of nitroglycerin. A single nucleotide polymorphism (SNP) in exon 12 [487Glu (GAA)/wild; Lys (AAA)/mutant], which is known to influence the individual constitution related to drinking, can be a risk factor of cancer, especially oropharyngolaryngeal and esophageal cancer, caused by the uptake of excess alcohol, and may affect the efficacy of nitroglycerin in the treatment of angina pectoris [1–5]. Here we show that this SNP can be correctly typed by the combination of our unique DNA amplification method, isothermal and chimeric primer-initiated amplification of nucleic acids (ICAN), and cycling probes [6–8]. This method does not need thermal cycling, an essential prerequisite for PCR, and analysis was carried out with a non-expensive UV transilluminator. The SNP typing shown in this study offers a good base to realize affordable typing in the near future.

2. Cycleave ICAN

ICAN consists of a pair of 5′-DNA-RNA-3′ chimeric primers, thermostable RNaseH, and strand-displacing DNA polymerase. It allows the amplification of target DNA encompassed by the primers as in PCR, but unlike PCR, under isothermal conditions at around 55 °C [6]. In ICAN, a pair of chimeric primers specifically hybridizes to the template, DNA extension occurs from the 3′ end of the primer, RNaseH introduces a nick in the RNA portion, and strand-displacing DNA extension occurs from the nicked site. In some cases, a chimeric primer in the reaction solution hybridizes to the template soon after a new strand starts extending from the nicked site, leading to the occurrence of multiple priming on one strand (multi-priming) [7]. The two strands extending from both primers separate from the original templates at a certain ratio and switch templates to strands extending from opposite primers (template-switching), as well as reaching the 5′ end of the original template [7]. Template-switching generates reaction intermediates which can facilitate specific amplification of a primer-encompassed target sequence. Both multi-priming and template-switching occur simultaneously until more than half of the primers in the reaction mixture are used up, leading to a yield of at least several times greater than PCR. The cycling probe is composed of DNA residues with a few RNA residues in the middle, and is labeled with a fluorescent molecule and a quencher molecule at each end. When this probe is present in the ICAN reaction mixture, it is cleaved and emits a fluorescent signal, as depicted in Fig. 1, and the signal intensifies as the amount of the amplified fragment increases. In this method, which we call Cycleave ICAN, RNaseH splits a hybridized probe to emit a fluorescent signal as well as introducing a nick in chimeric primers to drive the amplification reaction. The cycling probe can strictly distinguish SNPs in an amplified fragment [9–11].

3. Genomic DNA extraction from oral mucosa

We performed ALDH2 typing using DNA extracted from the oral mucosa of twelve adults (10 males and two females aged from 25 to

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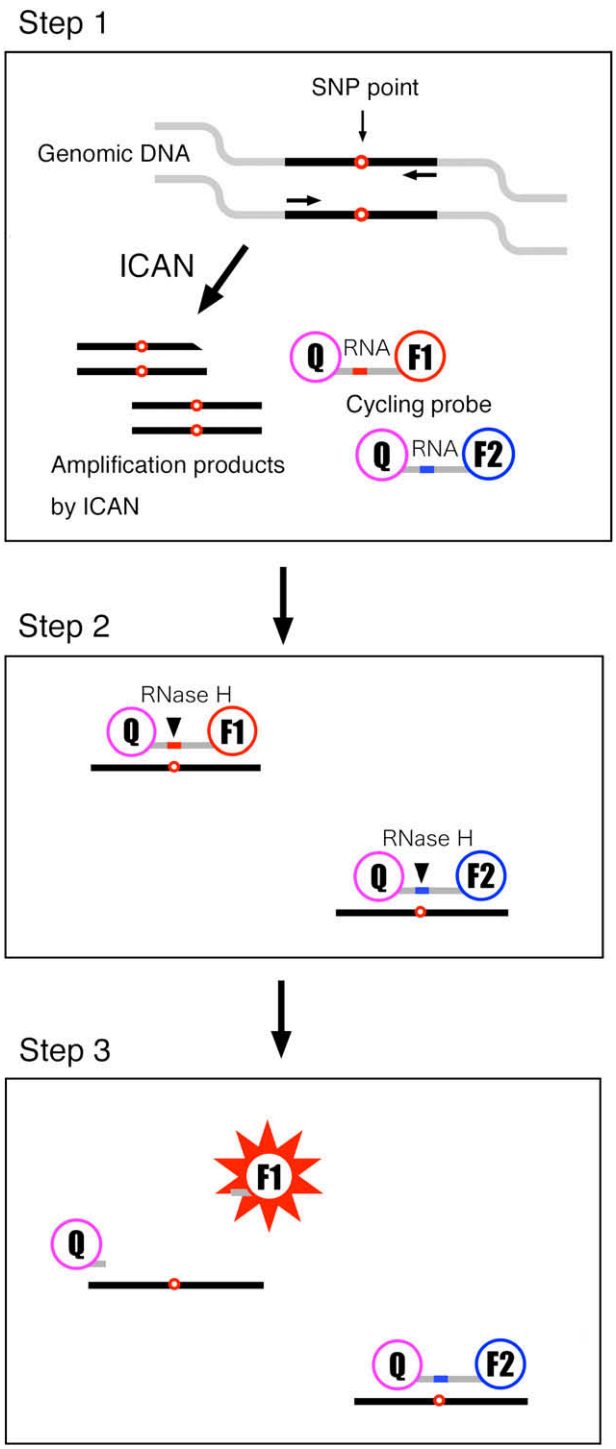

Fig. 1. Schematic representation of the principle of Cycleave ICAN. When the RNA residue of a cycling probe can form the correct hydrogen bond with the corresponding residue in the amplification product of ICAN (shown in red), the probe is cleaved with RNaseH and a fluorescent signal is emitted as a fluorescent dye and quencher molecule separate from each other.

51) as a template with Cycleave ICAN after obtaining their informed consent. We performed the same typing with real-time PCR for comparison. First, we prepared genomic DNA from oral mucosa obtained from the buccal surface with the procedure described below. Cells of the oral mucosa were collected with a Catch-All Sample Collection Swab (Epicentre, Madison, WI, USA) and suspended in 180 μl of PBS in a microtube. The suspension was mixed with 20 μl of proteinase K solution and 180 μl of lysis buffer in the product and incubated at 70 °C for 10 min, and 240 μl of 99.5% ethanol was added. The obtained lysate was applied to the cartridge of a FastPure®DNA Kit (Takara Bio Inc., hereafter abbreviated to TBI, Otsu, Shiga, Japan). The cartridge was washed twice and DNA was eluted with the corresponding buffers included in the product. The eluted DNA solution was used as a template for amplification with either Cycleave ICAN or real-time PCR (template DNA).

4. SNP typing with Cycleave ICAN and real-time PCR

For Cycleave ICAN, a mixture containing 20 pmol each of the chimeric forward (5′-AGTTGGGCGAGTACGGGcug-3′) and reverse (5′-CAGACCCTCAAGCCCCAaca-3′) primers, 10 pmol each of the cycling probe for the wild (ROX-GGCATACACTgAAG-Quencher) and mutant (FAM-GGCATACACTaAAG-Quencher) type, 0.4–16.2 μg of template DNA, 22 units of *Bca*BEST DNA polymerase, and 100 units of Tli RNaseH were added to make up to 25 μl reaction mixture. In parentheses, deoxyribonucleotides and ribonucleotides are shown in upper and lower case letters, respectively. The final composition of the reaction mixture was 32 mM Hepes–KOH buffer (pH 7.8), 100 mM potassium acetate, 0.11% bovine serum albumin, 1% dimethyl sulfoxide, 0.04% propylendiamine, 0.6 mM of each dNTP, and 5 mM $Mg(OAc)_2$. The reaction mixtures were kept at a constant temperature of 60 °C for 60 min with real-time PCR apparatus (Thermal Cycler Dice® Real Time PCR System, TBI) and DNA amplification was monitored by measuring the fluorescent signals of carboxyfluorescein (FAM) at 536 nm and 6-carboxy-X-rhodamine (ROX) at 624 nm. Real-time PCR was performed with 10 pmol each of the forward (5′-CAGGGTCAACTGCTATGATGT-3′) and reverse (5′-AGCCCCCAACAGACCCCAATC-3′) primers in 25 μl reaction mixture using a CycleavePCR® Core Kit (TBI). The same cycling probes as in the Cycleave ICAN were used for real-time PCR at the same concentration. The thermal cycling condition was 95 °C for 10 s followed by 45 cycles of thermal shift at 95 °C for 5 s, 55 °C for 15 s, and 72 °C for 15 s with the same PCR apparatus used for Cycleave ICAN. It took 90 min for Cycleave ICAN and 110 min for real-time PCR, counting from the preparation of DNA from oral mucosa to the end of the amplification reaction. The result of Cycleave ICAN obtained in real time with an "isothermal block" in the PCR apparatus showed that eight of twelve samples (A, D, E, F, I, J, K, L) were the wild type and the remaining four (B, C, G, H) were the hetero-mutant type (Fig. 2). These results were coincident with those of real-time PCR, suggesting that Cycleave ICAN enables differentiation between wild and hetero-mutant types with a real sample, i.e., genomic DNA from oral mucosa, when the shift of relative fluorescent signal intensity is monitored in real time throughout the whole reaction.

5. End-point assay with Cycleave ICAN

Next, we tested whether Cycleave ICAN allowed correct typing by measuring the absolute intensity of fluorescent signal only at the end point of the reaction instead of monitoring the whole reaction in real time. If this is possible, typing can be performed less expensively than with real-time monitoring. After the same reaction of Cycleave ICAN as described in the previous section was carried out for 1 h in an air incubator, fluorescence intensity was measured with a fluorescence detector, Fluoroskan Ascent FL (Labsystems, Waltham, MA). For the wild-type probe with ROX dye, the signal intensity measured at the wavelength of 612 nm ranged from 1.3 to 3.9 for all of the wild and hetero-mutant (positive) samples, and signal intensity, measured in the same way, was 0.4 for the double mutant (negative) control (column "Wild-type probe" in Table 1). For the mutant-type probe with FAM dye, signal intensity measured at the wavelength of 538 nm was either 1.3 or 1.4 for hetero-mutant (positive) samples, 1.5 for the double mutant

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