Article

Detection of c.375A>G, c.385A>T, c.571C>T, and sedel2 of FUT2 by real-time PCR in a single tube

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Abstract: The α(1,2)fucosyltransferase (Se enzyme) encoded by *FUT2* is involved in the secretor status of ABH(O) blood group antigens. The se^{del2} allele is one of the non-functional *FUT2* (*se*) alleles in which 9.3 kb containing the entire coding region of *FUT2* is deleted by *Alu*-mediated nonhomologous recombination. In addition to this allele, three SNPs of *FUT2*, c.375A>G, c.385A>T, and c.571C>T, appear to be prevalent in certain Oceanian populations such as Polynesians. Recently, we developed an endpoint genotyping assay to determine the se^{del2} zygosity, using a FAM-labeled probe for detection of the se^{del2} allele and a VIC-labeled probe for detection of *FUT2*. In this study, instead of the VIC probe, a HEX-labeled probe covering both c.375A>G and c.385A>T and a Cy5-labeled probe covering c.571C>T were added to the se^{del2} allele assay mixture to allow simultaneous detection of these four variations by endpoint genotyping for the se^{del2} zygosity and fluorescence melting curve analysis for c.375A>G, c.385A>T, and c.571C>T genotyping. The results obtained from 24 Samoan subjects with this method were identical to those obtained with previous methods. Therefore, it appears that present method can accurately determine these four variations simultaneously.

Keywords: *Alu*-mediated nonhomologous recombination; *FUT2*; se^{del2}; c.375A>G; c.385A>T; c.571C>T; Polynesian population

1. Introduction

The α(1,2)fucosyltransferase (Se enzyme) encoded by *FUT2* is involved in the secretor status of ABH(O) blood group antigens [1-3]. Secretors carry at least one functional *FUT2* (*Se*) allele, which encodes the active Se enzyme, and consequently expresses ABH antigens in their secretions. In contrast, individuals with only the Se enzyme-deficient (*se* or Se^{w}) alleles either do not express (non-secretors) or express these antigens in their secretions weakly (weak-secretors) [3]. *FUT2* is located in the region of chromosome 19q13.3, together with a pseudogene (*SEC1P*) with high sequence similarity [4,5].

Several single nucleotide polymorphisms (SNPs) of *FUT2* have been identified [6]. Among these, the nonsense SNP at c.428G>T (W143X, rs601338) is responsible for Se enzyme inactivation of the se^{428} allele, which is found at approximately 50% frequency in Europe, Africa, West Asia [6]. On the other hand, the missense SNP at c.385A>T (I129F, rs1047781) is responsible for partial inactivation of the Se enzyme in a representative Se^{w} allele [6]. This allele is present in East and Southeast Asians at about 50% frequency and is also common in certain Oceania populations such as Polynesians [7]. The missense SNP at c.302 C>T (I101P, rs200157007) constitutes the se^{302} allele that is common in South Asian populations [6]. The nonsense SNP at c.571C>T (R191X, rs1800028) constitutes the se^{571} allele that is relatively common in Polynesian and Taiwanese populations [7-10]. The synonymous SNP at c.375A>G (rs1800026) has been found in certain New Guineans (Melanesians) with relatively high frequency (more than 20%) and in Africans and Samoans (Polynesians) with relatively low frequency (about 2%) [7,11].

In addition, five *se* alleles resulting from non-allelic homologous recombination have been identified and shown to have a population-specific distribution [7,12]. The se^{fus} allele was due to recombination between *SEC1P* and *FUT2* and seems to be specific to the Japanese population [13], whereas the se^{del}, se^{del2}, se^{del3}, and se^{del4} alleles are deletions of the entire coding region of *FUT2* resulting from recombination of two interspersed repeat elements [7,12]. The se^{del} allele is a 10-kb deletion due to recombination between two *Alu* elements and appears to be characteristic of South Asian populations with frequencies ranging from 10–20%, while the se^{del2} allele is a 9.3-kb deletion due to recombination between

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two *Alu* elements different from the *se*^{del} allele and appears to be characteristic of certain Oceanian populations such as Samoans (Polynesians) and New Guineans (Melanesians) with frequencies ranging from 10–20% [7,11]. The *Alu* elements are the most abundant repeat elements, each approximately 300 bp in length, and occupy about 10% of the human genome. Based on its abundance and sequence similarity, the *Alu* elements are thought to be involved in genomic rearrangements in the human genome [14-16]. The *se*^{del3} and *se*^{del4} alleles are very rare, currently found in only one Chinese and one Peruvian [12].

TaqMan assays using dual-labeled fluorescence oligonucleotide probes (TaqMan probes) allow real-time PCR monitoring for DNA and RNA quantification and detection of SNPs [17]. This is because when the probe hybridizes to the complementary target DNA, it is cleaved by the 5'-3' exonuclease activity of *Taq* polymerase, causing the quencher to separate from the fluorophore and emit fluorescence [17]. Recently we developed an endpoint genotyping assay using two TaqMan probes which are a FAM-labeled probe for detection of the *se*^{del2} allele and a VIC-labeled probe for detection of the *FUT2* coding region [12]. The TaqMan probes are also available for fluorescence melting curve analysis (FMCA) to detect SNPs [18-21]. FMCA is one of the most robust SNP detection methods [18]. Unlike the TaqMan assay, it would be desirable for FMCA to have no degradation of the fluorescent probe. However, it has been reported that both *Taq* polymerases with and without 5'-3' exonuclease activity are suitable for FMCA under asymmetric PCR conditions, probably because suppressing primer extension of the sense strand of the probe inhibits probe hydrolysis [19].

In this study, instead of a VIC-labeled probe, a HEX-labeled probe covering c.375A>G and c.385A>T and a Cy5-labeled probe covering c.571C>T were added to the assay mixture of the FAM-labeled probe for detection of the *se*^{del2} allele, allowing determination of the *se*^{del2} zygosity by the endpoint genotyping, and three SNPs of *FUT2* by FMCA could be identified in a single tube.

2. Materials and Methods

The research protocol was reviewed and approved by the ethical committee of Kurume University School of Medicine (approval No. 22158).

2.1 DNA samples

Genomic DNA from 24 Samoans in Apia was used. Their *se*^{del2} zygosity and all SNPs in the *FUT2* coding region have been previously determined by conventional PCR for amplification of the 2.6-kb junction region of the *se*^{del2} allele and direct Sanger sequencing of PCR products [7].

2.2 Probes and primers used in this study

The nucleotide position numbers for *FUT2* and *SEC1P* follow those reported by Kelly et al. [4]. Because *FUT2* and *SEC1*P have high DNA sequence similarity we should select primers of *FUT2* that will not amplify *SEC1* [4].

For detection of the *se*^{del2} allele, we used the FAM-labeled hydrolysis probe (sedel2-probe; 5'-FAM-CCAGTCTGGCCAACAT-MGB-3') and a set of primers (sedel2-F primer; 5'- CCGCAATAGAAAGACGTGGA-3' and sedel2-R primer; 5'- CCAGGTTCAAGCGATTCTTC-3') that were the same as those described previously [12]. The primers and probes for detection of c.375A>G, c.385A>T, and c.571C>T are indicated in Table 1 and Figure 1. Primers for amplification of the *FUT2* fragment surrounding c.571C>T were designed using Primer3Plus (https://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi) [22]. Either a HEX-labeled 385-probe, identical with 411–435 bp of *SEC1P* and 369–393 bp of 385A allele of *FUT2* but one base different from the 385T allele and 375G allele (wild-type, 385A allele with 375G substitution), or a HEX-labeled 375-probe identical with the 411-435 bp of *SEC1P* and 369-393 bp of 375G allele of *FUT2* but one base different from the wild-type, 385A allele and two bases different from the 385T allele, was used for detection of c.375A>G and c.385A>T. In addition, a Cy5-labeled FUT2-571C probe was used for detection of c.571C>T. We also compared FMCA using a 281-bp PCR amplicon from 337 to 617 bp of *FUT2* and 76-bp PCR amplicon from 337 to 412 bp of *FUT2* plus a 79-bp amplicon from 539 to 617 bp of *FUT2*.

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Table 1. Primers and probes for detection of c.375A>G, c.385A>T and c.571C>T of *FUT2*.

Primer sequences	Position of *FUT2*	Position of *SEC1P*	Differences with *SEC1P*	Amplicon length
Detection of 375A>G, 385A>T				
FUT2-337F: 5′-TGGCAGAACTACCACCTGAA-3′	337 to 356 bp	379 to 398 bp	0	76 bp
FUT2-412R: 5′-CGGTGAAGCGGACGTACT-3′	395 to 412 bp	437 to 454 bp	6	
385-probe:5'-HEX GGAGGAATACCGCCACATCCCGGGG-BHQ1-3'	369 to 393 bp	411 to 435 bp	1	
375-probe:5'-HEX GGAGGAGTACCGCCACATCCCGGGG-BHQ1-3'	369 to 393 bp	411 to 435 bp	0	
Detection of 571T>C	539 to 556 bp	581 to 598 bp	5	79 bp
FUT2-531F:5′- GGCCGGGCACCTTTGTAG-3′	598 to 617 bp	640 to 659 bp	5	
FUT2-617R: 5'-ACCACCCCCTTCCACACTTT-3'	558 to 582 bp	600 to 624 bp	3	
571C-probe: 5′-Cy5-GGTCCATGTTCGCCGAGGGGACTAT-BHQ2-3′	539 to 556 bp	581 to 598 bp	5	
Detection of 375A>G, 385A>T, 571T>C				
FUT2-337F: 5′-TGGCAGAACTACCACCTGAA-3′	337 to 356 bp	379 to 398 bp	0	281 bp
FUT2-617R: 5'-ACCACCCCCTTCCACACTTT-3'	598 to 617 bp	640 to 659 bp	5	
375-probe and 571-probe				

"Differences with *SEC1P*" indicates the number of base differences in each of primer or probe with corresponding *SEC1P* sequences.

FUT2-337F primer and FUT2-412 R primer: same primers as previously used for 385A>T detection by unlabeled probe HRM analysis [23].

385-probe: same probe (TaqMan probe for c.385A>T, labeled by 5′-HEX and 3′-BHQ1) as previously used for detection of c.385A>T [24].

375-probe: same probe (formerly named SEC1P-FUT2 probe) as previously used for simultaneous detection of *se*^{fus} and c.385A>T [13].

BHQ: black hole quencher.

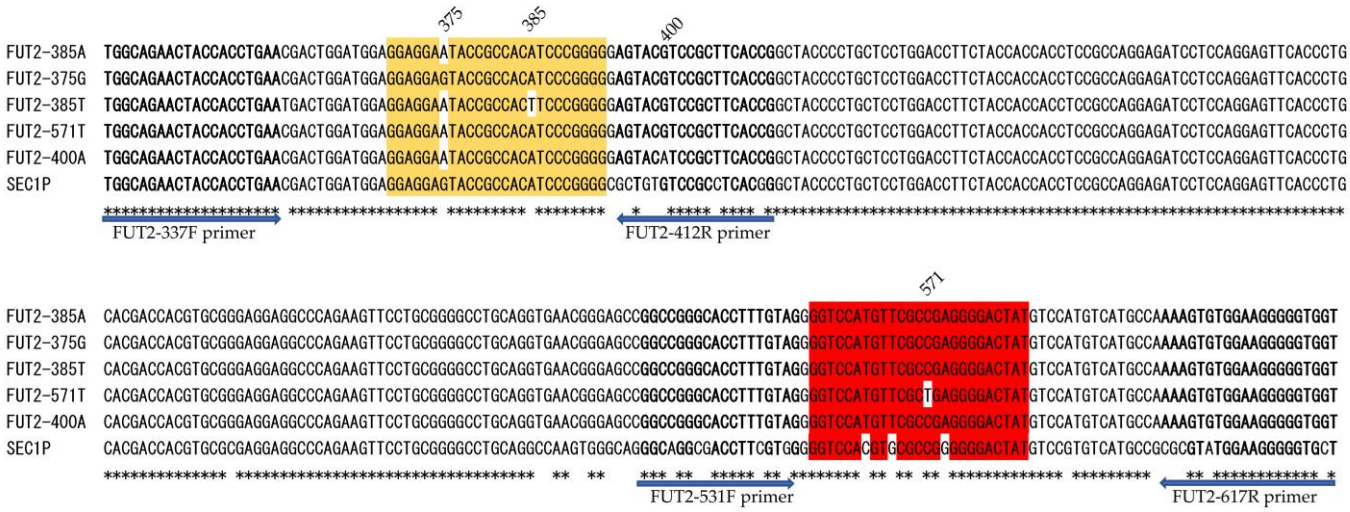

Figure 1. Alignment of DNA sequences of five *FUT2* alleles (FUT2-385A, -375G, -385T, -571T, -400A) and corresponding regions of *SEC1P* are indicated. Primer sequences are shown in bold, the 375-probe sequence is highlighted in orange and the 571-probe sequence is highlighted in red. Mismatched nucleotides in each probe are shown without highlighting. The positions and directions of the primers are indicated by arrows below the alignment. An asterisk shows an identical nucleotide among the six sequences.

2.3 Probe selection for detection of c.375A>G and c.385A>T by FMCA

To select probes for detection of c.375A>G and c.385A>T, asymmetric real-time PCR and FMCA were performed with the following reaction mixture (10 μL total): 5 ng genomic DNA, 5 μL of Premix Ex Taq™ (Probe qPCR) (Takara, Tokyo, Japan) containing *Taq* polymerases with 5'-3' exonuclease activity, 50 nM FUT2-337F primer, 500 nM FUT2-412R

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primer, and 200 nM HEX-labeled 385-probe or HEX-labeled 375-probe. FUT2-337F and FUT2-412R primers are identical to those used for 385A>T detection by unlabeled probe HRM analysis [23].

2.4 Evaluation of the number of amplicons in genotyping of c.375A>G, c.385A>T, and c.571C>T of FUT2 by FMCA

To compare one amplicon and two amplicons in genotyping of c.375A>G, c.385A>T, and c.571C>T of *FUT2* by FMCA, asymmetric real-time PCR and FMCA were performed with the following reaction mixture (10 μL total): 5 ng genomic DNA, 5 μL of Premix Ex Taq (Probe qPCR), 200 nM HEX-labeled 375-probe, and 200 nM Cy5-labeled 571 probe. In addition, 50 nM FUT2-337F primer and 500 nM FUT2-617R primer were included for the 281 bp amplicon. Alternatively, 50 nM FUT2-337F primer and 500 nM FUT2-412R primer for the 76 bp amplicon and 50 nM FUT2-531F primer and FUT2-617R primer for the 79 bp amplicon were included.

2.5 Detection of c.375A>G, c.385A>T, and c.571C>T by FMCA and detection of se^{del2} by endpoint genotyping in a single tube

To detect c.375A>G, c.385A>T, c.571C>T, and the se^{del2} allele in a single tube, real-time PCR, FMCA, and endpoint genotyping were performed with the following reaction mixture (10 μL total): 5 ng genomic DNA, 5 μL of Premix Ex Taq (Probe qPCR), 50 nM each of sedel2-F and sedel2-R primers, and 100 nM of sedel2-probe, 50 nM FUT2-337F primer, and 500 nM FUT2-617R primer, 200 nM HEX-labeled 375-probe, and 200 nM Cy5-labeled 571 probe.

2.6 Real-time PCR monitoring, FMCA, and endpoint genotyping

In this study, real-time PCR monitoring, FMCA, and endpoint genotyping were all performed using the LightCycler 480 instrument II (Roche Diagnostics, Tokyo, Japan). The thermal conditions for all PCRs were identical: preheating at 95°C for 30 sec, followed by 45 cycles of denaturation at 95°C for 5 sec, and annealing/extension at 63°C for 15 sec. The fluorescence data were collected at the end of the annealing/extension step of each cycle using a FAM (465 nm excitation and 510 nm emission), VIC/HEX/Yellow 555 (533 nm excitation and 580 nm emission), and/or Cy5/Cy5.5 (618 nm excitation and 660 nm emission) filters.

For melting curve genotyping, PCR products were heated to 95°C for 1 min, rapidly cooled to 40°C for 1 min, and then the fluorescence data were collected over the range from 50 to 80°C, increasing at 0.10°C/sec with 2 to 6 acquisitions/sec using the same filters. Endpoint genotyping, melting curve genotyping, and melting temperature (Tm) calculation were performed using LightCycler 480 gene scanning software with default settings.

3. Results

3.1. Probe selection for genotyping of c.375A>G and c.385A>T by FMCA

As mentioned above, in Oceanian populations including Samoans, a synonymous SNP at c.375A>G of FUT2 has been observed. In fact, one of our Samoan subjects is heterozygous for c.375A>G [7]. We first examined whether c.385A>T and c.375A>G could be separated using a 25 bp hydrolysis (TaqMan) probe, 385-probe, for the same sequence as the 385A (wild-type) allele used previously [24]. As shown in Figure 2A, the Tm value of the 385A allele was around 72°C and clearly separated from the 385T allele and the 375G allele, however, the Tm values of the 385T and 375G alleles were both around 67°C when using this probe. Although the peak height of the 375G allele seems to be slightly lower than that of the 385T allele, accurate separation is considered difficult. Therefore, we next examined the 375-probe, which has the same sequence as the 375G allele of FUT2 and SEC1P previously used for simultaneous detection of 385A>T and the se^{fus} allele [13]. The 385A allele peak with a Tm around 68°C could be separated from the 375G allele peak with a Tm around 74°C and the 385T allele peak with a Tm around 62°C (Figure 2B). Although the peak with a Tm around 74°C might also be generated if a corresponding region of the SEC1P was amplified, the primers used in this study amplify only FUT2 and not SEC1P, allowing us to examine the presence of the 375G allele and separate it

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from the 385A and 385T alleles. Therefore, in this study, we used the 375-probe instead of the 385-probe for detection of c.375A>G and c.385A>T.

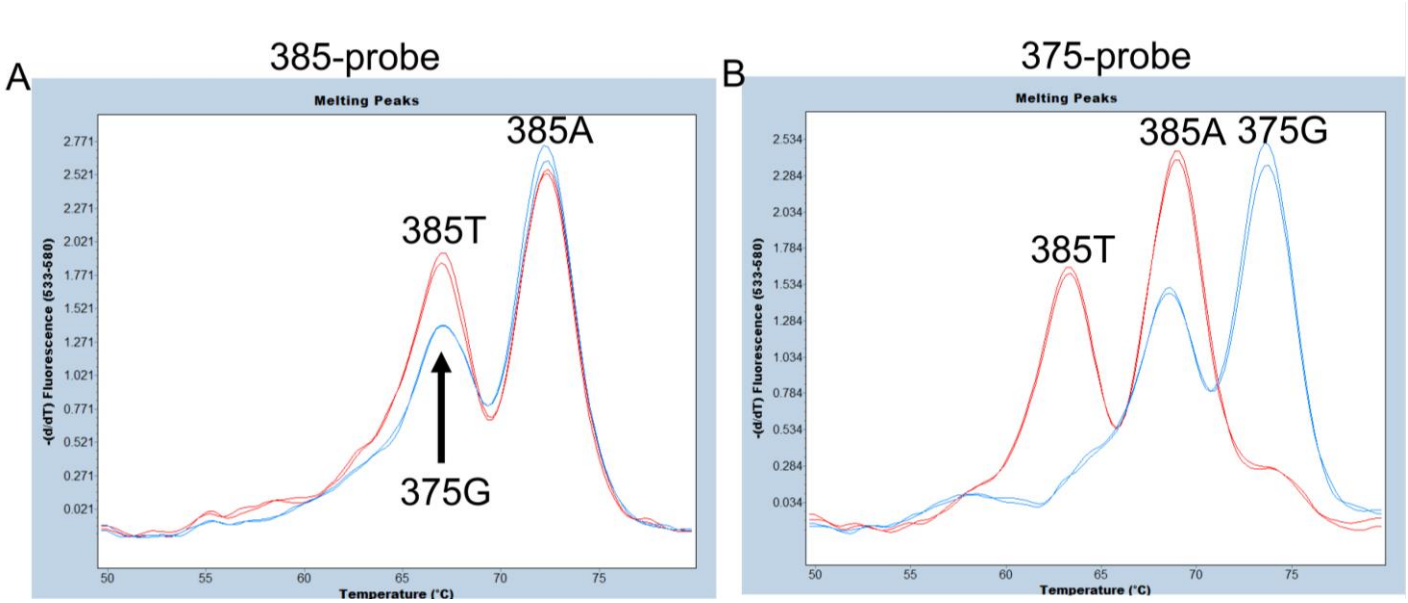

Figure 2. Melting peak profiles of the 385-probe (A) and of the 375-probe (B) for heterozygote of c.385A>T (385A/T) (red) and c.375A>G (375G/385A) (blue). Duplicated result using two samples were shown. (A) The peak height of 375G seems to be slightly lower than that of 385T, but accurate separation is considered difficult using a 385-probe. (B) The three melting peaks corresponding to 375G, 385A, and 385T alleles were completely separated using the 375-probe.

3.2 Evaluation of the number of amplicons in genotyping of c.375A>G, c.385A>T, and c.571C>T of FUT2 by FMCA

We then compared FMCA results using a 281 bp PCR amplicon from 337 to 617 bp of *FUT2* (one amplicon) and a 76 bp PCR amplicon from 337 to 412 bp of *FUT2* plus a 79 bp amplicon from 539 to 617 bp of *FUT2* (two amplicons). The primers used for PCR amplification are listed in Table 1. As shown in Figure 3A, we were able to separate the 571C allele signal with Tm around 72°C from the 571T allele signal with Tm around 64°C, and the peak heights of the 571-probe obtained with two amplicons and those with one amplicon are almost equivalent. Although none of the 24 Samoans are 571T/T subjects, one 571T hemizygote (571T/se^{del2} subject) showed only one melting peak corresponding to 571T. The melting peak pattern of this subject is considered identical to that of the 571T/T subject. Therefore, it would be possible to detect the 571T/T subject. On the other hand, the peaks of the 375-probe obtained with the two amplicons were higher than those obtained with one amplicon, but smaller peaks were observed with the two amplicons that were almost identical to the Tm value of the 375G allele. In addition, as shown in Figure 1, the FUT2-412R primer for two PCR amplicons contains a c.400G>A SNP (V134I, rs370886251), which is relatively common in Melanesians such as New Guineans and is rarely present in peripheral populations such as Polynesians but almost always absent in other populations [7,11]. In fact, one of the 24 Samoans is heterozygous for 400G/A (G is on 385T allele and A is on 385A allele) and the peak height corresponding to 385A is quite low in this subject when using two amplicons by FMCA (Figure 3C) probably due to a single base mismatch in the FUT2-412R primer. Therefore, there would be a risk of misdiagnosis with

a homozygote 385T allele of this subject. For these reasons, we decided to perform the FMCA with one amplicon instead of two amplicons in this study.

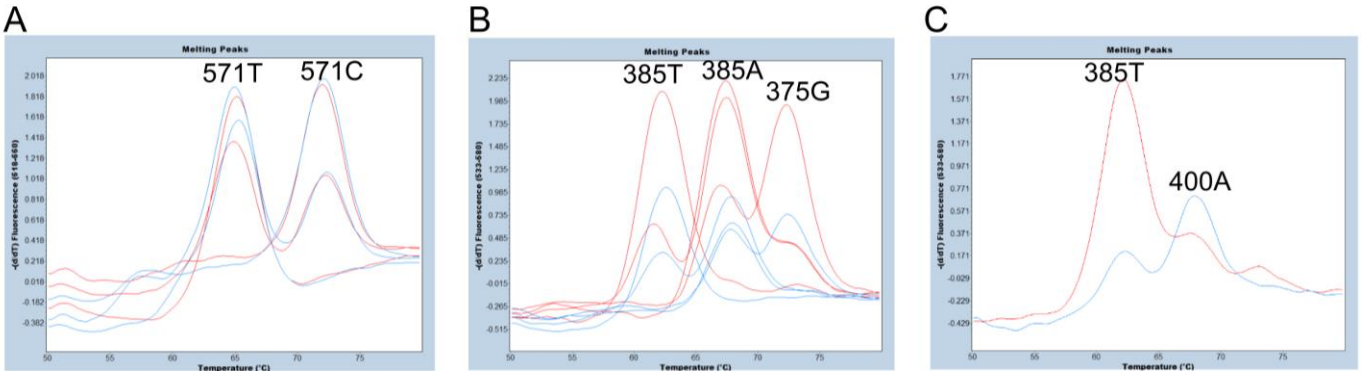

Figure 3. Melting peak profiles of the 571-probe (A) and the 375-probe (B and C) for selected subjects using one or two amplicons. (A) Melting peak profiles of the 571-probe obtained from 571C/C, 571C/T, 571T/*se*^{del2} subjects with two amplicons (red) and those with one amplicon (blue). The peak heights obtained with two amplicons and those with one amplicon are almost equivalent. (B) Melting peak profiles of the 375-probe obtained from 385A/A, 385A/T, 385T/T, and 385A/375G subjects with two amplicons (red) and those with one amplicon (blue). The peak heights obtained with two amplicons are higher than those with one amplicon. (C) Melting peak profiles of the 375-probe obtained from one heterozygote of 400G/A with two amplicons (red) and one amplicon (blue). The peak height corresponding to 385A (400A allele) is quite low in this subject when using two amplicons while the melting peak profile is similar to that of 385A/T heterozygote when using one amplicon.

In addition, as shown in Figure 1, the FUT2-412R primer for two PCR amplicons contains a c.400G>A SNP (V134I, rs370886251), which is relative common in Melanesian such as New Guineans and is rarely present in peripheral populations such as Polynesians but almost absent in other populations [7,11] . In fact, one of the 24 Samoans is heterozygous for 400G/A (G is on 385T allele and A is on 385A allele) and the peak height corresponding to 385A is quite low in this subject when using two amplicons by FMCA (Figure 3 C) probably because of a single base mismatch in the FUT2-412R primer. Therefore, there would be a risk of misdiagnosis with homozygote 385T allele of this subject. For these reasons, we decided to perform the FMCA with one amplicon instead of two amplicons in this study.

3.3. Genotyping of c.375A>G, c.385A>T, and c.571C>T of FUT2 by FMCA and endpoint genotyping of the se^{del2} *allele in a single tube*

Finally, we evaluated a simultaneous assay of FMCA genotyping of c.375A>G, c.385A>T, and c.571C>T of *FUT2* and endpoint genotyping of the *se*^{del2} allele using 24 Samoans whose *se*^{del2} zygosity and all SNPs in the *FUT2* coding region had been previously determined by direct sequencing of PCR products by Sanger sequencing [7]. As mentioned above, we clearly identified the 375G, 385A, and 385T alleles by the VIC/HEX/Yellow 555 filter (Figure 4A), and the 571C and 571T alleles by the Cy5/Cy5.5 filter (Figure 4B).

Figure 4. Melting peak profiles of the 375-probe (A) and of the 571-probe (B) for 24 Samoans. Duplicated results are shown. (A) The subjects with 385A/A (red), A/T (blue), and T/T (green) genotypes were completely separated. In addition, one 375G/385A heterozygote was clearly separated. Negative controls and one *se*^{del2} homozygote are indicated by light blue. (B) The subjects with 571C/C (blue), C/T (red), and T/T (green) genotypes were completely separated. Negative controls and one *se*^{del2} homozygote are indicated by light blue.

Endpoint genotyping was then performed using the same primer sets previously reported for detection of the *se*^{del2} allele and the HEX-labeled 375 probe or Cy5-labeled 571 probe instead of the FUT2-specific VIC probe for detection of the *FUT2* signal [12]. In the present assay, the *se*^{del2} allele was detected by a FAM-labeled probe and *FUT2* alleles were detected by a HEX-labeled probe or a Cy5-labeled probe. This method completely discriminated the three genotypes, one homozygote of the *se*^{del2} allele (only FAM signal), three heterozygotes of the *se*^{del2} allele (with FAM, HEX, and Cy5 signals), and 20 subjects without the *se*^{del2} allele (without FAM signal and with HEX and Cy5 signals) for 24 Samoans. Scatter plots of endpoint genotyping are shown in Figure 5A and B. The subjects with 385A/- (385A/*se*^{del2}) were separated from those with 385T/- (385T/*se*^{del2}) in a dual-color scatter plot of fluorescence signals because HEX/FAM signal ratios of

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385A/se^{del2} were approximately 3-fold higher than those of 385T/se^{del2} (Figure 5A). This may be because the 375-probe dissociates mostly from the T allele, whereas it binds completely to the A allele at 63°C, which is the temperature of annealing/extension that collects the real-time PCR fluorescence signal. On the other hand, no such differences were observed between the subjects with 571C/se^{del2} and 571T/se^{del2} (Figure 5B). At all events, the results of the se^{del2} zygosity determined by this method were also in perfect agreement with those by previous genotyping [7].

Figure 5. Endpoint genotyping of se^{del2}. Duplicated results for genomic DNA from 24 Samoans are shown. (A) The results of a dual-color scatter plot of fluorescence signals by 465–510 nm/533–580 nm. The subjects of wild types; without se^{del2} (green), se^{del2} heterozygotes; 385A/- (red), se^{del2} heterozygotes; 385T/- (pink), and se^{del2} homozygote (blue) were separated. Negative controls are indicated by gray. (B) The results of a dual-color scatter plot of fluorescence signals by 465–510 nm/618–660 nm. The subjects with wild types: without se^{del2} (green), se^{del2} heterozygotes; both 571C/- and 571T/- (red) and se^{del2} homozygote (blue) were separated. Negative controls are indicated by gray.

4. Discussion

Recently, we developed endpoint genotyping to determine the se^{del2} zygosity using primers and a FAM-labeled sedel2-probe for amplification and detection of se^{del2} and primers and VIC-labeled probe for *FUT2* [12]. In this study, we applied this method for determination of three SNPs of *FUT2* (c.375A>G, c.385A>T, and c.571C>T) as well as for determination of the se^{del2} zygosity. The present method appears to be advantageous in terms of cost and time savings compared to the previous method that determined only the se^{del2} zygosity because the present method could estimate the secretor status of Polynesian subjects in a single assay [12].

Given the characteristic distribution of *se* alleles due to non-allelic homologous recombination events, it is likely that the se^{fus} allele was generated in Japan and the se^{del} allele in South Asia [7,13]. On the other hand, the origin of the se^{del2} allele was unclear in some respects because the history of the Oceania population is complex. We first found this allele in a Samoan population (Polynesians, also called Remote Oceanian) and later in several New Guinean populations (Melanesians, also called Near Oceanian) [7,11]. Previous studies suggested that Polynesians have genetically interbred with East Asians (Taiwanese) and indigenous Melanesians many times [25-27]. The se^{del2} allele has been found in Papuan- and Austronesian-speaking peoples of Irian Jaya (West Papua, Indonesia) and in mixed highland and coastal Papua New Guineans, but not in New Guinean highlanders (Dani peoples) [11]. Polynesians had a high frequency of c.385A>T and c.571C>T, which are common in Taiwanese, while these two alleles were rare or almost absent in Melanesians with or without the se^{del2} allele [7,8,10,28]. In addition, the se^{del2} allele does not to appear to be present in peoples from East and Southeast Asians such as Taiwanese and Indonesians [9,10]. Therefore, the se^{del2} allele is thought to have originated in an Oceanian population, rather than in an Asian population. However, it is still unclear whether the se^{del2} allele originated from Near or Remote Oceania. It would be desirable to conduct a large-scale study of the distribution of the se^{del2} allele in various populations in Oceanian and neighboring areas in order to determine the distribution and origin of this allele.

In addition, the c.375A>G in *FUT2* is a very interesting SNP. This is because in gorillas and orangutans the nucleotide at position 375 in *FUT2* is G, while in chimpanzees and humans it is A. Therefore, it is assumed that the substitution from G to A occurred in the common ancestor of chimpanzees and humans, and that the re-substitution from A to G occurred once again in humans. Furthermore, c.375A>G appears to be relatively unique to Papuan-speaking Melanesian populations, although it was also present Austronesian-speaking Melanesian populations and Polynesian populations with relatively low frequency [11]. In addition, c.375A>G and c.571C>T have been recently found in two late Neanderthals who lived in Russia and Croatia about 500,000 to 800,000 years ago [29]. However, these two Neanderthals had c.375A>G accompanied by c.571C>T, whereas the Samoan and Taiwanese c.571C>T were not accompanied by c.375A>G [7-10,29]. Furthermore, one late Neanderthal in Altai and one Denisovan without c.571C>T also had c.375A>G as a homozygous state. One Denisovan had a c.400G>A as a heterozygous state. However, as with c.571C>T, the Denisovan c.400G>A was accompanied by c375A>G, but the Oceania c.400G>A was not accompanied by c375A>G [11,29]. The c.400G>A and c.375A>G were found at relatively high frequencies in Melanesians but were quite rare or not found in other populations [11].

Previous studies had suggested that a complex pattern of natural selection such as balancing selection or positive selection in different populations might act on the *FUT2* locus [6]. The selective force is presumed to be several

pathogens. In fact, non-secretors have been reported to be highly resistant to noroviruses and rotaviruses, and more recently, to COVID-19 infection, albeit weakly [30-32]. In addition, recent studies have suggested that secretor status affects susceptibility to a variety of clinical conditions, including some infectious diseases, inflammatory bowel disease, and plasma vitamin B_{12} levels [33-40]. Therefore, to understand the complex evolutionary history of the *FUT2* locus, it would be very important to know the origin of these alleles and their relationship to the late Neanderthals, Denisovans, and Melanesians. From these perspectives, the present method is useful for the investigation to estimate the origin of these variations by knowing the distribution of these variations.

5. Conclusions

In this study, we developed a method for simultaneous detection of c.375A>G, c.385A>T, and c.571C>T as well as the se^{del2} allele by FMCA and endpoint genotyping assay in a single tube. The present FMCA and endpoint genotyping method appeared to be a reliable high-throughput method for detecting c.375A>G, c.385A>T, and c.571C>T as well as the se^{del2} allele and for estimating secretor status in the Oceania populations. In addition, this method may be useful to examine the distribution and origin of these alleles.

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Data Availability Statement: The data presented in this study are available on request from the corresponding author.

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