Stop codon mutations in the flavin-containing monooxygenase 3 (*FMO3*) gene responsible for trimethylaminuria in a Japanese population

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Running title: **R500X and C197X FMO3 cause trimethylaminuria**

Abstracts

The reduced capacity of flavin-containing monooxygenase 3 (FMO3) to *N*-oxidize trimethylamine (TMA) is believed to cause a metabolic disorder. The aim of this study was to investigate the inter-individual variations of FMO3. Genomic DNA of case subjects that showed only 10-20% of FMO3 metabolic capacity among self-reported trimethylaminuria Japanese volunteers was sequenced. Functional analysis of recombinant FMO3 proteins was also performed. One homozygote for a novel single nucleotide substitution causing a stop codon at Arg500 was observed. The biological parents of this Proband A were heterozygous and showed > 90% TMA *N*-oxygenation metabolic capacity. Another Proband B had the Arg500Stop and Cys197Stop codons. The TMA *N*-oxygenation metabolic capacities of the father and brother of this Proband B were apparently observed by possessing Arg205Cys mutant that coded for decreased TMA *N*-oxygenase. Recombinant Arg500Stop FMO3 cDNA expressed in *Escherichia coli* membranes and a series of highly purified truncation mutants at different positions of the *C*-terminus of FMO3 showed no detectable functional activity toward typical FMO3 substrates. The results suggest that individuals homozygous for either of the nonsense mutations, Arg500Stop and/or Cys197Stop alleles, in the *FMO3* gene can possess abnormal TMA *N*-oxygenation.

Keywords: Flavin-containing monooxygenase; fish-like odor syndrome; trimethylamine; truncated FMO3; Japanese; trimethylaminuria.

Introduction

The flavin-containing monooxygenase (FMO, EC 1.14.13.8) is an NADPH-dependent enzyme that catalyzes the oxygenation of a wide variety of nucleophilic compounds containing sulfur, nitrogen or phosphorus atoms [1;2]. To date, eleven *FMO* genes have been identified in humans (*FMO1* to *FMO11p*) but only FMO1-5 are functionally active [3]. FMO3 is considered a prominent functional form expressed in adult human liver although FMO5 is also present [4;5]. In humans, a 20-fold inter-individual variation in FMO3 expression levels have been reported [6;7]. FMO3 may also play a role in processing some types of drugs such as the anticancer drug tamoxifen, the pain medication codeine, the antifungal drug ketoconazole, the addictive chemical nicotine found in tobacco, and diet-derived trimethylamine (TMA) [1;2]. Wild-type human FMO3 has 532 amino acids but there are genetic polymorphisms in the *FMO3* gene that code for naturally truncated forms that have no or almost no detectable amount of functional enzymatic activity [1;8-10]. Mutations in the *FMO3* gene are summarized in a Web-database using systematic and trivial names [8].

Trimethylaminuria, or fish-like odor syndrome, is a genetic disease characterized by excretion of excessive unmetabolized TMA [11;12]. Individuals suffering from trimethylaminuria have a decreased capacity to oxygenate free malodorous TMA to non-odorous trimethylamine *N*-oxide (TMAO) by the FMO3 [13;14] and this is the case for individuals with causative nonsense *FMO3* gene mutations found in North American and European populations [15;16]. Unpleasant and/or pungent malodor caused by excess TMA present in various bodily fluids of some affected individuals may lead to profound social problems [11;12].

Due to its strong linkage with trimethylaminuria, considerable work has been done related to the contribution of genetic polymorphisms of the *FMO3* gene of the coding region to inter-individual differences in FMO3 phenotype [17-19]. In order to identify novel mutations of FMO3 and/or haplotypes of the *FMO3* gene found in Japanese individuals suffering from

trimethylaminuria, we rsequenced the entire coding region of the *FMO3* gene using genomic DNA from individuals that, judged by self-reported analysis were suspected to be positive for trimethylaminuria and later showed low FMO3 metabolic capacity on the basis of urine testing of TMAO levels. In a preliminary report [20], there were some FMO3 variants like Cys197Stop, Asp198Glu or Arg205Cys observed in a Japanese population, but the characterization of these FMO3 mutants were not examined in detail.

Herein we report data supporting the involvement of two novel deleterious *FMO3* gene mutations causative of abnormal TMA *N*-oxygenation and trimethylaminuria in self-reporting Japanese individuals that were diagnosed with low FMO3 metabolic capacity based on urine testing. Subjects homozygous for either of the nonsense mutations, Arg500Stop and Cys197Stop alleles, in the *FMO3* gene suffered from trimethylaminuria.

Materials and methods

Chemicals

TMA and TMAO were obtained from Wako Pure Chemicals (Osaka, Japan). The tertiary amine substrate 5-DPT and its *N*-oxide were synthesized as described previously [1;21]. The other chemicals and reagents used were obtained in the highest grade available commercially.

Subjects

The ethics committees of Showa Pharmaceutical University and Hokkaido University approved this study. Volunteer subjects who responded to an Internet article for screening of urinary TMA and TMAO levels and for sequencing the *FMO3* gene included 90 males and 74 females ranging from 1 to 64 years of age. Informed consent was obtained from each subject or parent. The study participants collected their urine samples as described previously [22]. Urinary TMA and TMAO concentrations were determined by gas chromatography using a flame ionization detector as

described previously [23]. Urinary concentrations of free TMA or total TMA (μmol/mL of urine) were corrected for creatinine excretion (mmol/mL) [22]. Individuals that showed impaired FMO3 metabolic capacity, defined as the ratio of TMAO to total TMA (% of TMAO / (TMA+TMAO)), lower than 40% were considered to constitute abnormal TMA metabolism and possibly suffering from severe trimethylaminuria [16;22;24]. The values of urinary TMA and TMAO were shown as the average of at least three determinations obtained from first morning void urine.

DNA analysis

Genomic DNA prepared from peripheral lymphocytes [20] or buccal cells [25] of the study participants were analyzed. The sequence of the complete human *FMO3* gene described in GenBank (Accession Number AL021026) was used as a reference. Polymerase chain reaction (PCR) for the all exons and exon-intron junctions of the human *FMO3* gene was conducted in a 25 μL reaction mixture containing 50 ng of genomic DNA, 1.0 U LA-Taq DNA polymerase (Takarabio, Shiga, Japan), LA-PCR buffer, 2.0 mM $MgCl_2$, 0.2 mM dNTPs, 5.0 pmol of each sense and antisense primer reported previously [23]. The PCR conditions consisted of an initial denaturation at 94°C for 1 min, following by 35 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s, and extension at 72 °C for 45 s. The PCR products were directly sequenced on both strands using an ABI bigdye terminator cycle sequencing kit (Applied Biosystems, Foster City, CA, USA) with the sequencing primers [20]. The purified PCR products were analyzed on an ABI PRISM 3730*xl* DNA analyzer (Applied Biosystems).

Genotyping analysis for the novel g.30398 C>T mutation (Arg500Stop FMO3) in exon 9 was also carried out by a PCR-RFLP method with amplified DNA with the primers FMO3-9S and FMO3-9AS [23] and digestion by *Bss*SI at 37 °C for 2 h.

Recombinant wild type and modified FMO3 protein preparations

The FMO3 cDNA used was previously modified by a PCR procedure [23] using a 5'-primer (5'-AAAAAGCTTACCATGGGGAAGAAAG-3', that introduced an *Nco* I site prior to the start

codon) and a 3'-primer (5'-CTAGAGAAGCTTATGATTAGGTCAACAC-3', that introduced a *Hind* III site downstream of the stop codon). The full-length DNA sequence was confirmed again using DNA re-sequencing of both strands. To produce Arg205Cys FMO3, site directed mutagenesis was performed by the primer-directed enzymatic amplification method [23]. Briefly, G-base at position 706 bp in the FMO3 cDNA was substituted by a T-base using the primers, 5'-AGAACTCAGC**t**GCACAGCAGA-3' and 5'-TCTGCTGTGC**a**GCTGAGTTCT-3', to introduce the single nucleotide substitution, that coded for Arg205Cys in exon 5. Similarly, a C-base at position 1590 bp was substituted by a T-base for preparation of Arg500Stop FMO3. The resultant cDNAs were amplified by KOD polymerase (Toyobo, Osaka, Japan). The wild type and modified FMO3 cDNAs were introduced into the pTrc99A expression vector (Pharmacia Biotechnology, Milwaukee, MI, USA) and then transformed into *E. coli* strain JM109 as described previously [23]. The entire coding regions of the wild type and mutagenized FMO3 cDNAs including the mutated sites were verified by re-sequencing of both strands.

Membrane fractions were prepared from the bacterial pellets that the FMO3 cDNAs had been introduced into by a series of fractionations and high-speed centrifugation steps as described previously [23]. Briefly, *E. coli* JM109 transformed by the FMO3 expression vector was grown overnight at 37 °C in Luria-Bertani medium containing 50 μg/mL ampicillin. A 1.0 mL aliquot of the starter culture was inoculated into 100 mL of Terrific Broth medium containing 50 μg/mL ampicillin and 100 mM potassium phosphate buffer (pH 7.4) in a 300-mL triple-baffled flask and cultivated at 120 rpm at 30 °C. After the absorbance of the culture broth at a wavelength of 600 nm reached 0.3, 1 mM isopropyl-β-D-thiogalactoside was added and shaking was continued further for 24 h at 30 °C. The cells were then harvested by centrifugation at 10,000 g for 20 min. All subsequent steps were carried out at 4 °C. The cells were resuspended (*ca* 0.03 g/mL) in 50 mM Tris-acetate buffer (pH 7.5) containing 0.25 mM EDTA, 0.25 M sucrose, and 0.1 mg/mL lysozyme. The cell suspension was kept on ice for 30 min and then centrifuged at 9,000 g for 10 min. The

pellet was resuspended (*ca* 0.5 g/mL) in 100 mM potassium phosphate buffer (pH 7.4) containing 20% (v/v) glycerol, 6 mM magnesium acetate, 0.1 mM dithiothreitol, and 0.1 mM phenylmethylsulfonyl fluoride. Cells were disrupted using an ultrasonic processor and the resulting lysate was centrifuged at 9,000 g for 20 min. The supernatant was then further centrifuged at 100,000 g for 1 h. The pellet (membrane fraction) was resuspended in one volume of 10 mM Tris-HCl buffer (pH 7.4) containing 0.1 mMEDTA and 20% glycerol (v/v). The amount of recombinant FMO3 was determined by immunoquantification by comparison with a standard of human FMO3 (BD Gentest, Woburn, MA, USA) and by the FAD content [23;26].

Wild type human FMO3 and several truncated variants were also expressed in *E. coli* as maltose-binding fusion proteins using the expression vector pMAL-c2 [21;27]. The maltose-binding FMO3 proteins were purified by affinity chromatography as described previously [1;21;27].

Other Assays

Rates of *N*-oxygenation of TMA and 5-DPT by recombinant FMO3 forms were determined by gas chromatography and HPLC, respectively, as described previously [23;27]. The methods yielded precision and accuracy of < 10 % with linearity of time- (to 60 min) or protein- (to 1 mg/mL) dependent manner. The kinetic analysis of TMA *N*-oxygenation was performed using a nonlinear regression analysis program (KaleidaGraph, Synergy Software, Reading, PA, USA).

Results

DNA analysis of Proband A

FMO3 metabolic capacity was determined in self-reporting individuals that claimed to possess defective TMA *N*-oxygenation. Based on a low metabolic capacity of FMO3 ($\leq$ 40% urinary TMAO formation) causing severe trimethylaminuria symptoms [16;19], the frequency of subjects that showed less than 40% of FMO3 metabolic capacity was only 3.0% (5 individuals out

of 164 individuals) in a Japanese population.

We focused on one of the participants that showed low metabolic capacity for TMA *N*-oxygenation (10-20%). From Proband A who showed 10 % FMO3 metabolic capacity, a single nucleotide substitution at g.30398 C>T in the *FMO3* gene was observed that resulted in c.1498C>T and caused a stop codon at Arg500 in exon 9 (Fig. 1A). The Proband A was homozygous for this novel FMO3 stop codon mutation. To confirm the mutation of this *FMO3* gene, a simple polymerase chain reaction (PCR)-restriction fragment length polymorphism (RFLP) method was developed. As shown in Fig. 1B, the PCR product from the Arg500stop codon could not be digested by *Bss*SI. A more extensive analysis of DNA revealed that both parents of Proband A were heterozygous for the mutation at g.30398 C>T (Arg500Stop) (Fig, 1C). Analysis of urinary TMA *N*-oxygenation of both parents showed FMO3 metabolic capacity was greater than 90%, in contrast to 10 % metabolic capacity of Proband A (Fig, 1C).

DNA analysis of Proband B

We also observed the novel Arg500Stop mutation in the *FMO3* gene from Proband B that also possessed 21% metabolic capacity. In the course of sequencing the FMO3 DNA of the samples from this family (Fig. 2), we found that Proband B was heterozyous for the Arg500Stop allele and also had another allele (Fig.2A) that was reported recently as a single nucleotide polymorphism [20]. The latter *FMO3* mutation was the g.21243_21244 TG deletion (c.591_592TG>del) caused a frameshift that encoded a new stop codon. This *FMO3* allele had g.5906 C>T and g.5907 A>G in intron 2, g.20852 C>T in exon 4 (c.441C>T), a g.20960_20962 CTT deletion, g.21115 G>A in intron 4, g.21246 T>A in exon 5 (c.594T>A), g.27091 C>T in exon 7 (c.855C>T), and a g.29232 T insertion in intron 8, together with the g.21243_21244 TG deletion in exon 5 (c.591_592TG>del) that coded for Cys197Stop.

Other mutations

In addition, we found another haplotype containing g.21265 C>T in exon 5 (c.613C>T) from the father and brother of the Proband B that resulted in Arg205Cys FMO3 [20]. The individuals that were heterozygous for Arg205Cys FMO3 showed 84-86% of the FMO3 metabolic capacity *in vivo* (Fig. 2B). We recently reported two additional missense mutants, Thr201Lys FMO3 and Met260Val FMO3, as a preliminary single nucleotide polymorphism communication [28] from the other three subjects out of 164 individuals showing less than 40% FMO3 metabolic capacities.

The allelic frequencies of Cys197Stop, Arg205Cys, and Arg500Stop of the *FMO3* gene were estimated to be 2.1 %, (7 out of 328 alleles), 4.0 % (13 out of 328 alleles), and 2.7 % (9 out of 328 alleles), respectively, in this Japanese cohort. Additional Thr201Lys and Met260Val mutations showed less frequencies estimated to be 1.2 % (4 out of 328 alleles) and 0.6% (2 out of 328 alleles), respectively, in the same cohort.

Recombinant R205C or R500Stop FMO3

Kinetic parameters for TMA *N*-oxygenation functional activity were determined by nonlinear regression analysis of recombinant Arg205Cys FMO3 and were compared with those of wild type FMO3 expressed in bacterial membranes (Table 1). Apparent K_m values of wild type and Arg205Cys FMO3 were not significantly different, but the V_{max} value of Arg205Cys was approximately one-fourth of that of wild-type enzyme.

To evaluate functional activity of the FMO3 mutant encoding a stop codon, recombinant Arg500Stop FMO3 was expressed in *E. coli* membranes and analyzed for functional activity. The Arg500Stop FMO3 expressed in the membranes was not able to catalyze *N*-oxygenation of TMA or another tertiary amine substrate for FMO3, 10-[(*N*,*N*-dimethylaminopentyl)-2-(trifluoromethyl)]phenothiazene (5-DPT) (Table 2).

To further investigate the effects of stop codons on 5-DPT *N*-oxygenation, six highly purified FMO3 fusion proteins truncated at Phe510, Ser467, Leu437, Glu403, Asp339, and Glu305 were also studied. A series of highly purified FMO3 fusion proteins truncated at different positions (from Glu305 to Ser467) did not show any detectable enzymatic activities (< 0.01 nmol products/min/nmol FMO3), except for Phe510Stop FMO3 that had *N*-oxygenation functional activity (0.02 nmol products/min/nmol FMO3) that was only 1% that of purified wild type FMO3 fusion protein. These results collectively suggested that Cys197Stop FMO3 and Arg500Stop FMO3 possessed little or no detectable functional activity as well, suggesting specific regions of the *N*-terminus of FMO3 was required for functional activity.

Discussion

The structural organization of the human *FMO3* gene has been reported [29]. Human *FMO3* is the gene candidate considered to be solely responsible for TMA *N*-oxygenation and deoderation and is defective in the inherited disorder, fish-like odor syndrome or trimethylaminuria. Because the functional FAD- and $NADP^+$-binding domains of the human *FMO3* (532 amino acid) are encoded by exon 2 and exon 5, respectively [29], it is possible that mutation(s) located toward the *N*-terminus of the *FMO3* could be responsible for decreased expression of the gene and/or abnormal functional activity or instability of the encoded protein. Previously, inactive variants of FMO3 at positions in exon 2 and exon 5 have been reported [8;9]. In the present study, a new mutation (Arg205Cys FMO3 in exon 5) was identified that possessed functional activity but decreased TMA *N*-oxygenase activity (Table 1). The frequency of this allelic variation may contribute to mild trimethylaminuria observed in the Japanese population, in association with a loss-of-function mutation on the other allele.

In addition, a novel Arg500Stop mutation (Fig. 1) responsible for severe trimethylaminuria

was discovered in the Japanese population of the present study. Arg500Stop FMO3 encodes a 499 amino acid protein (out of 532 amino acids, or only 94 % of the whole FMO3 structure). The Proband A that was a homozygote for this novel allele (Fig. 2) did not efficiently *N*-oxygenate dietary-derived TMA to TMAO. The observations about abnormal or impaired TMA *N*-oxygenation based on *in vivo* phenotype caused by the stop codon mutation in the *FMO3* gene was supported by *in vitro* evidence that showed no detectable functional activity of Arg500Stop FMO3 expressed in bacterial membranes (Table 2). Important contribution of *C*-terminus of human FMO3 for its function was clearly indicated but might be inconsistent with early findings of active recombinant FMO2 form in which the C-terminal 26 amino acids were deleted [30]. A molecular modeling of human FMO3 (532 amino acids) is worth examining to understand the contribution of *C*-terminus based on the recently reported crystal structure of yeast FMO (447 amino acid) [31], in spite of the low sequence identity (~20%) and short length (~80%) of the whole human FMO3 structure.

Furthermore, that no detectable functional activity of highly purified cDNA-expressed FMO3 proteins truncated at several *C*-terminal positions was observed in this study suggests that the *C*-terminus of the human FMO3 is important for monooxygenation action. Taken together with the present results, the Cys197stop FMO3, found in Proband B (Fig. 2), should also be inactive for typical tertiary amine substrates.

The Japanese individuals did not possess any of the previously reported *FMO3* gene mutations that typically cause severe trimethylaminuria symptoms and low metabolic capacity of FMO3 ($\leq$ 40% urinary TMAO formation) [16;19]. We recently reported that relatively low FMO3 metabolic capacity associated with liver damage could be another causal factor for mild trimethylaminuria, independent of the *FMO3* genotype present [32]. On the other hand, the present study is the first report that the stop codons exist as mutants of *FMO3* in Japanese severe trimethylaminuria patients, although further studies are needed to be conducted to clarify whether or

not these mutants and/or haplotypes are specific for Japanese suffering from trimethylaminuria. We recently reported two additional missense mutants, Thr201Lys FMO3 and Met260Val FMO3, as a preliminary single nucleotide polymorphism communication [28] from DNA of the other three subjects in this Japanese cohort showing less than 40% FMO3 metabolic capacities. Although these variants have not been fully characterized yet in terms of FMO3 function, recombinant Thr201Lys FMO3 and Met260Val FMO3 expressed in the bacterial membranes had impaired catalytic function in our preliminary study. Recently reported *FMO3* upstream variant haplotypes [33] and/or alternative processing [34] might also contribute to decreased *FMO3* expression and incidence of trimethylaminuria. Regardless, the present results suggest that truncation mutations of the *FMO3* gene that result in stop codons might be one of the causes of fish-like odor syndrome or trimethylaminuria in a Japanese cohort suffering from self-reported malodor, albeit at low frequencies (~2-4 %). The frequency of these mutations should be low in healthy Japanese populations because they could not be found at all in approximately 100 alleles tested in control individuals (data not shown). The results from the present study also suggest that harboring at least one wild-type or decreased- but not inactive allele of the *FMO3* gene could have FMO3 metabolic capacity and this may be relevant to diagnosis of trimethylaminuria.

In conclusion, individuals homozygous for either of the nonsense mutation Arg500stop or Cys197Stop alleles in the *FMO3* gene can possess abnormal TMA *N*-oxygenation and have trimethylaminuria. Heterozygotes for the nonsense mutations will exhibit trimethylaminuria symptoms only if they have, on the other chromosome, a mutation that substantially impairs enzyme activity (in which case they will have severe trimethylaminuria) or partially impairs enzyme activity (in which case they will have mild trimethylaminuria). The findings of the present study provide fundamental information for the importance of future investigations of the human *FMO3* gene associated with trimethylaminuria (fish-like odor syndrome).

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Table 1

Trimethylamine *N*-oxygenation activity of wild type and Arg205Cys FMO3 cDNA expressed in *E. coli*

FMO3	Trimethylamine *N*-oxygenation activity	
	K_m (μM)	V_{max} (nmol products/min/nmol FMO3)
Wild type	40 ± 8	16 ± 1
Arg205Cys	40 ± 5	4.2 ± 0.5

The *N*-oxygenation of trimethylamine (10-500 μM) was determined by gas chromatography. Kinetic parameters were calculated from the fitted curve by non-linear regression (mean ± SE).

Table 2

Functional activity of wild type and Arg500Stop FMO3 cDNA expressed in *E. coli*

FMO3	*N*-Oxygenation activity (nmol products/min/nmol FMO3)	
	Trimethylamine	5-DPT
Wild type	14.1	2.1
Arg500Stop	< 0.1	< 0.01

The *N*-oxygenation of trimethylamine (100 μM) and 5-DPT (250 μM) were determined by gas chromatography and HPLC, respectively, in triplicate.

5-DPT, 10-[(*N*,*N*-dimethylaminopentyl)-2-(trifluoromethyl)]phenothiazene.

Legend for figures

Fig. 1. (A) Nucleotide sequences of wild type and variant of exon 9 of the *FMO3*. Both strands were sequenced. The sequences are shown only for sense strands for exon 9 of genomic DNA from the Proband A. (B) Genotyping analysis for the novel g.30398 C>T mutation (Arg500Stop FMO3) in exon 9 by PCR-RFLP with *Bss*SI. Genomic DNA was amplified with primers FMO3-9S and FMO3-9AS [23] and was digested by *Bss*SI. (C) Pedigree analysis for the presence of novel Arg500Stop mutation in the *FMO3* gene of the Proband A. The numbers in parentheses showed the results of both free TMA concentration (μmol TMA/mmol creatinine) and FMO3 metabolic capacity (% of TMAO to total TMA + TMAO) from analysis of urine. The PCR products before and after digestion with *Bss*SI were separated on 2 % agarose gel. U, uncut; D, digested with *Bss*SI.

Fig. 2. (A) Nucleotide sequences of wild type and variant of exon 5 of the *FMO3*. Both strands were sequenced. Antisense stands for exon 5 of genomic DNA from the Proband B are shown in a reversed manner, because of upstream deletions in sense strands. (B) A family study for Proband B who was heterozygous for Cys197Stop and Arg500Stop FMO3 mutations. See legend of Fig. 1 for details.

Figure 1

Fig. 1

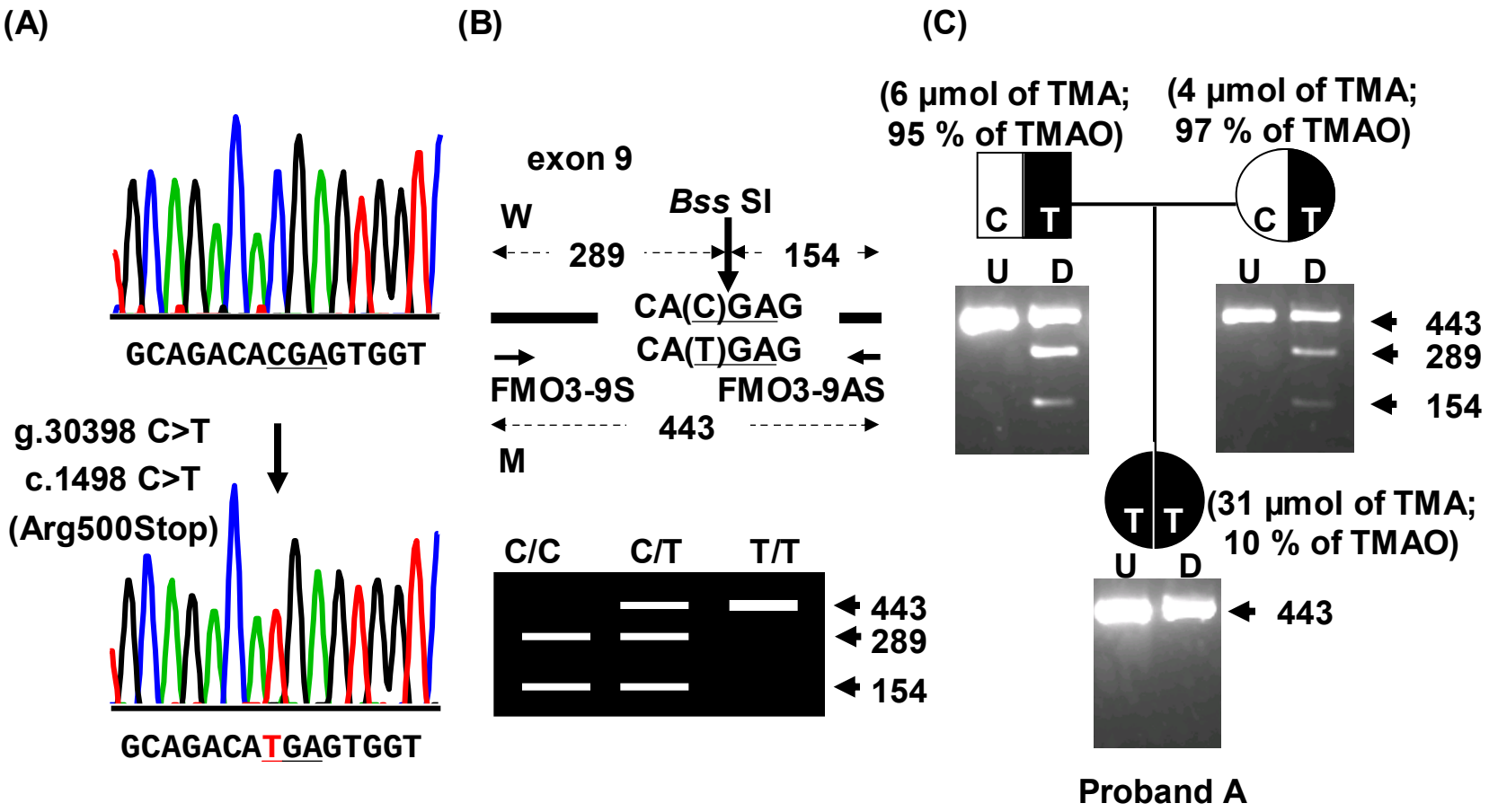

Figure 2

Fig. 2