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Significance of each of three missense mutations, G484A, G667A, and G808A, present in an inactive allele of the human Lewis gene (*FUT3*) for α(1,3/1,4)fucosyltransferase inactivation

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Recently, we found three novel missense mutations, G484A (Asp162Asn), G667A (Gly223Arg), and G808A (Val270Met), present in a Lewis-negative allele (*le*^{484,667,808}) from an African (Xhosa) population. To define the relative contribution of each of the three mutations in the *le*^{484,667,808} allele for inactivation of the *FUT3*-encoded enzyme, we made chimeric *FUT3* containing each of the three mutations. A transient expression study indicated that COS7 cells transfected with the *FUT3* construct containing the G484A mutation expressed the Lewis antigen and had about 20% enzyme activity as compared with COS7 cells transfected with the wild type *FUT3* allele, whereas COS7 cells transfected with the *FUT3* construct containing either the G667A mutation or the G808A mutation did not express the Lewis antigen and showed no detectable α(1,3/1,4)fucosyltransferase activity. These results suggest that the G667A and/or the G808A missense mutations of *FUT3* alleles are responsible for the inactivation of the *FUT3*-encoded enzyme.

***Keywords: FUT3,* α(1,3/1,4)fucosyltransferase, Fuc-TIII, Lewis-negative allele, Chimera, Mutation**

Introduction

The Le^a and Le^b antigens of the Lewis histo-blood group system are synthesized by a Lewis α(1,3/1,4)fucosyltransferase (Fuc-TIII), which is encoded by the *FUT3* gene [1-9]. The Le(a-b-) phenotype fails to express Le^a and Le^b antigens on erythrocytes and in secretions, because of homozygous status for null alleles (*le*) of *FUT3.* Five main missense mutations in *FUT3,* T59G (Leu20 to Arg), T202C (Trp68 to Arg), C314T (Thr105 to Met), G508A (Gly170 to Ser), and T1067A (Ile356 to Lys), have been identified in a number of Le(a-b-) individuals in various populations [2-9]. In addition, we found three novel missense mutations, G484A (Asp162 to Asn), G667A (Gly223 to Arg), and G808A (Val270 to Met), and three novel Lewis-negative alleles, the *le*^{484,667} and the *le*^{484,667,808} in African (Xhosa), and the *le*^{202,314,484} in Caucasian populations of South Africa [8]. In the present study, we examined if any one of the mutations, G484A, G667A, and G808A, was responsible for the inactivation of Fuc-TIII enzyme by making chimeric *FUT3* constructs containing each of the three mutations and by transfecting them into COS7 cells. We found that G667A and/or G808A alternations were the major inactivating point mutations for the *le*^{484,667} and *le*^{484,667,808} alleles, whereas the G484A mutation showed about 20% activity compared with wild type.

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Materials and methods

Genomic DNA of three individuals (W9, B93, and B57), previously characterized for *FUT3* genotypes as *le*^{202,314,484}/*Le, le*^{484,667} /*le*^{484,667} and *le*^{484,667,808}/*Le,* respectively [8], were used for this study. DNA fragments (1.6 kb) containing the 1.2-kb open reading frame of *FUT3* from three individuals were amplified by PCR as described [10]. Then, the PCR products were digested by restriction enzymes *Hin*dIII (artificially produced site in the upper primer) and *Xba*I (artificially produced site in the lower primer), and were subcloned into plasmid pRc/CMV (Invitrogen, San Diego, California) for DNA sequencing, constructing chimera and expression studies.

To construct a plasmid that contains the G484A mutation (pRC/CMV-*484A*), the plasmid pRc/CMV containing

Le (wild type) allele or $le^{202,314,484}$ allele was digested with *Hin*dIII and *Xba*I, and the resulting DNA fragments (1.6 kb) were subcloned into the plasmid pUC119. The pUC119-*484A* was constructed by ligation of a 1.2-kb fragment of pUC119-$le^{202,314,484}$ and a 4.4-kb fragment of pUC119-*Le,* obtained after treatment by restriction enzymes *Eco*RV and *Xba*I (bases 1 to 336 belong to the wild type and bases 337 to 1552 belong to the $le^{202,314,484}$). Then, the recombinated chimera containing only the G484A mutation was ligated into the plasmid pRc/CMV after digestion by *Hin*dIII and *Xba*I.

To prepare an *FUT3* construct containing the G667A mutation (pRc/CMV-*667A*), the PCR method was chosen. Figure 1 shows the whole process to create pRC/CMV-*667A* by two PCR amplifications, because there is no suitable sites for endonuclease between bases 484 and 667. Therefore, the purpose of the first PCR is to make a primer for the second PCR. The first PCR amplification was performed in a reaction mixture of 50 μl *rTaq* buffer, containing 1 ng of pRc/CMV-$le^{484,667}$ as template, 2.5 unit *rTaq* DNA polymerase (Toyobo, Osaka, Japan), 200 μM dNTP, 1.5 mM $MgCl_2$, and 12.5 pmol of each primer (Le-500U, 5′-CGC CCT ACG GCT GGC TGG AGC CGT G-3′, nucleotides (nt) 500 ~ 524 bp and Le-nestL, 5′-CTC TCT AGA GCC CAG GCA CCA TGC CGG CCT CTC-3′, nt 1109 ~ 1085 bp). PCR conditions were 30 cycles of denaturing at 94°C for 30 sec, annealing at 60°C for 30 sec, and extension at 72°C for 1 min. The second PCR was carried out in a reaction mixture of 50 μl *ExTaq* buffer, containing 1.5 unit *ExTaq* DNA polymerase (Takara, Shiga, Japan), 200 μM dNTP, 2.5 mM $MgCl_2$, and the primers (25 cycles of denaturing at 98°C for 10 sec, annealing at 60°C for 1 min and extension at 72°C for 1 min). One μl of 1000-fold diluted PCR product (1.6 kb) obtained from genomic DNA of an individual homozygous for the *Le* wild type allele was used as template. The Le-nestU primer, 5′-CTC AAG CTT GTC ATC ACT GAC CCT CAC TCC TC-3′ (nt −53 ~ −29 bp) was used for the upper primer (10 pmol). The reaction mixture of the first PCR product containing the G667A mutation was centrifuged by a Suprec-02 tube (Takara) to remove the primers of the first PCR and then 25 μl of the first PCR reaction mixture was used as lower primer (Figure 1). The resultant PCR product was digested by restriction enzymes, *Hin*dIII and *Xba*I, and then subcloned into plasmid pRc/CMV, and the pRc/CMV-*667A* was selected.

To construct a plasmid that contains the G808A mutation (pRc/CMV-*808A*), pRc/CMV-*Le,* and pRc/CMV-$le^{484,667,808}$ were digested by *Hin*dIII and *Eco*NI. Then, a 0.7-kb fragment from the wild type allele was ligated into digested pRc/CMV-$le^{484,667,808}$ (6.3 kb). This created a pRc/CMV-*808A* chimera (bases 1 to 683 belong to the wild type and bases 684-1552 belong to the $le^{484,667,808}$).

To select the *FUT3* constructs, which contain no misincorporation generated by PCR, DNA sequencing was performed with a dRhodamine terminator cycle sequencing ready reaction kit and the ABI PRISM 310 genetic analyzer (Perkin Elmer Japan ABI, Chiba, Japan).

Mammalian expression vectors containing *FUT3* constructs (2 μg) were transfected into COS7 cells (3×10^5 cells) by use of a TransIT polyamine transfection reagents kit (Mirus, Madison, USA). After 48 h of culture, the expression of the Lewis antigen on cell surfaces was examined using anti-Le^a monoclonal antibody (Kokusai Reagents, Kobe, Japan) and fluorescence-labeled anti-mouse IgM antibody, as described previously [2], and enzyme activities in cell extracts were determined. Cell extracts were prepared by resuspending cell pellets in 1%

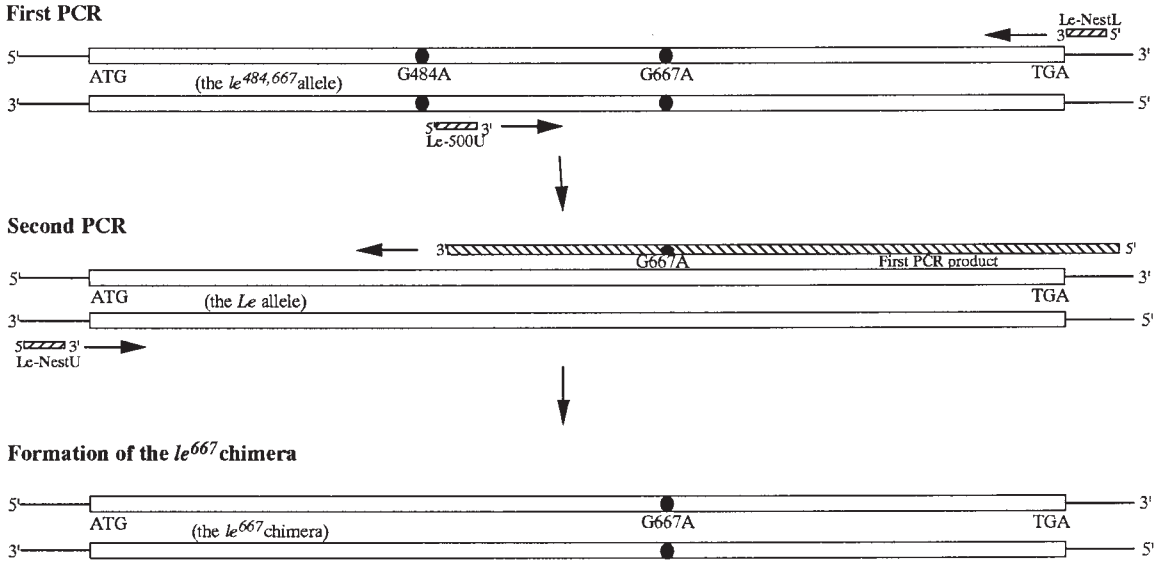

Figure 1. The strategy of constructing G667A chimera by PCR.

Triton X-100 and 10% glycerol. The α(1,3/1,4)fucosyltransferase (Fuc-TIII) activity was measured in 50 μl of 30 mM Tris-HCl buffer (pH 7.2), containing 60 μM GDP-L-[U-^{14}C]fucose (44,000 dpm), 5 mM ATP, 10 mM $MnCl_2$, cell extract (25 μl containing about 25 μg protein), and 5 mM lacto-*N*-biose I or *N*-acetyllactosamine as an acceptor. After incubation at 37°C for 20 min, the products were separated from GDP-[^{14}C]fucose by a Dowex 1-X8 (chloride form). A parallel control reaction without acceptor was performed for each reaction. To normalize the transfection efficiency, the pGL2 control vector (Promega, Madison, Wisconsin) was co-transfected, and the activity of luciferase in cell extracts (15 μl containing about 2 μg protein) was measured using a luciferase assay system (Promega). The transfection efficiency was similar in each dish in the one experiment but was different from experiments to experiments (from 6×10^5 RUL to 3×10^6 RUL).

Results

To determine if any one of the G484A, G667A, and G808A is a main deleterious mutation in the *le*^{484,667,808}, chimeric *FUT3* constructs which contained each of the three mutations were made as described in Materials and methods. All constructs contained the complete coding region of *FUT3* attached to the cytomegalovirus promoter and were verified to contain only the expected single point mutation by PCR-RFLP and sequencing.

To evaluate the ability of each of the mutations to express Fuc-TIII, we transfected each plasmid containing the G484A mutation (pRc/CMV-*484A*), the G667A mutation (pRc/CMV-*667A*), or the G808A mutation (pRc/CMV-*808A*) into COS7 cells, in which α(1,3/1,4)fucosyltransferase is absent. As shown in Table 1, COS7 cells transfected by the wild type construct (pRc/CMV-*Le*) showed a substantial amount of α(1,3/1,4)fucosyltransferase activity, whereas those transfected by pRc/CMV and pRc/CMV-*le*^{484,667,808}, which was confirmed as a Lewis-negative allele in the previous study [8], displayed no α(1,3/1,4)fucosyltransferase activity. Cell extracts transfected with the pRc/CMV-*484A* had about 20% of the enzyme activity of the pRc/CMV-*Le*-transfected cell extracts, whereas cell extracts transfected with the pRc/CMV-*667A* or pRc/CMV-*808A* had no detectable enzyme activity (Table 1). We also tested the expression of the Lewis antigen on cell surfaces using anti-Le^a after transfection with each construct. COS7 cells transfected with the wild type allele (pRc/CMV-*Le*) expressed the Lewis antigen on cell membranes, though the Lewis antigen was not detected on cells transfected with the plasmid pRc/CMV or pRc/CMV-*le*^{484,667,808}. COS7 cells transfected with pRc/CMV-*667A* or pRc/CMV-*808A* did not express the Lewis antigen. However, COS7 cells transfected with pRc/CMV-*484A* expressed the Lewis antigen on cell membranes, and the Lewis antigen level expressed on cell membranes was comparable to that of COS7 cells transfected with the wild type (data not shown). These results suggested that both G667A and G808A mutations of *le*^{484,667} and *le*^{484,667,808} alleles were responsible for the complete inactivation of Fuc-TIII, whereas the G484A mutation, like the T59G in the *le*^{59,508} [2, 5] and *le*^{59,1067} [5, 6] and the C314T in the *le*^{202,314} [9], did not completely disrupt the enzyme activity.

Discussion

Complex alleles containing more than one mutation have been reported in several disorders [11]. These complex alleles may represent the combination of a common mutation with a deleterious mutation, the combination of two "partial" deleterious mutations, the presence of two deleterious mutations derived by crossover with a pseudogene, or the presence of two deleterious mutations that, presumably, had occurred independently. Clinical studies in cystic fibrosis have indicated a beneficial modulating effect of a second mutation on the common F508 mutation allele [12]. We have also reported two missense mutations (T460C

Table 1. α(1,3/1,4) fucosyltransferase activity in COS7 cells transfected with plasmids containing chimeric *FUT3* and *FUT3* alleles

	Enzyme activity (nmol/mg/h)	
Transfected vector	*Lacto-N-biose I*	*N-acetyllactosamine*
pRc/CMV-*wild type*	20.2 ± 3.5	1.9 ± 0.3
pRc/CMV-*484A*	3.9 ± 0.5	0.22 ± 0.04
pRc/CMV-*667A*	nd	nd
pRc/CMV-*808A*	nd	nd
pRc/CMV-*484A-667A-808A*	nd	nd
pRc/CMV	nd	nd

Values are the mean ± s.d. of three experiments.
nd = not detected.

Table 2. Comparisons of amino acid sequences among the α-3-fucosyltransferase family (*FUT3 ~ FUT7*)

	Amino Acid Sequences																																				
Source of protein	*Cassette one*													*Cassette two*														*Cassette three*										*Reference*
						◆													◆										◆									
FUT3-484-667-808	M	S	Y	R	S	N	S	D	I	F	T	P		K	V	D	V	Y	R	R	-	S	H	-	K		L	E	A	W	A	M	P	V	V	L	G	P	[8]
		*	*	*		*	*	*		*		*				*	*		*										*				*	*	*		*	*	*	
FUT3-Human	M	S	Y	R	S	D	S	D	I	F	T	P		K	V	D	V	Y	G	R	-	S	H	-	K		L	E	A	W	A	V	P	V	V	L	G	P	[1]
FUT3-Chimpanzee	M	S	Y	R	S	D	S	D	I	F	T	P		K	V	D	V	Y	G	R	-	S	H	-	K		L	E	A	W	A	V	P	V	V	L	G	P	[14]
FUT4-Human	L	S	Y	R	A	D	S	D	V	F	V	P		T	V	D	V	F	G	R	G	G	P	G	Q		L	L	A	G	A	V	P	V	V	L	G	P	[15]
FUT4-Chicken	M	S	Y	R	R	D	S	D	V	F	V	P		P	I	D	V	Y	G	-	-	A	R	G	M		F	A	A	S	A	V	P	V	V	L	G	P	[16]
FUT4-Mouse	L	S	Y	R	T	D	S	D	V	F	V	P		S	V	D	V	F	G	R	T	G	P	G	R		F	L	A	G	A	V	P	V	V	L	G	P	[17]
FUT4-Rat	L	S	Y	R	T	D	S	D	I	F	V	P		S	V	D	V	F	G	R	A	G	P	G	Q		F	L	A	G	A	V	P	V	V	L	G	P	[18]
FUT5-Human	M	S	Y	R	S	D	S	D	I	F	T	P		K	V	D	V	Y	G	R	-	S	H	-	K		L	E	A	W	A	V	P	V	V	L	G	P	[19]
FUT5-Chimpanzee	M	S	Y	R	S	D	S	D	I	F	T	P		K	V	D	V	Y	G	R	-	S	H	-	K		L	E	A	W	A	V	P	V	V	L	G	P	[14]
FUT6-Human	M	S	Y	R	S	D	S	D	I	F	T	P		K	V	D	V	Y	G	R	-	S	H	-	K		L	E	A	W	A	V	P	V	V	L	G	P	[20]
FUT6-Chimpanzee	M	S	Y	R	S	D	S	D	I	F	T	P		K	V	D	V	Y	G	R	-	S	H	-	K		L	E	A	W	A	V	P	V	V	L	G	P	[14]
FUT7-Human	L	S	Y	R	R	D	S	D	I	F	V	P		R	V	D	V	F	G	R	-	A	N	G	R		L	V	A	G	T	V	P	V	V	L	G	P	[21]
FUT7-Mouse	L	S	Y	R	R	D	S	D	I	F	V	P		Q	V	D	V	F	G	R	-	A	S	G	R		L	A	A	G	A	V	P	V	A	L	G	P	[22]
FUTb-Bovine	M	S	Y	R	R	D	S	D	I	F	M	P		Q	V	D	V	Y	G	R	-	F	H	-	T		L	Q	A	W	A	V	P	V	V	L	G	P	[23]

Cassette one, two, and three represent areas of 12 amino acids containing G484A, G667A, and G808A mutation, respectively. *, conserved amino acids; ◆, substituted amino acids in the *Ie*^{484,667,808}.

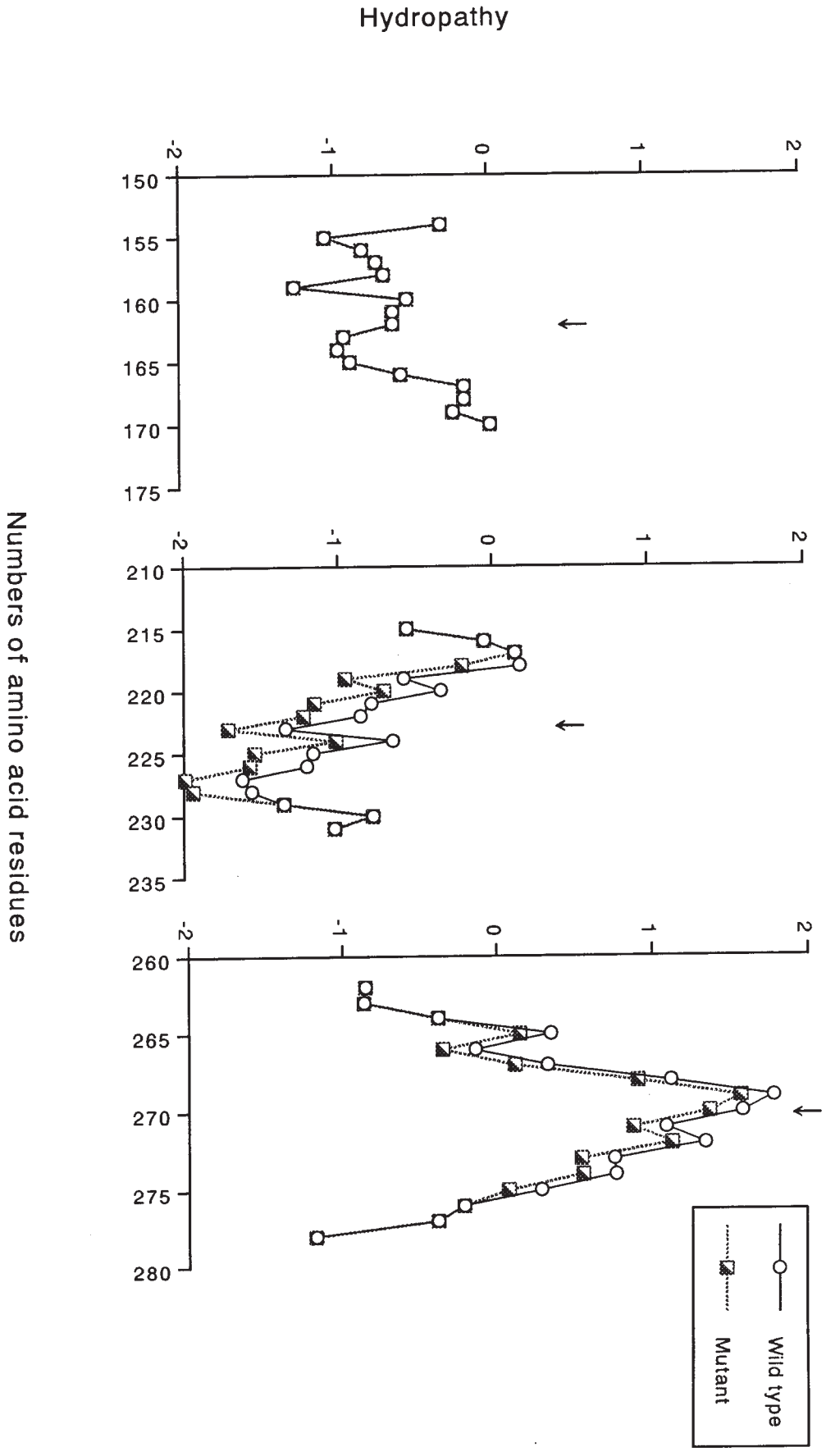

Figure 2. The hydropathy plots of the wild type Fuc-TIII and of the mutated Fuc-TIII (Asp162 to Asn, Gly223 to Arg and Val270 to Met) enzymes.

and G1042A) of the H α(1,2)fucosyltransferase gene (*FUTI*) of a Japanese individual with the para-Bombay phenotype and confirmed that these two mutations are synergistically responsible for complete H enzyme inactivation [13]. In *FUT3,* alleles containing two mutations are a common character in Lewis-negative alleles [2, 5, 8, 9]. Several studies have documented a deleterious mutation in the Lewis allele carrying two or three mutations. The T202C [9] and G508A [2] mutations in $le^{202,314}$ and $le^{59,508}$ were determined to be responsible for the Lewis-negative phenotype through constructing chimera containing a single point mutation. In a previous study, in addition to two mutations found in a Lewis-negative allele ($le^{484,667}$), we also detected *FUT3* alleles carrying three missense mutations ($le^{202,314,484}$ and $le^{484,667,808}$) [8]. Based on *in vitro* studies of transient expression of chimeric *FUT3,* the G484A mutation was demonstrated to be a partial deleterious mutation (about 20% activity of the wild type), and the G667A and G808A mutations were shown to be deleterious mutations (Table 1). The deleterious effect of the G484A mutation on the activities of α(1,4)- and α(1,3)-fucosyltransferases seemed to be different (19% and 12% activities of the wild type, respectively). However, this might be due to a relatively low activity of α(1,3)fucosyltransferase (about 1/10 activity of that of α(1,4)fucosyltransferase).

An analysis of evolutionary conservation is also consistent with the observed effects on α(1,3/1,4)fucosyltransferase activity. Comparison of the amino acid sequences among members of the α-3-fucosyltransferase family (Fuc-TIII ~ Fuc-TVII) and among various species [1,8,14-23] reveals that Asp at codon 162, Gly at codon 223, and Val at codon 270 (numbers of codons were followed by the human *FUT3* codon) are all completely conserved (Table 2). The substitutions of Asn for Asp, Arg for Gly, and Met for Val are nonconservative substitutions, being consistent with a major deleterious effect of these mutations on enzyme activity.

The relative significance of the three substitutions, Asp162Asn (G484A), Gly223Arg (G667A), and Val270Met (G808A), present in the $le^{484,667,808}$ in comparison to the Lewis wild type enzyme, was analyzed by secondary structure Choi-Fasman prediction (MacMolly Tetra software). The relative significance of the three substitutions at amino acids 162, 223, and 270 versus the wild type enzyme are illustrated in hydropathy plots (Figure 2). The results suggest that the changes in amino acids at codons 223 (Gly223 to Arg) and 270 (Val270 to Met) have a relatively large effect on the secondary structure of the protein and that Asp162 to Asn has no effect on this character of the Fuc-TIII enzyme. The change in hydropathy might be responsible for the inactivation of the enzyme. Although the change of Asp162 to Asn had no effect on the hydropathy character of the Fuc-TIII enzyme, this substitution of a neutral amino acid for an acidic amino acid may change the structure and/or charge of the enzyme, resulting in a partial deleterious effect on the enzyme activity.

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