



## Molecular analysis of three *FUT3* gene single nucleotide polymorphisms and their relationship with the lewis erythrocytary phenotype in a human population of japanese-ancestry living in Tomé Açu, a town in the Brazilian Amazon

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### Abstract

The Lewis blood group system involves two major antigens, Le<sup>a</sup> and Le<sup>b</sup>. Their antigenic determinants are not primary gene products but are synthesized by the transfer of sugar subunits to a precursory chain by a specific enzyme which is the product of the *FUT3* gene (*Lewis* gene). The presence of three *FUT3* gene single nucleotide polymorphisms (SNPs) (*59T > G*; *508G > A* and *1067T > A*) was related to the Lewis phenotype of erythrocytes from 185 individuals of Japanese ancestry living in the town of Tomé-Açu in the Brazilian Amazon region. This relationship was detected using a serological hemagglutination test and the *Dot*-ELISA assay along with the molecular technique PCR-RFLP. We found that the three SNPs investigated in this study only accounted for a proportion of the Lewis-negative phenotype of the erythrocytes.

*Key words:* Lewis blood groups, *FUT3*, Japanese, PCR-RFLPs, SNPs.

Received: May 9, 2006; Accepted: September 13, 2006.

### Introduction

The *Lewis* (*FUT3* or *Le*) gene is located on the short arm of chromosome 19 (Mollicone *et al.*, 1990, 1992) and codes for  $\alpha$ 1-3/4-L-fucosyltransferase III (FucT III, also called the Lewis enzyme), an enzyme with 361 amino acids which catalyzes the transfer of a fucose unit to the subterminal GlcNAc unit of type 1 precursory chains (De Vries *et al.*, 2001). The Lewis blood group system is comprised of two major antigens, one being the monofucosylated Le<sup>a</sup> antigen formed by the action of FucT III on a type 1 precursor and the other the difucosylated Le<sup>b</sup> antigen produced by the epistatic interaction between the *FUT3* and *secretor* (*FUT2*) gene products on a type 1 precursor in tissues such as salivary glands, digestive mucosa and respiratory mucosa (Watkins, 1980; Oriol *et al.*, 1986).

The Lewis antigens are not synthesized in erythrocyte progenitors, erythrocytes acquiring their Le<sup>a</sup> and Le<sup>b</sup> epitopes by adsorbing Lewis antigenic glycosphingolipids from

plasma. Accordingly, typing of Lewis phenotypes is difficult and is sometimes misjudged because of weak hemagglutination as a result of the low specificities of anti-Le<sup>a</sup> and anti-Le<sup>b</sup> antibodies and the low number of antigens on red cells, resulting in low titers. (Nishihara *et al.*, 1993; Liu *et al.*, 1996).

The situation is even more complex due to the presence of *FUT3* single nucleotide polymorphisms (SNPs) which can result in nucleotide mutations that generate enzymes which may have different catalytic activities or even be inactive (Nishihara *et al.*, 1993, 1994; Kudo *et al.*, 1996; Cakir *et al.*, 2002; Cooling and Gu, 2003; Soejima *et al.*, 2004; Jost *et al.*, 2005). The most frequent *FUT3* SNPs are the *202T > C*, *314C > T*, *508G > A* and *1067T > A* polymorphisms (*le* genes) which affect and inactivate the catalytic domain of FucT III enzyme (Nishihara *et al.*, 1993, 1994; Kudo *et al.*, 1996), while a further SNP, *59T > G*, is known to cause the substitution of an amino acid in the transmembrane region but its effect on enzymatic activity has not yet been defined (Elmgren *et al.*, 1996).

The *FUT3* *202T > C*; *314C > T* and *59T > G* polymorphisms have been reported in all populations in which

this gene has been studied (Elmgren *et al.*, 1993, 1996; Mollicone *et al.*, 1994; Nishihara *et al.*, 1994; Kudo *et al.*, 1996; Ørntoft *et al.*, 1996; Liu *et al.*, 1999; Cakir *et al.*, 2002), while the *508G > A* and *1067T > A* polymorphisms have been identified at a high frequency in Japanese populations (Koda *et al.*, 1993; Nishihara *et al.*, 1994).

In 1929, the first Japanese colonists to immigrate to the Northern region of Brazil arrived in the state of Pará and settled predominantly in areas near the town of Tomé Açu, there initially being 189 people divided into 43 families which settled permanently in the area supported by incentives provided by the state government for developing agricultural activities. As of 2006, about two percent of the inhabitants of Tomé Açu are of Japanese ancestry (SEPOF, 2006).

The aim of the present study was to establish the frequency of three *FUT3* SNPs and investigate the expression of red blood cell Lewis antigens in a population of Japanese ancestry in the Northern region of Brazil.

## Materials and Methods

### Participants

Blood samples were obtained from 185 apparently healthy individuals randomly selected (134 females, 51 males; median age 46, range 20 to 88 years) of Japanese ancestry living in the town of Tomé-Açu (02°25'0" S; 48°09'09" W) in the northeastern region of the Brazilian state of Pará. The participants in this study were either born in Japan or had both parents and all grandparents born in Japan. There were no kinship ties between the participants and their paternal origin was from 22 different Japanese cities situated on the three main Japanese islands of Hokkaido, Honshu and Kyushu.

This study was approved by the NMT - Federal University of Pará Ethics Research Committee (protocol number 005/2000) and all individuals gave their informed consent to participate in the study.

### Peripheral blood samples

From each participant we collected 5 mL of peripheral blood in tubes containing EDTA. Erythrocytes were subjected to Lewis blood group typing, and genomic DNA was extracted from white blood cells using the QIAamp DNA Blood Mini kit (QIAGEN, Inc. Valencia, CA, USA).

### Typing of Lewis red blood group phenotypes

Lewis blood group phenotypes of erythrocytes were determined using anti-Le<sup>a</sup> and anti-Le<sup>b</sup> monoclonal antibodies (Ortho Diagnostic System, Raritan, NJ, USA) and hemagglutination tests according to the manufacturer's instructions. If a Lewis negative reaction had occurred in previous hemagglutination tests, the phenotypic expression of Lewis antigen on erythrocytes was reexamined using the dot-blot-ELISA assay (Pflug *et al.*, 1989) and the same

batches of monoclonal antibody used for the hemagglutination tests. The red cell suspensions were prepared at a concentration of 1:2, 1:4 and 1:10 in distilled water and 5 µL of appropriate dilution were spotted onto nitrocellulose membranes (Schleicher & Schuell, Dassel - Germany). Appropriate controls were used at all stages of testing.

### SNP Screening

The *FUT3* genotype was assigned based on the detection of the *59T > G*, *508G > A* and *1067T > A* SNPs by the PCR restriction fragment length polymorphism (PCR-RFLP) method. The primers used (Invitrogen, São Paulo - Brasil) being listed in Table 1.

The DNA amplification were performed in a final volume of 25 µL of LA Taq buffer (Perkin Elmer, USA) containing 2.5 mM MgCl<sub>2</sub>, 20 mM of dNTPs, 25 pmol of each primer, 1 µL of Taq polymerase (Perkin Elmer, USA), and 40 ng of sample genomic DNA as template. The PCR cycles for the first, second and third set of primers in Table 1 consisted of initial denaturing at 94 °C for 5 min followed by 35 cycles of denaturing at 94 °C for 30 s, annealing at 65 °C for 30 s and extension at 72 °C for 1 min.

To detect the *FUT3 1067T > A* SNP, one µL of the product from the first PCR was used as the template for a nested PCR (denominated the 'second PCR' in Table 1) which was carried out under the same conditions the first PCR, except that the fourth primer set in Table 1 was used and the annealing temperature was 60 °C.

The *FUT3* PCR products were digested by three different restriction endonucleases: *MspI* for the *59T > G* SNP products; *PvuII* for the *508G > A* SNP products; and *HindIII* for the *1067T > A* SNP products. The *FUT3* digestion products were separated on 7% (w/v) polyacrylamide gel (GibcoBRL, New York - USA) for the *59T/59G* and *508G/508A* SNP products and 10% (w/v) polyacrylamide gel for the *FUT3 1067T/1067A* SNP products. The electrophoresis was carried out using 10x TEB buffer (90 mM Tris; 2 mM EDTA, 90 mM boric acid pH 8.6), at 150 V, 25 mA, for 5 h and then silver stained (17% solution of silver nitrate). The primers, restriction enzymes and fragment sizes produced after PCR and digestion are listed in Table 1.

### Statistical analysis

Data was analyzed by calculating the Hardy-Weinberg Equilibrium and applying the chi-squared ( $\chi^2$ ) heterogeneity test using the CLUMP computer program (Sham and Curtis, 1995) and BioEstat 3.0 (Ayres *et al.*, 2003). The frequencies of the haplotypes were estimated using the ARLEQUIN version 2.000 software.

## Results

We were successful in determining the Lewis genotype using PCR-RFLP detection of the *59T > G*, *508G > A*

**Table 1** - *FUT3* gene single nucleotide polymorphism (SNP), PCR primer sequences and fragment sizes in base pairs (bp) before and after digestion by each restriction enzyme. The *1067T > A* SNP was treated with restriction enzyme only after the second PCR.

SNPs	Primers (5'-3')	Fragment sizes after PCR (bp)	Enzyme	Fragment sizes after digestion (bp)	
First PCR					
<i>59T &gt; G</i>	CCATGCCGCGCTGTCTGGCCGCC AGTGGCATCGTCTCGGGACACACG	93	<i>Msp</i> I	<i>59T</i> = 93	<i>59G</i> = 25+68
<i>508G &gt; A</i>	ACTTGGAGCCACCCCTAACTGCCA TGAGTCCGGCTTCCAGTTGGACACC	206	<i>Pvu</i> II	<i>508G</i> = 206	<i>508A</i> = 102+104
<i>1067T &gt; A</i> (1° PCR)	ATGATGGAGACGCTGTCCCGGTACAAGTT CGGCCTCTCAGGTGAACCAAGAAGCT	400	-	-	-
Second PCR					
<i>1067T &gt; A</i> (2° PCR)	CGCTCCTCAGCTGGGCACTGGA CGGCCTCTCAGGTGAACCAAGAAGCT	109	<i>Hind</i> III	<i>1067T</i> = 109	<i>1067A</i> = 24+85

and *1067T > A* SNPs. In addition, we detected five different *FUT3* haplotypes, estimated using the ARLEQUIN program (Table 2).

Our study of Lewis phenotype showed that 44 of the 185 individuals evaluated using the hemagglutination test and dot-ELISA assay were negative for Lewis antigen expression in erythrocytes (Table 3).

The complexity of the polymorphism profiles obtained in our study is supported by the observation that 20 out of 44 of the individuals with the Lewis negative phenotype had the *FUT3 59G* allele associated with the *FUT3 508A* or *FUT3 1067A* alleles in *le<sup>59, 508</sup>/le<sup>59, 508</sup>*, *le<sup>59, 508</sup>/le<sup>59, 1067</sup>* and *le<sup>59, 1067</sup>/le<sup>59, 1067</sup>* genotypes. The others 24/44 individuals with the Lewis negative phenotype presented the *Le/Le*, *Le/le<sup>59, 508</sup>* and *Le/le<sup>59, 1067</sup>* genotypes (Table 3).

We also compared the *FUT3* Lewis genotype frequencies found in the current study with those reported by other authors and found that the frequencies obtained by us in the present study (Table 4) were similar to the frequency distributions ( $\chi^2_4 = 2.603$ ;  $0.90 > p > 0.80$ ) reported by Liu *et al.* (1996) in a Japanese population, although the authors

**Table 2** - *FUT3* gene haplotypic frequencies and their standard deviations (SD) identified using the Arlequin program.

Haplotypes	Frequency $\pm$ SD
<i>Le</i>	0.6405 $\pm$ 0.0250
<i>le<sup>59</sup></i>	0.0189 $\pm$ 0.0071
<i>le<sup>59, 508</sup></i>	0.2622 $\pm$ 0.0023
<i>le<sup>59, 1067</sup></i>	0.0676 $\pm$ 0.0131
<i>le<sup>59, 508, 1067</sup></i>	0.0108 $\pm$ 0.0054
Total	1.0000

did not cite the location of the population. But were significantly different to the frequencies reported by Liu *et al.* (1999) for two Chinese populations, one from Shenyang ( $\chi^2_4 = 15.603$ ;  $p < 0.05$ ) and the other from Guangzhou ( $\chi^2_4 = 15.993$   $p < 0.05$ ).

In our study the observed *Le/Le* genotype frequency was 39.46% (Table 4), significantly lower ( $p < 0.001$ ) compared with the 61.72% (Table 4) reported by Liu *et al.*

**Table 3** - Association between the *FUT3* gene SNP genotype frequencies and the Lewis (*Le*) erythrocyte phenotypes for a sample population (n = 185) of Brazilians of Japanese ancestry. The *le<sup>59</sup>* genotype represents the *59G* allele while *le<sup>59, 508</sup>* represents the association of the *59G* and *508A* alleles on the same chromosome and *le<sup>59, 1067</sup>* the association of the *59G* and *1067A* alleles on the same chromosome.

Lewis genotype	Frequency (n) of erythrocyte phenotypes			Total	
	Le (a+b-)	Le (a-b+)	Le (a-b-)	Frequency (n)	%
<i>Le/Le</i>	10	57	6	73	39.46
<i>Le/le<sup>59</sup></i>	1	1	0	2	1.08
<i>Le/le<sup>59, 508</sup></i>	12	44	17	73	39.46
<i>Le/le<sup>59, 1067</sup></i>	4	11	1	16	8.65
<i>le<sup>59, 508</sup>/le<sup>59, 508</sup></i>	0	0	12	12	6.49
<i>le<sup>59, 1067</sup>/le<sup>59, 1067</sup></i>	0	0	4	4	2.16
<i>le<sup>59, 508</sup>/le<sup>59, 1067</sup></i>	0	0	4	4	2.16
<i>le<sup>59</sup>/le<sup>59, 1067</sup></i>	0	1	0	1	0.54
Total	27	114	44	185	100

**Table 4** - Comparison of the genotype frequencies of the three *FUT3* gene SNPs analyzed in the current study (n = 185) and in other populations described in the literature. The *le*<sup>59</sup> genotype represents the *FUT3 59G* allele while *le*<sup>59, 508</sup> represents the association of the *FUT3 59G* and *FUT3 508A* alleles on the same chromosome and *le*<sup>59, 1067</sup> the association of the *FUT3 59G* and *FUT3 1067A* alleles on the same chromosome.

Genotypes	Population											
	Japanese <sup>1</sup>		Japanese <sup>2</sup>		Chinese <sup>3</sup>		Chinese <sup>4</sup>		African <sup>5</sup>		Caucasian <sup>6</sup>	
	Frequency											
	n	%	n	%	n	%	n	%	n	%	n	%
Le/Le	73	39.46	55	36.91	79	61.72	72	49.32	23	33.33	47	67.14
Le/ <i>le</i> <sup>59</sup>	2	1.08	0	0	2	1.56	5	3.43	0	0	2	2.85
Le/ <i>le</i> <sup>59, 508</sup>	73	39.46	47	31.54	30	23.44	32	21.92	31	44.93	1	1.43
Le/ <i>le</i> <sup>59, 1067</sup>	16	8.65	23	15.44	8	6.25	21	14.39	2	2.90	16	22.86
<i>le</i> <sup>59, 508</sup> / <i>le</i> <sup>59, 508</sup>	12	6.49	15	10.07	2	1.56	1	0.68	10	14.49	0	0
<i>le</i> <sup>59</sup> / <i>le</i> <sup>59, 508</sup>	0	0	0	0	0	0	2	1.37	0	0	0	0
<i>le</i> <sup>59, 1067</sup> / <i>le</i> <sup>59, 1067</sup>	4	2.16	3	2.01	1	0.70	4	2.74	0	0	1	1.43
<i>le</i> <sup>59, 508</sup> / <i>le</i> <sup>59, 1067</sup>	4	2.16	5	3.36	5	3.91	6	4.11	3	4.35	1	1.43
<i>le</i> <sup>59</sup> / <i>le</i> <sup>59, 1067</sup>	1	0.54	0	0	0	0	1	0.68	0	0	1	1.43
Le/ <i>le</i> <sup>1067</sup>	0	0	1	0.67	1	0.78	0	0	0	0	1	1.43
<i>le</i> <sup>59, 1067</sup> / <i>le</i> <sup>1067</sup>	0	0	0	0	0	0	1	0.68	0	0	0	0
<i>le</i> <sup>1067</sup> / <i>le</i> <sup>1067</sup>	0	0	0	0	0	0	1	0.68	0	0	0	0
Total	185	100	149	100	128	100	146	100	69	100	70	100

<sup>1</sup>Japanese ancestry population from the Brazilian town of Tomé Açú (current study); <sup>2</sup>Japanese population (Liu *et al.*, 1996); <sup>3</sup>Chinese Shenyang population (Liu *et al.*, 1999); <sup>4</sup>Chinese Guangzhou population (Liu *et al.*, 1999); <sup>5</sup>South African Xhosa population (Pang *et al.*, 1998); <sup>6</sup>South African Caucasian population (Pang *et al.*, 1998).

(1999) for the same genotype in a population from Shenyang, China.

### Discussion

In our study, the genotype frequencies of the *FUT3 59T > G*, *508G > A* and *1067T > A* SNPs was similar to data published for other Asiatic populations, especially Japanese populations (Table 4). This was clearly observable for the *FUT3 59G* and *FUT3 508A* alleles, predominant among oriental populations (Liu *et al.*, 1996), which were present at a high frequency in our population sample (Table 3), reflecting the ethnic origin of the individuals sampled. The frequencies seen in our study were also different from the frequencies obtained for other populations, such as the European population studied by Pang *et al.* (1998) and the Chinese population investigated by Liu *et al.* (1999).

The *FUT3 59G* allele isolated (*le*<sup>59</sup>), found in three individuals in our study, was not described by Liu *et al.*, (1996) in a study involving Japanese populations but was observed at a low frequency in European (Pang *et al.*, 1998) and Chinese (Liu *et al.*, 1999) populations. Conversely, the *FUT3 508A* allele isolated (*le*<sup>508</sup>) was not detected by us but was described by Liu *et al.* (1996) in an individual of Japanese origin. Because they are rare, both the *FUT3 59G* and *FUT3 508A* alleles, may be present in some populations but absent in others of the same ethnic group, due to the low frequency of these alleles, or alternatively, due to the phe-

nomenon of population isolation and other processes that restrict gene flow.

Based on previous studies (Nishihara *et al.*, 1993, 1994; Kudo *et al.*, 1996, Liu *et al.*, 1999), we inferred that individuals who presented the *FUT3 59G* allele associated with the *FUT3 508A* or *FUT3 1067A* allele would present a cis-type chromosomal disposition forming the *le*<sup>59, 508</sup> or *le*<sup>59, 1067</sup> haplotypes. This is because isolated *FUT3 508A* or *FUT3 1067A* alleles, although described, are uncommon, and their association with the *FUT3 59G* allele in a trans arrangement would be extremely rare. When estimating allele and genotype frequencies, this possibility can be discarded, but to eliminate totally this risk direct DNA sequencing is needed, which we did not carry out in our study.

The fact that in our study all the individuals carrying genotypes *le*<sup>59, 508</sup>/*le*<sup>59, 508</sup>, *le*<sup>59, 508</sup>/*le*<sup>59, 1067</sup> and *le*<sup>59, 1067</sup>/*le*<sup>59, 1067</sup> presented a negative Lewis phenotype, confirms that these mutations, especially those situated in the catalytic domain, eliminate enzymatic activity through changing the protein conformation of the enzyme, leading to the inactivity of the *FUT3* gene product. The *le*<sup>59</sup>/*le*<sup>59, 1067</sup> genotype identified by us in a Lewis-positive individual suggests that the *FUT3 59G* allele alone is not capable of eliminating the enzymatic activity of the *FUT3*, although some studies (Elmgren *et al.*, 1993, 1996; Kudo *et al.*, 1996; Liu *et al.*, 1996, 1999) suggest that the presence of this allele may result in an increased susceptibility to the occurrence of the false Lewis-negative phenotype.



Comparison between the three *FUT3* polymorphisms of individuals with Lewis-negative phenotype erythrocytes revealed that only 20 out of 44 presented the *le*<sup>59, 508</sup> and/or *le*<sup>59, 1067</sup> haplotypes related to *FUT3* inactivation in both *FUT3* alleles (Table 4). This implies that there were other *FUT3* polymorphisms which were not detected in our study. However, on the other hand, such a discrepancy may be the result of the difficulty in characterizing the Lewis phenotype in erythrocytes, which may lead to false negatives because of specific biological conditions, as has been mentioned in this paper.

Our findings indicate that the frequency of the Lewis-negative trait, obtained by molecular analysis of the three *FUT3* SNPs studied, was about 10.8% in the population sample studied, which is close to the serological phenotype frequencies previously described for Asiatic populations (Lin-Chu *et al.*, 1988; Liu *et al.*, 1996, 1999).

Lewis antigens are particularly important in kidney transplants because rejection is lower in transplants involving Lewis-compatible donors and receptors (Oriol *et al.*, 1978; Williams *et al.*, 1978; Oriol and Danilovics, 1980; Myser *et al.*, 1985; Blajchman *et al.*, 1985; Delmotte *et al.*, 2002), and the molecular test described in this paper may be helpful in avoiding the false negative results sometimes observed with the serological methods available for typing Lewis antigens.

## Acknowledgments

The authors wish to thank the people who participated in this study and the Brazilian agency CAPES for financial support.

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## Internet Resources

- Arlequin - A software for population genetics data analysis, <http://anthro.unige.ch/software/arlequin/>.
- SEPOF (Secretaria Executiva de Estado de Planejamento, Orçamento e Finanças), [http://www.sepof.pa.gov.br/estatistica/ESTATISTICASMUNICIPAIS/Mesorr\\_Nordeste/TomeAcu/TomeAcu.pdf](http://www.sepof.pa.gov.br/estatistica/ESTATISTICASMUNICIPAIS/Mesorr_Nordeste/TomeAcu/TomeAcu.pdf) (April 10, 2006).

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