Significance of Individual Point Mutations, T202C and C314T, in the Human Lewis (FUT3) Gene for Expression of Lewis Antigens by the Human α(1,3/1,4)-Fucosyltransferase, Fuc-TIII*

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The Lewis α(1,3/1,4)-fucosyltransferase, Fuc-TIII, encoded by the FUT3 gene is responsible for the final synthesis of Lea and Leb antigens. Various point mutations have been described explaining the Lewis negative phenotype, Le(a−b−), on erythrocytes and secretions. Two of these, T202C and C314T originally described in a Swedish population, have not been found as single isolated point mutations so far. To define the relative contribution of each of these two mutations to the Lewis negative phenotype, we cloned and made chimeric FUT3 constructs separating the T202C mutation responsible for the amino acid change Trp68 to Arg, from the C314T mutation leading to the Thr105 to Met shift. COS-7 cells were transfected and the expression of Fuc-TIII enzyme activity and the presence of Lewis antigens were determined. There was no decrease in enzyme activity nor of immunofluorescence staining on cells transfected with the construct containing the isolated C314T mutation compared with cells transfected with a wild type FUT3 allele control. No enzyme activity nor immunoreactivity for Lewis antigens was detected in FUT3 constructs containing both mutations in combination. The T202C mutation alone decreased the enzyme activity to less than 1% of the activity of the wild type FUT3 allele. These results demonstrate, that the Trp68 to Arg substitution in human Fuc-TIII is the capital amino acid change responsible for the appearance of the Le(a−b−) phenotype on human erythrocytes in individuals homozygous for both the T202C and C314T mutations.

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FUT1 to FUT7 are the Genome Data Base (GDB) registered names of the cloned human fucosyltransferase genes, accessible in the EMBL/GenBank data libraries under accession numbers: M35531, U17894, X55378, M58956/M58977/S65161/M65030, MR1485, L01698/M88825, and X78031/U09112/U11282.

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The Lewis histo-blood group system comprises different complex carbohydrate structures, which participate in different biological processes such as embryogenesis, tissue differentiation, tumor metastasis, inflammation, and bacterial adhesion (1). Lea and Leb (2, 3) are the major Lewis antigens found on human erythrocytes. These fucosylated glycosphingolipids are synthesized by exocrine epithelial cells (4) and are secondarily passively adsorbed onto erythrocytes in the peripheral circulation giving these blood cells their Lewis phenotype (5).

It was shown as early as the 1950’s, that the Lewis phenotype of erythrocytes is influenced by the ABH secretor status of the individual (6), and the erythrocyte phenotype is the result of the epistatic interaction of the Lewis (Le-le) and the salivary ABH secretor (Se-se) loci (7). Fucosyltransferases (Fuc-Ts) encoded by genes at these loci compete and interact with each other and with other glycosyltransferases to determine the individual’s final Lewis and secretor phenotypes.

Since 1990, seven human fucosyltransferase genes (FUT1–7) have been cloned, sequenced, and characterized according to acceptor specificities (8–18). The human α(1,2)-Fuc-T gene family comprises FUT1 encoding for the H enzyme and FUT2 encoding for the human secretor α(1,2)-fucosyltransferase (9). FUT3–7 encode for α(1,3)-Fuc-Ts. Two of these human enzymes, Fuc-TIII and Fuc-TV encoded by the FUT3 and FUT5 genes, respectively (10, 14), also express α(1,4)-fucosyltransferase activities (15, 19, 20).

Five main missense mutations have been identified in FUT3. Together they explain the majority of Lewis negative phenotypes on erythrocytes and in secretions. In Indonesians (21) the T1067A mutation, and in Japanese (22, 23) the T1067A and G508A mutations have been identified. These mutations were also found in studies on Swedish individuals (24). In the latter population, two additional FUT3 mutations T202C and C314T (24, 25) were identified, and they constitute the Lewis negative allele Lebα02,314, typically occurring in Swedish Le(a−b−) individuals. These latter mutations have so far not been found separately except for the rare Lebα02,202–214 (24). The T59G missense mutation has been described in studies on Indonesians (21), Japanese (22, 23), and in Sweden (24). The corresponding amino acid is located in the transmembrane region of
Le(a–b–) Phenotype Determined by the FUT3 T202C Mutation

TABLE I

| Antigens | pcDNA1 alone | FUT3/1067/ | FUT3/202/–314 | FUT3/202/2 | FUT3/314 | FUT3/wt |
|----------|-------------|-----------|-------------|-----------|---------|--------|
| Anti-Lea | 0           | 0         | 0           | 0         | 22      | 26     |
| SSEA-1   | 0           | 0         | 0           | 0         | 30      | 25     |
| Anti-sial-Lea | 0       | 0         | 0           | 0         | 4       | 30     |
| KM93     | 0           | 0         | 0           | 0         | 6       | 24     |
| Anti-Leb | 0           | 0         | 0           | 0         | 6       | 14     |
| O95      | 0           | 0         | 0           | 0         | 4       | 25     |
| Anti-sial-Leb | 0       | 0         | 0           | 0         | 6       | 49     |
| 19.9     | 0           | 0         | 0           | 0         | 49      | 33     |

Experimental Procedures

Individuals and Samples—Two individuals (numbers 3 and 5), previously characterized for Lewis erythrocyte phenotype and FUT3 genotype (24, 25) were chosen for this study. Individual number 3 was phenotyped on erythrocytes as Le(a–b–) and genotyped as homozygously mutated for the T202C and C314T FUT3 mutations. Number 5 was phenotyped as Le(a–b–) and had the wild type sequence of FUT3 (10). DNA from one Le(a–b–) individual designated 529 (26), carrying the FUT3 inactive allele lo<sup>c</sup>, was chosen as a negative control.

Molecular Cloning of FUT3 Coding Regions and the Corresponding Expression of Constructs with Wild, Mutated, and Chimeric FUT3 Sequences—PCR was used to amplify the coding region, and immediately adjacent 5′- and 3′-flanking regions of the FUT3 gene of individuals numbers 3, 5, and 529. Genomic DNA (0.25–0.5 μg) was amplified in a PTC-100-96V thermal cycler (MJ Research, Inc., Watertown, MA) using PCR mixtures and reagents as described in Ref. 24 with 25 pmol of each primer. The PCR program included hot start at 85 °C for 10 min, followed by 33 cycles with 15 s at 95 °C, 15 s at 60 °C, and 3 min at 68 °C. The last extension step was elongated 10 min at 68 °C. The sense primer (EL-29 s) 5′-ccagcatcttcttcagatctctgacc-3′ anneals to nucleotides −1 to −20 (10) and contains additional nucleotides (lowercase) at its 5′ end, including an EcoRI and a HindIII (underlined) restriction site. The antisense primer (EL-25as) 5′-aagatctcagcgcaaaagattc-3′ is complementary to nucleotides 1159–1144 (10), and the additional nucleotides (lowercase) at its 5′ end include an EcoRI and a NotI (underlined) restriction site. The 1209-bp PCR products were purified on NuSieve-agarose gels (FMC BioProducts, Rockland, ME) according to Ref. 27 and ligated into the pCRII TA-cloning vector (Invitrogen Corp., San Diego, CA) or the pTA cloning vector (R&D Systems, Abingdon, United Kingdom). Positive clones were selected by blue-white screening. Sequencing of TA clones was done as described (24, 25). One clone without PCR induced errors of each individual was chosen for subcloning of its FUT3 insert into the mammalian expression vector pcDNA1/Amp (Invitrogen). For subcloning of FUT3 inserts, HindIII (New England Biolabs, Beverly, MA) and NotI (Boehringer Mannheim Scandinavia AB, Bromma, Sweden) were used for double digests of the three selected TA clones and pcDNA1/Amp. The latter was also treated with alkaline phosphatase (Boehringer Mannheim). The FUT3 inserts and pcDNA1/Amp were purified on SeaPlaque-agarose gels (FMC BioProducts) and the purified FUT3 fragments were subcloned into the purified restricted (HindIII-NotI) expression vector using T4 DNA ligase (Boehringer Mannheim).

The chimeric FUT3 vector constructs were made by restriction cleavages of the two selected TA-clones of individuals numbers 3 and 5, respectively. Double digest (HindIII-NotI) created a 1188-bp fragment, which was purified on NuSieve-agarose gel and subsequently cleaved by BsaOI (Promega, Madison, WI). BsaOI cleaved only at nucleotide position 288 in the coding region of FUT3 and created two different fragments, 313 and 875 bp, respectively. These fragments were purified on NuSieve-agarose gels and the 313-bp fragment of individual number 3 was ligated to the 875-bp fragment of individual number 5 together with restricted (HindIII-NotI) pcDNA1/Amp. This created a pcDNA1/Amp–FUT3 construct with the isolated T202C mutation. To make the construct with the isolated C314T mutation, the longer fragment of individual number 3 and the shorter fragment of individual number 5 were ligated into the restricted expression vector. E. coli Strains DH5<sup>a</sup> or HB101 Were Transformed by the Different pcDNA1/Amp–FUT3 constructs and cultured in large scale. All different plasmid constructs were prepared and purified by two consecutive CsCl/ethidium bromide equilibrium ultracentrifugations (28) and sequenced completely once more (24, 25), using AmpliTaq DNA polymerase FS kit (Perkin-Elmer), to verify the presence of a full-length FUT3 insert in the right direction to the cytomegalovirus promoter and the expected point mutations of each construct.

Transfection—COS-7 cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 20% heat-inactivated fetal calf serum and transfected with 10 μg of expression vector constructs using the DEAE-dextran method (29). The cells were selected for transfection with pcDNA1 (CLONTECH Laboratories, Inc.) without insert as a negative control and with the different pcDNA1/Amp–FUT3 constructs. These different constructs were the FUT3 wild type, the T202C and C314T mutations in combination, the isolated T202C mutation, the isolated C314T mutation, and finally the T1067A isolated mutation. One μg of expression vector containing the coding region of the bacterial chloramphenicol acetyltransferase (pcDM5-CAT) (30), was simultane-ously transfected to allow for normalization of transfection efficiency. Transfected cells were harvested after a 48-h growth period.

Immunofluorescent Expression of Lewis Antigens on the Surface of Transfected COS-7 Cells—The transfected cells were trypsinized, washed, and labeled with different antibodies. Mouse monoclonal anti-Le<sup>a</sup> antibodies used were: 82H5 (Chembiomed, Alberta Research Council, Edmonton, Canada) and SSEA-1 (Valbiotech, Paris, France). Mouse monoclonal anti-sialyl-Le<sup>a</sup> used was KM93 (Valbiotech). Mouse monoclonal anti-sialyl-Le<sup>b</sup> was 19.9 (from J. Bara (INSERM U55, St. Antoine Hospital, Paris, France)).

After 30 min incubation with the first antibody, the cells were washed with phosphate-buffered saline and incubated another 30 min with affinity purified fluorescein isothiocyanate-conjugated sheep anti-mouse immunoglobulins as second antibodies (Pasteur Diagnostics, Marnes la Coquette, France). After labeling with the second antibody the cells were washed with phosphate-buffered saline and fixed with 10 μl of 4% phosphate-buffered saline-parafomaldehyde. Five μl of Mowiol 4:80 ( Hoechst, Frankfurt am Main, Germany) were added be-
fore mounting under coverslides. The cells were observed and counted under a Leitz SM-LUX epifluorescence microscope (34, 35).

**Fucosyltransferase Assay—**Enzyme activity was measured at 37 °C in 30-μl aliquots of the 1% Triton X-100 protein extracts of COS-7 cells transfected with the pcDNA1 vector alone and containing the different FUT3 constructs with the T202C, C314T, and T1067A mutations. Fucosyltransferase activities were determined at initial velocity (14 h for the 202 mutated construct) for GDP-fucose and H type 1.

Enzyme kinetics were determined at initial velocity for GDP-fucose and H type 1. Incorporation of [14C]fucose (cpm) onto the H type 1 oligosaccharide acceptor as a function of incubation time, by 30 μg of protein homogenates of COS-7 cell extracts transiently transfected with pcDNA1 alone, with the constructs of FUT3 wild type or mutated at positions 202, 314, or 1067 (in parentheses). These acceptors were: (i) the disaccharide precursor of type 1, 1-O-(CH2)8COOCH3 (Chembiomed) by the Sep-Pak C18 product isolation procedure (36, 37). Enzyme kinetics were determined at initial velocity (15 min for the 314 mutated and the wild type FUT3 constructs and 14 h for the 202 mutated construct) for GDP-fucose and H type 1.

**RESULTS**

**Molecular Cloning of Wild, Mutated, and Chimeric FUT3 Sequences—**One clone of each pcDNA1/Amp-FUT3 construct used for transfection studies was sequenced. This confirmed that all constructs contained the complete coding region of the human FUT3 gene in the right direction to the cytomegalovirus promoter. The pcDNA1/Amp-FUT3 (wt) construct was verified to have the FUT3 wild type sequence. The pcDNA1/Amp-FUT3(1067) and the pcDNA1/Amp-FUT3(202–314) constructs contained the mutations T1067A, and T202C and C314T, respectively. The chimeric constructs, pcDNA1/Amp-FUT3(202) and pcDNA1/Amp-FUT3(1067) contained each of the isolated mutations on a FUT3 wild type background, and also the correct sequence over the BsaI restriction site, respectively.

Expression of pcDNA1/Amp-FUT3 Wild, Mutated, and Chimeric Constructs—Immunofluorescence results are summarized in Table I. Carbohydrate epitopes Le+, sialyl-Le+, Lea+, and sialyl-Lea+ were all found on COS-7 cells transiently transfected with the pcDNA1/Amp-FUT3(314) construct. The pcDNA1/Amp-FUT3(wt) construct was used as a positive control and the percentage of positive cells and the intensity of the fluorescence were of the same order of magnitude for both these constructs. However, a dramatic decrease (>100-fold) of Vmax was observed for the 202 mutated construct as compared with the other two constructs (Table IV).

**Table II**

| Transfected constructs | Incorporation of [14C]fucose (cpm) |
|------------------------|----------------------------------|
|                        | Galβ1,3GlcNAc | Fucα1,2Galβ1,3GlcNAc |
| pcDNA1 alone           | 0              | 0                     |
| FUT3(1067)             | 0              | 0                     |
| FUT3(202–314)          | 0              | 0                     |
| FUT3(202)              | 50             | 310                   |
| FUT3(314)              | 20,500         | 51,000                |
| FUT3(wt)               | 18,800         | 49,700                |

**DISCUSSION**

The importance of the Le−202–314 allele either in homozygous or in heterozygous state in conjunction with an additional Le− allele, for the appearance of the Le(a−b−) phenotype on erythrocytes was originally demonstrated in a Swedish population (24). This has also recently been observed in studies on French individuals from the Reunion island. The significance of this allele was recently confirmed in expression and enzymatic studies on transfected COS-7 cells (38), where some of our own unpublished FUT3-primers were used for PCR amplifications.

**Table III**

| Transfected constructs | Incorporation of [14C]fucose (cpm) |
|------------------------|----------------------------------|
|                        | 15 min  | 1 h         | 2 h         | 6 h         | 21 h        |
| pcDNA1 alone           | 0       | 0           | 0           | 0           | 0           |
| FUT3(1067)             | 0       | 0           | 0           | 0           | 0           |
| FUT3(202–314)          | 0       | 15          | 68          | 150         | 380         |
| FUT3(202)              | 140     | 380         | 830         | 2,540       | 8,680       |
| FUT3(314)              | 13,340  | 51,000      | 62,500      | 81,400      | 79,600      |
| FUT3(wt)               | 11,260  | 46,000      | 60,200      | 80,200      | 79,000      |
mutation from the C314T mutation, we have now shown that the former mutation (T202C) is the essential one for the \( Le(a-b-) \) phenotype on transfected COS-7 cells and most probably also on human erythrocytes.

The \( T \) to \( C \) mutation at nucleotide 202 induces a major amino acid change, tryptophan to arginine at amino acid position 68. This Trp\(^{68} \) position of the Fuc-TIII enzyme is in a highly hydrophobic area (10, 39–41) and a shift to a charged hydrophilic amino acid such as Arg in this position may induce changes in the folding of the protein which might be responsible for the dramatic decrease of the \( V_{\text{max}} \) of the mutated enzyme. The relative significance of the two substitutions at amino acids 68 and 105 versus the wild type enzyme are illustrated in the Hopp and Woods hydropathicity plots in Fig. 1 (42). From these plots it is obvious that the Trp\(^{68} \) → Arg has a major effect while the Thr\(^{105} \) → Met has only a minor effect on this character of the Fuc-TIII enzyme. However, it is also worth noticing that the naturally occurring allele, \( le^{202-314} \), in our extended enzymatic studies encodes for a protein that was about 20 times less active than that encoded for by the 202 mutated construct and more than 200 times less active than the wild type enzyme. Thus, there seems to be a synergistic negative effect on the protein activity of these two mutations which contrasts to the single point mutation at nucleotide 1067 resulting in a protein without any measurable enzyme activity. The molecular explanation for these results will have to await experimental data on the three-dimensional structure of the human Lewis Fuc-TIII enzyme and its interaction with the Golgi membrane, neighboring proteins, GDP-fucose, and the acceptor substrates.

It was hypothesized in this latter study that the C314T mutation of the \( le^{202-314} \) allele might be the more important of the two mutations for introducing the Lewis negative phenotype on erythrocytes.

In an earlier study the acceptor specificities of Fuc-TIII, Fuc-TV, and Fuc-TV\(^{I} \) were shown to be dependent on the variation of 11 amino acid residues in a region corresponding to the Fuc-TIII amino acids 103–153 (39). The Fuc-TIII amino acid Thr\(^{105} \) is indeed located in this region but it was not identified as one of these 11 amino acids affecting the enzyme substrate specificity. A more recent study using truncated forms of Fuc-TIII and Fuc-TV demonstrated that only constructs containing amino acids 62–361 of Fuc-TIII and 76–374 of Fuc-TV were active, whereas shorter forms of the enzymes were inactive (40). This supports the results of the present study that amino acid Trp\(^{68} \) of Fuc-TIII (and the corresponding Trp81 of Fuc-TV) are indeed amino acids necessary for enzymatic activity of these fucosyltransferases.

We have in parallel cloned and sequenced the corresponding \( FUT3, FUT5, \) and \( FUT6 \) genes of the chimpanzee\(^{3} \) and the bovine \( FUT8 \) gene (35), and aligned these sequences with the human \( FUT3, FUT5, \) and \( FUT6 \) sequences. The tryptophan residue at position 68, which is affected by the T202C mutation of human Fuc-TIII, is conserved in the corresponding positions of the three human, three chimpanzee, and bovine Fuc-Ts. In contrast, the threonine residue at position 105, affected by the C314T mutation of human Fuc-TIII, is conserved in the chimpanzee Fuc-TIII, but not in the human or chimpanzee Fuc-TV\(^{s} \) or Fuc-TV\(^{I} \)s, nor in the bovine Fuc-T enzyme where an alanine residue was identified at the corresponding positions.

By using chimeric \( FUT3 \) constructs, separating the T202C

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\(^{3}\) M. Costache, P. A. Apoil, A. Cailleau, A. Elmgren, G. Larson, S. Henry, A. Blancher, D. Jordachescu, R. Oriol, and R. Mollicone, manuscript in preparation.
Le(a⁻b⁻) Phenotype Determined by the FUT3 T202C Mutation

J. A., and Struhl, K. (1987) Current Protocols in Molecular Biology, John Wiley & Sons, Inc., New York
29. Davis, L. G., Dibner, M. D., and Battey, J. F. (1986) Basic Methods in Molecular Biology, Elsevier Science Publishing Co., New York
30. Berger, S. L., and Kimmel, A. R. (1987) Methods in Enzymology: Guide to Molecular Cloning Techniques, Academic Press, NY
31. Good, A. H., Yau, O., Lamontagne, L. R., and Oriol, R. (1992) Vox Sang. 62, 180–189
32. Chester, M. A., Johnson, U., Lundblad, A., Löw, B., Messeter, L., and Samuelsson, B. (1990) Proceedings of the Second International Workshop on Monoclonal Antibodies against Human Red Blood Cells and Related Antigens, April 1–4, 1990, Lund, Sweden
33. Mollicone, R., Cailleau, A., Imberty, A., Gane, P., Perez, S., and Oriol, R. (1996) Glycoconj. J. 13, 263–271
34. Candelier, J. J., Mollicone, R., Mennesson, B., Bergemer, A. M., Henry, S. M., Coulin, P., and Oriol, R. (1993) Lab. Invest. 69, 449–459
35. Oulmouden, A., Wierinckx, A., Petit, J.-M., Costache, M., Palcic, M. M., Mollicone, R., Oriol, R., and Julien, R. (1997) J. Biol. Chem. 272, 8764–8773
36. Mollicone, R., Ghazal, A., Francois, A., Ratcliffe, M., and Oriol, R. (1990) Eur. J. Biochem. 191, 169–176
37. Palcic, M. M., Heerze, L. D., Pierce, M., and Hindsigal, O. (1988) Glycoconj. J. 5, 49–63
38. Ørntoft, T. F., Vestergaard, E. M., Holmes, E., Jakobsen, J. S., Grunnet, N., Mortensen, M., Johnson, P., Bross, P., Gregersen, N., Skorstengaard, K., Jensen, U. B., Bolund, L., and Wolf, H. (1996) J. Biol. Chem. 271, 32260–32268
39. Legault, D. J., Kelly, R. J., Natsuka, Y., and Lowe, J. B. (1995) J. Biol. Chem. 270, 20987–20996
40. Xu, Z., Vo, L., and Macher B. A. (1996) J. Biol. Chem. 271, 8818–8823
41. Breton, C., Oriol, R., and Imberty, A. (1996) Glycoconjug. J. 13, vii-xii
42. Hopp, T. P., and Woods, K. R. (1981) Proc. Natl. Acad. Sci. U. S. A. 78, 3824–3828