Two new *FUT2* (fucosyltransferase 2 gene) missense polymorphisms, 739G → A and 839T → C, are partly responsible for non-secretor status in a Caucasian population from Northern Portugal

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Secretor status is defined by the expression of H type 1 antigen on gastric surface epithelium and external secretions. The H type 1 structure, and other fucosylated carbohydrates (Le^a, sialyl-Le^a, Le^b, Le^x, sialyl-Le^x and Le^y), can serve as ligands for several pathogens, including *Helicobacter pylori*, and are cancer-associated antigens. Secretor individuals are more susceptible to some bacterial and viral infections of the genito-urinary and digestive tracts. The aim of the present study was to examine *FUT2* (fucosyltransferase 2 gene) polymorphisms in a Caucasian population of non-secretor individuals ($n = 36$) from northern Portugal and to evaluate the activity of the mutant *FUT2* enzymes. The secretor status was determined by UEAI [*Ulex europaeus*

(gorse) lectin] histochemistry in gastric mucosa, and *FUT2* polymorphisms were studied by restriction-fragment-length polymorphism and direct sequencing. The majority of non-secretors (88.9 %) were homozygous for 428G → A polymorphism; 5.6 % were homozygous for 571C → T and 5.6 % were homozygous for two new missense polymorphisms, 739G → A (2.8 %) and 839T → C (2.8 %). By kinetic studies it was demonstrated that the two new *FUT2* mutants (739G → A and 839T → C) are almost inactive and are responsible for some non-secretor cases.

Key words: α -1,2-fucosyltransferase, non-secretor, polymorphism.

INTRODUCTION

The secretor status is defined by the presence of H type 1 antigen in body secretions such as milk and saliva [1]. H type 1 antigen belongs to both the Lewis and the ABO(H) histo-blood-group systems and is expressed in erythrocyte membranes and in several epithelial tissues, namely the gastric mucosa, the upper respiratory tract and the lower genito-urinary tract [1,2]. Although the synthesis of H type 1 antigen is dependent on the sequential action of several glycosyltransferases, the secretor enzyme (*FUT2*), an α -1,2-fucosyltransferase, is responsible for the transfer of fucose in an α -1,2 linkage to form the terminal H type 1 structure [3–6]. The cell-surface fucosylated oligosaccharides participate in several biological processes, such as embryogenesis, tissue differentiation, tumour metastasis, inflammation and bacterial adhesion [7].

Various pathogens, such as *Helicobacter pylori*, *Escherichia coli* and Norwalk virus, have been shown to attach themselves to either α 1,2-fucosylated glycans or to precursor structures masked by fucosylation [8–10] on epithelial cells. Accordingly, several studies pointed out either the secretor or the non-secretor status as risk factors for pathogens that bind to fucosylated or precursor glycans respectively [1,11–16].

FUT2 gene polymorphisms have been described in several worldwide human populations, and some of them are responsible for non-secretor status [2,17–21]. The aim of the present study was to examine *FUT2* polymorphisms in a Caucasian population of non-secretor individuals from Northern Portugal and to evaluate the implications of the mutant *FUT2* enzymes.

MATERIALS AND METHODS

Population

In the present study we evaluated 36 asymptomatic or dyspeptic Caucasian individuals from northern Portugal [22]. All individuals were non-secretors, determined by UEAI [*Ulex europaeus* (gorse) lectin] staining in gastric epithelium. The screening of *FUT2* 428G → A polymorphism was made by RFLP (restriction-fragment-length polymorphism). Individuals that were not homozygous for *FUT2* 428G → A polymorphism were sequenced for the whole coding region of the *FUT2* gene. All individuals gave their informed consent, and the study had the ethical approval of the institutions involved.

PCR-RFLP

PCR was performed using DNA extracted from blood cells as a template. The amplification of a 195 bp *FUT2* fragment was performed by using the forward primer: 5'-GAGGAATACCGCCACATCCCGGGGGAGTAC-3' and the reverse primer: 5'-AGCCGGCCGGGCACCTTTGTAGGGGTCCAT-3'. The 25 μ l final reaction mixture comprised 2 μ l of DNA, 2.5 μ l of 10 $\times$ *Taq* polymerase buffer, 25 pmol of each primer, 3 μ l of dNTPs (10 mM) and 1 μ l of *Taq* polymerase (Amersham). After 5 min of DNA denaturation at 95 °C, PCR was performed in 35 cycles: DNA denaturation, 94 °C, 30 s; annealing at 70 °C, 30 s, and elongation at 72 °C, 1 min; with a final extension at 72 °C for 10 min. The PCR products were verified by electrophoresis in 1.5 % (w/v) agarose/TBE (90 mM Tris/85 mM boric acid/20 mM

Abbreviations used: *FUT2*, fucosyltransferase 2; RFLP, restriction-fragment-length polymorphism; RT, reverse transcription; UEAI, *Ulex europaeus* (gorse) lectin; wt, wild-type.

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EDTA buffer, pH ??) gel and then extracted using a Gel Band DNA Extraction Kit (Amersham). The screening for 428G → A polymorphism was performed by digestion using the restriction endonuclease *Ava*II. The mutated sequence was not digested, and the normal sequence gave rise to two fragments (59 bp and 136 bp).

PCR and sequencing

All non-secretor individuals that did not show homozygosity for the 428G → A inactivating polymorphism ($n = 4$) were screened for rarely described or for new polymorphisms by sequencing the whole coding region of the *FUT2* gene.

The full-length *FUT2* coding sequence was amplified using the forward primer: 5'-ATGCTGGTCGTTTCAGATGCCTTTC-3' and the reverse primer: 5'-CTGTCCCCCTTACTCAAGCACTAA-3'. PCR was performed according to the conditions described above. PCR products were purified using a Gel Band DNA Extraction Kit (Amersham) and used as templates for the sequencing reactions. Each sequencing reaction was performed in a final volume of 5 μ l. The reaction mixture contained 2 μ l of DNA template, 3 pmol of primer and 2 μ l of TRR (terminator ready reaction) mix (ABI Prism® 3100 Sequencer; Applied Biosystems). The sequencing PCR was performed for 30 cycles: DNA denaturation, 94 °C, 30 s; annealing at 50 °C, 30 s, and elongation at 60 °C, 4 min; with a final extension at 60 °C for 20 min. The sequenced products were analysed in the ABI Prism® sequencer.

Construction of expression vectors

To evaluate enzyme activity of wt (wild-type) and polymorphic variants of *FUT2* gene, expression vectors were constructed. To build the wt construct, the full-length *FUT2* gene was amplified using the forward-full-length primer: 5'-ATGAAGCTTATGCTGGTCGTTTCAG-3' and the reverse-full-length primer: 5'-ATGCGGGCCGCGAGTGCTTGAGTAAGG-3' (*Hind*III and *Not*I restriction sites respectively are underlined). The PCR product (1032 bp) was restricted with the endonucleases *Hind*III and *Not*I, gel-purified and inserted into *Hind*III/*Not*I doubly digested pcDNAI. The mutant vector pcDNAI-*FUT2*-739G → A was made using the pcDNA-*FUT2*wt for site-directed mutagenesis using a nested PCR protocol. In the first PCR, two sets of primers were used to construct the mutant, namely the forward-full-length/reverse primer: 5'-GACACCTCCACAGTGATGTGGTGT-3' and forward primer/reverse-full-length primer: 5'-ACACCACATCACTGTGGGAGGTGTC-3'. In the second PCR the full-length mutant was amplified by using forward-full-length primer and reverse-full-length primer. The mutant vector pcDNAI-*FUT2*-839C → T was made by amplifying the mutant gene directly from an individual carrying the 839C → T polymorphism in homozygosity, using forward-full-length primer and reverse-full-length primer. The mutants were cloned into pcDNAI using the same protocol as that described above. The sequence of all constructs was confirmed by direct sequencing.

COS-7 cells

For the transient transfection with the expression vectors, COS-7 cells were grown in Dulbecco's modified Eagle's medium supplemented with 10 % (v/v) foetal-bovine serum. Transfection was performed using the cationic lipid reagent Tfx50 (Promega). A control plasmid encoding β -galactosidase was co-transfected to allow normalization of transfection efficiency. Cell extracts were prepared in 1 % (v/v) Triton X-100/10 % (v/v) glycerol/25 mM sodium phosphate, pH 6, 72 h after transfection. Samples were assayed for β -galactosidase (Promega) and α -1,2-

fucosyltransferase. Protein concentrations from cell extracts were determined by using the BCA (bicinchoninic acid) assay reagent (Pierce Chemical Co.).

α -1,2-Fucosyltransferase activity

Fucosyltransferase assays were performed on a 20 μ l reaction volume containing 3 μ M GDP-[¹⁴C]fucose, 5 mM ATP, 25 mM sodium phosphate, pH 6.0, 40 μ g of total protein from cell extracts and phenyl β -D-galactoside or asialofetuin as acceptor substrates. All assays were performed in duplicate, along with control reaction mixtures lacking acceptor. Reaction mixtures were incubated at 37 °C for 2 h, and termination was achieved by adding 1 ml of water to each microtube. The purification of hydrophobic fucosylated phenyl β -D-galactoside products was made by passage through a C₁₈ reverse-phase column. Radio-labelled asialofetuin products were purified by filtration through microfibre membranes (GF/C; Whatman) and radioactivity measured by liquid-scintillation counting.

To determine the K_m value for GDP-fucose, the reaction mixtures were supplemented with different concentrations of GDP-fucose over the range 3–400 μ M. In these reaction mixtures the phenyl β -D-galactoside concentration was constant (25 mM). The K_m value for phenyl β -D-galactoside was calculated by testing different concentrations (5–50 mM) of the acceptor in reaction mixtures either containing 3 μ M GDP-[¹⁴C]fucose or lacking GDP-fucose. The K_m for asialofetuin was determined by using a range of concentrations from 0.5 to 8 mg/ml.

RESULTS

Four *FUT2* polymorphisms were found in our population (428G → A, 571C → T, 739G → A and 839T → C)

The 428G → A polymorphism was found in homozygosity in 32 (89 %) of non-secretor individuals (Figure 1A). Individuals that were normal for 428G → A polymorphism ($n = 4$; 11 %) (Figure 1B) showed other polymorphisms after sequencing: 571C → T ($n = 2$); 739G → A ($n = 1$), and 839T → C ($n = 1$). The 571C → T polymorphism inactivates enzyme activity and was previously described in Asian populations [21,23]. The polymorphisms 739G → A and 839T → C generate missense polymorphisms [739G → A (²⁴⁷Gly → Ser) and 839T → C (²⁸⁰Phe → Ser)] and are described for the first time in our population.

FUT2-²⁴⁷Gly → Ser and *FUT2*-²⁸⁰Phe → Ser proteins show impaired α -1,2-fucosyltransferase activity

After transfection, COS-7 cells showed high amounts of β -galactosidase that was used as a control for transfection: Cos-7 *FUT2* wt (0.7×10^{-2} unit); Cos-7 *FUT2*-739G → A (10^{-2} unit); Cos-7 *FUT2*-839T → C (0.7×10^{-2} unit) and Cos-7 mock (1×10^{-2} unit). Cos wt cells had 0.006×10^{-2} unit of β -galactosidase. The values for β -galactosidase are very similar in the cells transfected with the different constructs.

The expression of all variants of *FUT2* gene (*FUT2* wt, *FUT2*-739G → A and *FUT2*-839T → C) was confirmed by RT (reverse transcription) PCR (Figure 2). Mock and Cos-7 wt cells had no expression of *FUT2* gene.

Kinetic analysis showed that *FUT2* wt has an apparent Michaelis–Menten constant (K_m) of 50 μ M for GDP-fucose and a V_{max} of 21.23 pmol/h (Figure 3A). The mutants *FUT2*-²⁴⁷Gly → Ser and *FUT2*-²⁸⁰Phe → Ser had higher K_m values (178.6 and 90.9 μ M respectively) and lower V_{max} values (8.66

Figure 1 UEA1 histochemistry and *FUT2* genotype for 428G → A polymorphism in different individuals

(A) Negative for UEA1 and homozygous for 428G → A; (B) negative for UEA1 and normal for 428G → A; (C) positive for UEA1 and normal for 428G → A; and (D) positive for UEA1 and heterozygous for 428G → A. All images have the gastric surface on top.

Figure 2 Detection of *FUT2* expression by RT-PCR

Expression of *FUT2* and of GAPDH, used as an internal control, was detected by RT-PCR in Cos-7 cells either not transfected (Wt), mock transfected (mock) or transfected with variants of human *FUT2* (*FUT2* Wt, *FUT2*-739G → A and *FUT2*-839T → C).

Figure 3 Determination of apparent Michaelis-Menten constant (K_m) and V_{max} for GDP-fucose, phenyl β -D-galactoside and asialofetuin in *FUT2* wt, *FUT2*-²⁴⁷Gly → Ser and *FUT2*-²⁸⁰Phe → Ser

(A) K_m and V_{max} for GDP-fucose in *FUT2* wt; (B) K_m and V_{max} for GDP-fucose in *FUT2*-²⁴⁷Gly → Ser; (C) K_m and V_{max} for GDP-fucose in *FUT2*-²⁸⁰Phe → Ser; (D) K_m and V_{max} for phenyl β -D-galactoside in *FUT2* wt; and (E) K_m and V_{max} for asialofetuin in *FUT2* wt. The polymorphic enzymes *FUT2*-²⁴⁷Gly → Ser and *FUT2*-²⁸⁰Phe → Ser showed higher K_m values and lower V_{max} values for GDP-fucose compared with the *FUT2* wt enzyme. It was not possible to calculate the K_m and V_{max} for phenyl β -D-galactoside and asialofetuin in *FUT2*-²⁴⁷Gly → Ser and *FUT2*-²⁸⁰Phe → Ser, since the reaction mixtures showed very low enzyme activity.

Figure 4 Transfer of GDP-fucose in different concentrations of acceptor and donor substrates for FUT2 wt, mock, FUT2-²⁴⁷Gly → Ser and FUT2-²⁸⁰Phe → Ser

(A) Incorporation of GDP-Fucose in different concentrations of cold GDP-fucose onto phenyl-β-D-galactoside; (B) incorporation of GDP-fucose in different concentrations of phenyl-β-D-galactoside, and (C) incorporation of GDP-fucose in different concentrations of asialofetuin. FUT2 wt enzyme showed high α-1,2-fucosyltransferase activity whereas FUT2-²⁴⁷Gly → Ser and FUT2-²⁸⁰Phe → Ser enzymes were very similar to mock.

and 4.37 pmol/h respectively), showing a lower α-1,2-fucosyltransferase activity under these conditions compared with FUT2 wt (Figures 3B and 3C). The K_m determined for the synthetic acceptor phenyl β-D-galactoside was 8.79 mM and the V_{max} was 0.89 pmol/h (Figure 3D). The α-1,2-fucosyltransferase activity assays using asialofetuin as the acceptor substrate revealed a K_m of 3.33 mg/ml and a V_{max} of 1.4 pmol/h for FUT2 wt (Figure 3E). It was not possible to determine the phenyl β-D-galactoside and asialofetuin K_m and V_{max} values for the mutants (FUT2-²⁴⁷Gly → Ser and FUT2-²⁸⁰Phe → Ser), since the reaction mixtures showed very low enzyme activity. Under all the reaction conditions tested, the cell extracts from mutants Cos-7 FUT2-²⁴⁷Gly → Ser and Cos-7 FUT2-²⁸⁰Phe → Ser exhibited levels of activity very similar to mock and lower than the cell extracts from Cos-7 FUT2 wt (Figure 4).

DISCUSSION

In the present study we have shown that all the non-secretors cases, determined by histology of gastric epithelium using UEAI, were homozygous for one nonsense (428G → A or 571C → T) or missense (739G → A or 839T → C) polymorphism located in the catalytic domain, generating nearly inactive FUT2 enzymes.

The 428G → A polymorphism was found in an allelic frequency of 0.89 in the population of UEAI-negative individuals in the present study. The 428G → A polymorphism was also shown to be the main one responsible for non-secretors in Caucasian populations from the United States [24], Sweden [17,19], Denmark [25] and South Africa [18]. In the case of 571C → T, 739G → A and 839T → C polymorphisms, it was not possible to calculate the allelic frequency, since only four individuals were

screened. As far as we are aware, the 571C → T polymorphism was found in one Caucasian individual from South Africa, in an allelic frequency of 0.005 [18]. Furthermore, it is associated in a relatively low allelic frequency with non-secretors from Indonesia (0.03 [21]), Polynesia (0.13 [17]), Asian [aboriginal Taiwanese (from 0.02 to 0.2 [21]), Chinese (0.005, 0.007 and 0.008 [20,21,26]), Thai (0.006 [21]), Filipino (0.014 and 0.13 [21,27]) and Japanese (0.005 [2]) populations. The two new polymorphisms (*FUT2*-739G → A and *FUT2*-839T → C), identified for the first time in the present study, were found to be almost inactive by kinetic studies. The 739G → A mutation, at least, is not characteristic of the Portuguese population, since we found it in the heterozygous state in two Chinese individuals out of 50 (results not shown). Both *FUT2*-739G → A and *FUT2*-839T → C polymorphisms lead to an exchange between two neutral amino acids, ²⁴⁷Gly → Ser and ²⁸⁰Phe → Ser respectively. These changes can lead to conformational deviations in the enzyme catalytic domain that are incompatible with α-1,2-fucosyltransferase activity. Unfortunately, it is not possible to determine the approximate conformation of the polymorphic enzymes, since the *FUT2* crystallized structure is not known.

The GDP-fucose *K_m* determined for *FUT2* wt (50 μM) is lower than that described by other authors (197 μM) for α-1,2-fucosyltransferase supposedly encoded by the *Secretor* locus [24,28]. The higher affinity of our enzyme for the substrate GDP-fucose may be attributable to the fact that we cloned the full-length *FUT2* and not only the *secretor* enzyme. The polymorphic enzymes (*FUT2*-²⁴⁷Gly → Ser and *FUT2*-²⁸⁰Phe → Ser) had higher *K_m* values, namely 178.6 and 90.9 μM respectively, showing a lower affinity for GDP-fucose. The *K_m* value determined for phenyl β-D-galactoside (8.79 mM) is very close to the values (15.1 and 11.4 mM) determined for α-1,2-fucosyltransferase in other studies [24,28]. Although asialofetuin is a heterogeneous substrate, the α-1,2-fucosyltransferase activity showed a very good correlation, and the *K_m* was 3.33 mg/ml. For both polymorphic enzymes (*FUT2*-²⁴⁷Gly → Ser and *FUT2*-²⁸⁰Phe → Ser) it was not possible to calculate the *K_m* for phenyl β-D-galactoside or for asialofetuin, since the reaction mixtures showed a very low enzyme activity. These results may have been obtained because the α-1,2-fucosyltransferase activity of these mutant proteins, if it exists, is very low, despite occurring in a linear way.

In conclusion, we have described two new polymorphisms in the *FUT2* gene (*FUT2*-739G → A and *FUT2*-839T → C) that lead to the expression of two inactive *FUT2* variants (*FUT2*-²⁴⁷Gly → Ser and *FUT2*-²⁸⁰Phe → Ser), and are responsible for some non-secretor cases in a Portuguese population.

We thank the Fundação para a Ciência e a Tecnologia and Programa Operacional Ciência, Tecnologia, Inovação do Quadro Comunitário de Apoio III. We thank the Estaleiros Navais de Viana do Castelo, Viana do Castelo, Portugal, and Departamentos de Cirurgia B, Gastroenterologia e imunohemoterapia, Hospital S. João, Porto, Portugal, where patients' data and samples were collected. This work was supported by Praxis/P/BIO/12072/1998 project from Fundação para a Ciência e a Tecnologia, Fundação Calouste Gulbenkian (FC-54918), Luso American Foundation for Development (project 173/2002), and by a grant from the Nantes University Hospital (DRC 02/2P).

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Received 13 May 2004/1 July 2004; accepted 13 July 2004

Published as BJ Immediate Publication 14 July 2004, DOI 10.1042/BJ20040803