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Genetic variability of the fructosamine 3-kinase gene in diabetic patients

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Abstract

Background: Nonenzymatic glycation appears to be an important factor in the pathogenesis of diabetic complications. Fructosamine 3-kinase (*FN3K*), initially identified in erythrocytes, appears to be responsible for the removal of fructosamine from proteins, suggesting a protective role in nonenzymatic glycation. Recently, genetic variants in the *FN3K* gene have been studied in diabetic patients. The aim of our study was the molecular characterization of the *FN3K* gene in a representative group of Italian patients with type 1 (T1DM) and 2 (T2DM) diabetes mellitus and in a cohort of healthy controls.

Methods: Seventy diabetic subjects (35 type 1 and 35 type 2) with stable glycemic control and 33 healthy control subjects were evaluated using PCR and direct sequencing of the *FN3K* gene. Denaturing high performance liquid chromatography (DHPLC) was used in controls for screening for the presence of the genetic variants previously found in diabetic patients.

Results: Seven different genetic variants were identified, five of them already reported and two new: the p.R187X and p.Y239C mutations identified in two females affected by T2DM. No significant association was found between certain polymorphisms and diabetes conditions. Preliminary haplotype studies are also reported. With respect to genotypes, we noted that some were not present in all the investigated cohort, and some were found related to higher glycated hemoglobin compared to others, although not at a significant level, probably because of the small number of subjects investigated.

Conclusions: In conclusion, this study identified two new mutations and additional variants within the *FN3K* gene. This is the first study on *FN3K* in Italy. Future work is needed to achieve a better understanding of the FN3K enzyme and its possible clinical utility in the management of diabetic patients.

Keywords: diabetes; fructosamine 3-kinase (FN3K); glycated hemoglobin; mutation analysis.

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Introduction

Fructosamine 3-kinase gene (*FN3K*) (NC_000017.10), located on chromosome 17q25.3 and organized in six exons, codes for a 34 kDa protein expressed in every human tissue. The greatest expression of the protein is in diabetes-susceptible organs, such as the kidneys, heart and nervous tissue (1). This enzyme is involved in the control of intracellular nonenzymatic glycation, a complex process that proceeds through many stages and ultimately leads to the formation of irreversible end products collectively designed as advanced glycation end products (AGEs) (2). The FN3K enzyme reverses this process by phosphorylating fructoselysine residues to fructoselysine-3-phosphate (FL3P) at the expense of ATP (3). This process destabilizes the fructoseamine linkage, leading to the spontaneous decomposition of FL3P to lysine, 3-deoxyglucosone, and inorganic phosphate (4). Fructosamines and AGEs can impair the function of enzymes and structural proteins (5) and are thought to participate in the pathogenesis of long-term diabetic complications (6). For this reason, FN3K can be considered a repair enzyme. In addition, inhibition of FN3K in intact erythrocytes leads to an increase in the rate of accumulation of glycated hemoglobin (7) and animal models, such as $FN3K^{-/-}$ mice have hemoglobin-bound fructosamines that are 2.5-fold higher than concentrations observed in $FN3K^{+/+}$ or $FN3K^{+/-}$ mice (8).

Very few studies have reported genetic variants in *FN3K* and its enzymatic activity. Delpierre and co-workers reported an association between the erythrocyte FN3K enzymatic activity and some polymorphisms in the *FN3K* gene in a Belgian cohort including 31 type 1 diabetic patients and 26 controls (9). More recently, in type 2 diabetic subjects, the *C* allele of the polymorphism c.900 *C/G* (rs1056534) located in exon 6 has been associated with lower hemoglobin A_{1c} (HbA_{1c}) concentrations and with a later onset of type 2 diabetes mellitus (T2DM) disease compared to the *G* allele (10).

The aim of the present study was the molecular characterization of the *FN3K* gene in a group of Italian individuals previously enrolled in the ADAG (A_{1c}-derived average glucose) study (11), with stable glycemic control, to verify the

possible effects of genetic variants of *FN3K* on the glycation of HbA_{1c}. The results obtained were then compared to the results reported by Delpierre's group on a Belgian population (9). In addition, association studies and preliminary haplotype analysis are also described.

Materials and methods

Patients and controls

All the individuals were recruited at the outpatient Diabetic Center of the Department of Medical and Surgical Sciences at the University of Padova.

Diabetic subjects consisted of a subgroup of patients enrolled in the ADAG study (11). Thirty-five patients were type 1 diabetes mellitus (T1DM), and 35 T2DM. The presence of T1DM and T2DM was assessed according to WHO criteria (12, 13). The diabetic patients had to have stable glycemic control, as shown by two HbA_{1c} values in the 6 months prior to enrolment, with a variation of no more than 1%. Furthermore, they had to be able to perform self-monitoring of blood glucose and continuous glucose monitoring. Patients affected by any condition that could cause changes in glycemic concentrations, as disease requiring steroid therapy or plans for pregnancy during the study, as well as patients affected by any condition that could influence HbA_{1c} concentrations (anemia, high erythrocyte turnover, blood loss, transfusions, hemoglobinopathies, liver and kidney disease, infections) were excluded from the study. All diabetic patients were between 18 and 70 years of age and were judged to be able to complete the protocol of the ADAG study.

The control cohort consisted of 33 healthy blood donors with no history of diabetes.

Blood samples were collected in EDTA tubes and stored at –80°C until use.

Written informed consent was obtained from all participants, also with regards to genetic testing. The study was approved by the local Ethic Committee and was conducted in accordance with the Declaration of Helsinki (14).

Biochemical analysis

Measurement of glycemia and HbA_{1c} were performed during a 12-week period according to the procedures of the ADAG study. Briefly, average fasting glucose concentrations were calculated by combining weighted results from at least 2 days of continuous glucose monitoring performed four times, with a seven-point daily self-monitoring capillary (fingerstick) glucose performed over at least 3 days per week. Concerning the HbA_{1c} values, these were obtained at baseline and monthly over four consecutive months and were analyzed by four different methods, including by HPLC, two immunoassays and an affinity assay in a centralized laboratory in the Netherlands. The mean HbA_{1c} value obtained by all these measurements was considered for each patient (11).

Molecular analysis

DNA was extracted from 200 μL of EDTA-whole blood using the QIAamp DNA Blood Mini Kit (QIAGEN, Hilden, Germany). All diabetic patients underwent *FN3K* gene sequence analysis. All six exons and the corresponding intron/exon boundaries were amplified using PCR. The PCR reactions were performed in 25 μL volumes containing 60 ng genomic DNA using 1.5 U of AmpliTaq Gold® DNA polymerase (Applied Biosystems, Foster City, CA, USA). Primers sequences, annealing temperatures and magnesium chloride ($MgCl_2$) concentrations used for each PCR amplification are reported in Supplemental Table 1 (online). Direct sequence analysis was performed using the BigDye™ Terminator Cycle Sequencing Kit Version 1.1 (Applied Biosystems) on a 3730 DNA automated analyzer (Applied Biosystems).

Denaturing high performance liquid chromatography (DHPLC) was performed on samples from the control group for screening for the presence of the genetic variants previously identified in diabetic subjects. DHPLC was performed using a DHPLC WAVE MD 4000 Plus (Transgenomic, Omaha, Nebraska, USA) and a DNASep column with buffer A [0.1 M triethyammonium acetate (TEAA) at pH 7.0] and buffer B (0.1 M TEAA solution in 25 vol/vol acetonitrile, pH 7.0). Elution of DNA from the column was followed by monitoring the absorbance at 260 nm. The optimum temperatures for analysis of each fragment were predicted using Navigator™ software (Transgenomic) and confirmed empirically (supplemental Table 1). Samples displaying abnormal elution profiles were re-amplified and subjected to direct sequencing. The sequencing data that was obtained was compared to the consensus sequence of *FN3K* NM_022158.

Statistical analysis

The allelic frequencies for each polymorphism were calculated in all groups. Hardy-Weinberg equilibrium (HWE) of the identified polymorphisms in the control group was estimated using the χ^2-test. The association between *FN3K* gene polymorphisms and susceptibility to T1DM or T2DM was then evaluated with the χ^2-test. Results were considered statistically significant for p-values <0.05. Haplotypes and their frequencies were estimated by using the HAPSTAT software (http://www.bios.unc.edu/~lin/hapstat/) under HWE.

Results

The baseline characteristics of patients and control subjects under our investigation are reported in Table 1.

The molecular characterization of the *FN3K* gene through direct sequencing allowed for the identification of seven genetic variants, as reported in Table 2.

Four variants, one missense, one nonsense and two synonymous, were identified in exons 2, 5 and 6; three were intronic, involving IVS2 and IVS4. Two variants are reported here, for the first time: the c.559 *C/T* in exon 5, with the substitution of an arginine with a stop codon (p.R187X), and the c.716 *A/G* in exon 6, with the substitution of a tyrosine with a cysteine (p.Y239C). Both variants were identified in two females affected by T2DM and not in control subjects and T1DM. The remaining two variants c.187 *A/C* (p.R63R) and c.900 *C/G* (p.S300S), already reported in NCBI, did not cause an amino acid change in the coded protein.

Among the intronic variations, IVS2+26 *A/G* and IVS2+31 *A/T* were present in intron 2 and IVS4-9 *delTTG*, consisting of a deletion of three nucleotides, in intron 4. Genotypes and allele frequencies distributions of all genetic variants were evaluated in T1DM, T2DM and control subgroups, as summarized in Table 2.

Table 1 Main characteristics of T1DM and T2DM patients and 33 control subjects of the present study.

	Type 1 diabetic subjects	Type 2 diabetic subjects	Control subjects
n	35	35	33
Male/female	15/20	17/18	23/10
Age, years (mean ± SD)	40 ± 20	59 ± 20	42 ± 13
Duration of disease, years	16 ± 9	14 ± 9	–
Fasting glycemia, mmol/L (mean ± SD)	12.1 ± 5.7	9.5 ± 3.2	4.2 ± 0.6
HbA_{1c}, % (mean ± SD)	8.4 ± 1.6	7.8 ± 1.4	5.6 ± 0.3

Table 2 Genotypes and allele frequencies of *FN3K* genetic variants identified in the studied groups.

Ex/IVS[a]	Variant	AA[b] change	RefSNP ID[c]		T1DM (n = 35)	T2DM (n = 35)	Controls (n = 33)
Ex 2	c.187 *A/C*	R63R	rs2253149	*AA*	/	/	/
				AC	/	/	/
				CC	35	35	33
				A	/	/	/
				C	1	1	1
IVS 2	IVS2 + 26 *A/G*	/	rs2253132	*GG*	/	/	/
				GA	/	/	/
				AA	35	35	33
				G	/	/	/
				A	1	1	1
IVS 2	IVS2 + 31 *A/T*	/	rs2253131	*AA*	/	/	/
				AT	10	7	5
				TT	25	28	28
				A	0.143	0.100	0.076
				T	0.857	0.900	0.924
IVS 4	IVS4-9 del*TTG*[d]	/	rs72318398	+ / +	25	30	22
				+ */del*	10	5	11
				del/del	/	/	/
				+	0.857	0.929	0.833
				del	0.143	0.071	0.167
Ex 5	c.559 *C/T*[e]	R187X	/	*CC*	35	34	33
				CT	/	*1*	/
				TT	/	/	/
				C	1	0.986	1
				T	0	0.014	0
Ex 6	c.716 *A/G*[e]	Y239C	/	*AA*	35	34	33
				AG	/	1	/
				GG	/	/	/
				A	1	0.986	1
				G	0	0.014	0
Ex 6	c.900 *C/G*	S300S	rs1056534	*CC*	3	5	3
				CG	21	19	17
				GG	11	11	13
				C	0.386	0.414	0.348
				G	0.614	0.586	0.652

[a]Ex: exon; IVS: intron. [b]AA: aminoacid. [c]RefSNP ID: http://www.ncbi.nlm.nih.gov/snp/. [d]For polymorphism IVS4-9 del*TTG* the ''+'' symbol indicates the wild type allele, ''*del*'' the allele characterized by the deletion. [e]Genetic variant previously unpublished, identified in T2DM females.

The three polymorphisms (IVS2 + 31 *A/T*, IVS4-9 *delTTG*, c.900 *C/G*) for whom the allelic frequencies could be calculated were in HWE. Using the χ^2-test, no significant association was found between these three variants and T1DM or T2DM, probably because of the small number of subjects studied.

Seven haplotypes were identified within T1DM, T2DM and control groups. However, we report in Table 3 those haplotypes obtained by the three variants IVS2 + 31 *A/T*, IVS4-9 *delTTG* and c.900 *C/G* since they were present in the wild-type, heterozygous and homozygous state. We were not able to observe any statistically significant difference

Table 3 Haplotypes and their respective frequencies found in T1DM, T2DM and control groups.

Haplotype	1[a]	2[b]	3[c]	T1DM	T2DM	Controls
I	*T*	+	*C*	0.3857	0.4000	0.3485
II	*T*	+	*G*	0.3286	0.4181	0.4413
III	*T*	*del*	*G*	0.1429	0.0533	0.1345
IV	*A*	+	*G*	0.1429	0.0819	0.0436
V	*A*	*del*	*G*	0.0000	0.0181	0.0322

[a]Variant IVS2+31 *A/T*. [b]Variant IVS4-9 del*TTG*: ''+'' symbol indicates the wild type allele, ''*del*'' the allele characterized by the deletion. [c]Variant c.900 *C/G*.

between the three cohorts. Nonetheless, it is interesting to note a slightly higher prevalence of haplotype III in T1DM, with respect to T2DM, as well as for haplotype IV.

Concerning the genotypes, our findings are summarized in Table 4. Also, in this case, the two monomorphic loci (c.187 *A/C* and IVS2+26 *A/G*) have been removed and we have considered only genotypes with the combination of the three variants IVS2+31 *A/T*, IVS4-9 *delTTG* and c.900 *C/G*. We were able to identify eight different genotypes and their relationship with the mean HbA_{1c} values in type 1 and type 2 diabetic subjects, obtained directly from the ADAG study over 4 months of observation for each patient (Figure 1). In this context, it is interesting to note that some genotypes, (such as D in type 1 diabetic patients, or C and G in type 2 diabetics) seem to be related to higher HbA_{1c} values.

Discussion

FN3K represents part of a cellular defense and/or repair system to control nonenzymatic glycation of proteins. By opposing one of the chemical consequences of hyperglycemia, it can act as a protective factor in the development of diabetic complications, differing from individual to individual and being, at least partly, genetically determined (15). Indeed, the entire deglycation pathway is likely to be more complex because other reactions, such as transglycation, seem to play a role as well (3, 5). However, FN3K has been recently demonstrated to be able to reduce the glycation of intracellular islet proteins, even though not being required for the maintenance of β-cell survival and function. FN3K seems not to be involved in protection against β-cell glucotoxicity (16).

Table 4 Genotypes and their respective frequencies found in T1DM, T2DM and control groups.

Genotype	1[a]	2[b]	3[c]	T1DM	T2DM	Controls
A	*TT*	+/+	*CC*	0.0857	0.1143	0.0909
B	*TT*	+/+	*CG*	0.4000	0.4571	0.3636
C	*TT*	+/+	*GG*	0.0571	0.0857	0.1212
D	*TT*	+/*del*	*CG*	0.0857	/	0.1515
E	*TT*	+/*del*	*GG*	0.0857	0.0857	0.1212
F	*TA*	+/+	*CG*	0.1143	0.0857	/
G	*TA*	+/+	*GG*	0.0571	0.0571	0.0909
H	*TA*	+/*del*	*GG*	0.1143	0.0571	0.0606

[a]Variant IVS2+31 *A/T*. [b]Variant IVS4-9 del*TTG*: ''+'' symbol indicates the wild type allele, ''*del*'' the allele characterized by the deletion. [c]Variant c.900 *C/G*.

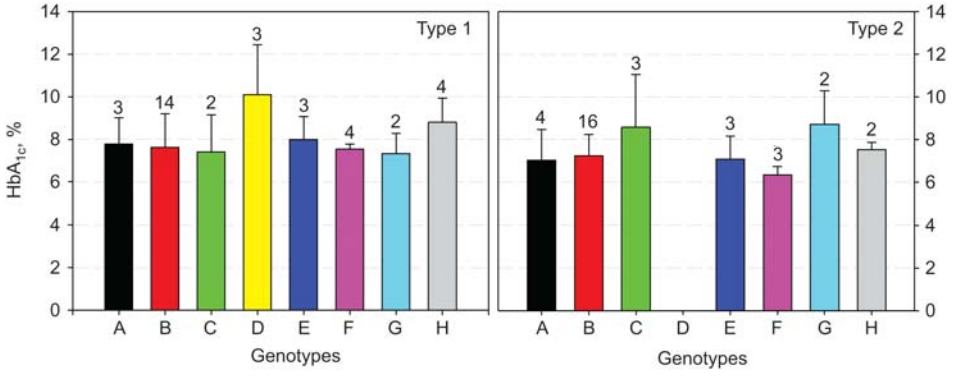

Figure 1 Glycated hemoglobin concentrations in type 1 (left) and type 2 (right) diabetic patients according to their *FN3K* genotypes. The genotype composition is reported in Table 4. Mean ± SD are reported per each group. The number of subjects per each genotype is reported above each bar.

The activity of FN3K is reported to vary in different subjects up to four-fold, and this variability seems to be significantly associated with several genetic variants present in the *FN3K* gene (9). Unfortunately, we have not been able to implement a robust method for assessing FN3K activity, essentially because the substrate used by Delpierre at al. (9) is not commercially available, and the previous described methods, outlined by the same group of investigators, seemed to not be sufficiently sensitive for clinical use.

In this report a well clinically characterized diabetic subpopulation was analyzed by directly sequencing the coding region and the intronic boundaries of the *FN3K* gene. DHPLC analysis for screening *FN3K* genetic variants in the control group was chosen because of its practicability and speed of execution. As a matter of fact, DHPLC is now considered a high capacity low-cost mutation scanning method (17) and its use for the analysis of more than 250 genes to uncover the presence of mutations has been well described in the literature (18).

With regards to the two new mutations identified, the p.R187X mutation, located in exon 5, introduces a premature stop codon causing a truncated protein lacking part of exon 5 and the entire exon 6. In contrast, the p.Y239C mutation is present in a conserved region of exon 6 and results in substitution of a tyrosine for cysteine, an amino acid known to form disulfur bonds, possibly causing structural changes and consequently, functional changes within the protein. In addition, exon 6 has been reported to be highly conserved among different species, probably because it contains the ATP binding site, fundamental to the proper function of the enzyme (19). We believe that the above mutations can represent a new step in the identification of a genetic risk factor for T2DM. Interestingly, only females showed such mutations, but this finding has to be interpreted with caution because of the limited number of subjects investigated. Thus, we plan in the near future to collect more T2DM samples to confirm such an observation.

The other genetic variants identified here have already been reported. Polymorphism c.187 *A/C* (rs2253149) was detected in the *CC* homozygous state in diabetic patients and control subjects, as well as polymorphism IVS2+26 *A/G* (rs2253132), found in the homozygous state *AA* in all the subjects. No data on allele frequency for these variants are reported, and we speculate that alleles *C* and *A* are only present in the Italian population.

For polymorphisms IVS2+31 *A/T* (rs2253131) and IVS4-9 *delTTG* (rs72318398), the homozygous *AA* and *del/del* genotype are absent, probably due to the low number of subjects. The first variant, located in intron 2, was also described by Delpierre et al. (9) and allele *T* seems to be present with higher frequency in both Italian and Belgian subpopulations. The polymorphism c.900 *C/G* (rs1056534) was also described by Delpierre et al. (9) and found in both diabetic and control individuals, in both the heterozygous and homozygous states. Even though no amino acids are changed, this variant was found to be associated with erythrocyte FN3K enzymatic activity (9). In addition, in T2DM subjects, the c.900 *CC* alleles have been shown to be associated with lower HbA_{1c} concentrations (10).

Our results did not display any direct relationship among each single variant identified and diabetes, and that is why we performed haplotype and genotype studies. It may be interesting to note that even though a small number of T2DM patients were analyzed, A genotype (containing c.900 *CC* alleles) seems to be associated with low concentrations of HbA_{1c}, whereas C and G genotypes (both containing c.900 *GG* alleles) seem to be associated with higher HbA_{1c} values, consistent with results from Mohás et al. (10). However, as shown in Figure 1, E and H genotypes also share c.900 *GG* alleles and are not associated with higher $HbA_{1c.}$ Such an observation could be explained by the genetic context in which the c.900 *C* or *G* allele lays. Indeed, the presence of +/+ or +/*del* alleles for the variant IVS4-9 *delTTG*, for example, modifies the genetic background, thus, possibly explaining the different values of HbA_{1c} found.

We did not perform any statistical valuation different from the estimation of the frequency because of the small number of subjects enrolled. Further investigation is needed to assess the real meaning of genotype and haplotype results, including the slight differences observed in haplotype frequencies, especially for haplotypes III and IV.

Concerning the selection of patients, we have focused our analysis on *FN3K* by choosing a particular group of subjects, i.e., a cohort of patients enrolled in a clinical study in which all patients were maintained under very stable glycemic control. In order to obtain a robust relationship between HbA_{1c} and the mean average glucose during the preceeding months, we chose to study patients with stable glycemic control, as evidenced by two HbA_{1c} values within one percentage point of each other, during the 6 months before the recruitment. Under such circumstances, we believe that possible effects of genetic determinants affecting the degree of glycation of human hemoglobin and other proteins, such as genetic variants of *FN3K*, could have more chances of expressing their phenotype.

Our work also confirmed in Italy the presence of some *FN3K* variants previously reported by Delpierre et al. (9) in T1DM patients only. In addition, we have also extended our analysis to T2DM patients and have identified additional genetic variants (c.187 *A/C*, IVS4-9 *delTTG*), and two new mutations (c.559 *C/T*, c.716 *A/G*). We hope that by increasing the number of enrolled subjects (T1DM, T2DM and controls) we will able to show the effective role of the variants/haplotypes here identified in the clinical phenotype. In particular, we are interested in studying the relationship between the *FN3K* genetic background and typical diabetic complications, such as micro- and macro-angiopathies, or neuropathies.

Conflict of interest statement

Author's conflict of interest disclosure: The authors stated that there are no conflicts of interest regarding the publication of this article. Research support played no role in the study design; in the collection, analysis, and interpretation of data; in the writing of the report; or in the decision to submit the report for publication.

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