Opinion Paper

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Possible role of fructosamine 3-kinase genotyping for the management of diabetic patients

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Abstract: Diabetes mellitus is a global pandemic and continues to increase in numbers and significance. Several pathogenic processes are involved in the development of such disease and these mechanisms could be influenced by genetic, epigenetic and environmental factors. Non-enzymatic glycation reactions of proteins have been strongly related to pathogenesis of chronic diabetic complications. The identification of fructosamine 3-kinase (FN3K), an enzyme involved in protein deglycation, a new form of protein repair, is of great interest. FN3K phosphorylates fructosamines on the third carbon of their sugar moiety, making them unstable and causing them to detach from proteins, suggesting a protective role of this enzyme. Moreover, the variability in FN3K activity has been associated with some polymorphisms in the *FN3K* gene. Here we argue about genetic studies and evidence of FN3K involvement in diabetes, together with results of our analysis of the *FN3K* gene on a Caucasian cohort of diabetic patients. Present knowledge suggests that FN3K could act in concert with other molecular mechanisms and may impact on gene expression and activity of other enzymes involved in deglycation process.

Keywords: deglycation; diabetes; fructosamine 3-kinase (FN3K); glycated hemoglobin (HbA_{1c}); glycation; single nucleotide polymorphisms.

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Introduction

Diabetes mellitus is one of the world's oldest diseases and is the major epidemic of this century, having increased by 50% over the past 10 years [1]. Typically, diabetes is presented as a common, heterogeneous, complex disease in which both predisposing genetic and environmental factors interact together and cause hyperglycemia.

Long-term exposure to excessive glucose concentrations can lead to deleterious results. In human diabetes, chronic elevation of blood glucose concentration leads to many long-term complications, including microvascular and macrovascular diseases resulting in significant morbidity and mortality [2]. The link between the elevated concentration of glucose and the development of these complications is not yet clear and many hypotheses have been proposed [3–5]. One of the leading proposals is the "Non-enzymatic Glycation Hypothesis" which postulates that the deleterious effects of chronic hyperglycemia are a result of excessive non-enzymatic modification of proteins and some phospholipids by glucose and its byproducts [5, 6].

Protein glycation refers to the binding of glucose or other reducing sugars to proteins. Particularly, glycation with aldoses takes place with the ε-amino group of lysine residues or with the N-terminus of the protein, resulting in the formation of a Schiff base product. This thermodynamically unstable compound rearranges to form ketoamine derivatives, the Amadori products, that can undergo a series of further rearrangements (dehydration, cyclization, fragmentation and oxidation) to form a wide and heterogeneous group of complex compounds called advanced glycation end products (AGEs) [7]. Both fructosamines and AGEs are involved in the etiology of diabetic complications and can impair structural and biological properties of proteins in living organisms, with a complex mechanism at least in part independent of hyperglycenia [8, 9].

Glycation is a common and spontaneous reaction, occurring in vivo. About 5% of total hemoglobin in normoglycemic subjects has a fructosamine bound to the amino-terminus of its β-chains (HbA_{1c}). It is well known that HbA_{1c} represents an important tool for the clinical management of diabetes and evidence confirms its important role as a retrospective indication of the quality of glycemic control [10, 11]. Moreover, since 2009, HbA_{1c} has also been recommended for the diagnosis of diabetes [12].

Fructosamine 3-kinase: a deglycating enzyme

Discovery and properties

Glycation has long been considered as irreversible, although some findings were suggesting the existence of intracellular deglycation mechanisms. Repair is of outstanding importance for life and three different types of enzymes are known to metabolize ketoamines: oxidases, isomerases and kinases [13, 14]. However, only the latter is used to deglycate proteins under physiological conditions.

The starting point for the discovery of fructosamine 3-kinase (FN3K), a protein distantly related to aminoglycoside kinases and, even more distantly, to protein kinases [15], was the identification of fructose 3-phosphate in human and animal tissues [16]. The investigation on the mechanism of its synthesis in erythrocytes showed the involvement of an ATP-dependent kinase. This enzyme exhibited a very low affinity for its substrate (K_m ≥30 mmol/L) and a low metabolic capacity, suggesting that it could act on some substrates different from fructose, even if on compounds with a closely related structure [17]. Two independent groups led to the conclusion that "fructose 3-kinase" phosphorylates fructosamines with a K_m in the micromolar range, i.e., 4–5 orders of magnitude lower than the K_m for fructose (≈50÷100 mmol/L). Then, the enzyme was purified from human erythrocytes [18] and its cDNA was cloned [15].

Human FN3K is a monomeric protein of 309 amino acids encoded by a gene (NC_000017.10) located on chromosome 17q25.3. *FN3K* gene may have arisen by an event of duplication of an ancestral gene, FN3K-related protein (*FN3K-RP*). The gene encoding FN3K-RP is located next to the one encoding FN3K, and share a 65% sequence homology with *FN3K* and an identical genome organization [19, 20]. Two distinct, but related genes, encoding orthologs of *FN3K* and *FN3K-RP* are present in mammals and chicken genomes, whereas there is only one *FN3K/FN3K-RP* homolog in the genomes of fishes and urochordates [20], suggesting that the gene duplication event occurred during fish radiation.

Both FN3K and FN3K-RP phosphorylate psicosamines and ribulosamines, but only the former act on fructosamines [19]. As expected, FN3K is more active in tissues containing proteins with long (half-)lives, such as erythrocytes, lens and brain [19]. Remarkably, FN3K activity is elevated in erythrocytes from rat, mouse and man, in whom the intracellular concentration of glucose is close to that of plasma. However, FN3K activity is low in erythrocytes from chicken and pig, where the glucose concentration is very low [21].

Furthermore, starvation and diabetes do not change the level of expression of *FN3K* in different tissues, and no regulation of *FN3K* expression was observed in human fibroblasts treated with condition mimicking the diabetic state [21].

Function and involvement in deglycation

FN3K appears to represent a part of a cellular defence and/or repair system to control non-enzymatic glycation of proteins. Indeed, the enzyme phosphorylates not only fructosamines, but also their C3-epimers psicosamines, as well as ribulosamines and erythrulosamines, even if psicosamines are much poorer substrates than the other ketoamines [18]. In details, FN3K would be able to break down the second intermediate of the non-enzymatic glycation cascade by phosphorylating fructoselysine to a fructoselysine-3-phosphate (FL3P). The latter compound spontaneously decomposes by β-elimination, regenerating an unmodified lysine along with inorganic phosphate

and 3-deoxyglucosone, then readily detoxified to inert products, such as 3-deoxyfructose or 2-deoxy-3-ketogluconic acid [15, 18].

FN3K involvement in deglycation was first provided by finding that its competitive inhibitor, deoxymorpholinofructose (DMF), increases about two-fold the rate of accumulation of glycated hemoglobin when erythrocytes are incubated in presence of 200 mmol/L glucose [22]. Definitive evidence for FN3K being responsible for deglycation was provided in animal models: *FN3K*$^{-/-}$ mice showed a level of hemoglobin-bound fructosamines of about 2.5-fold higher than those observed in *FN3K*$^{+/+}$ or *FN3K*$^{+/-}$ mice [23]. However, FN3K has been recently demonstrated to be able to reduce the glycation of intracellular islet proteins, but does not affect pancreatic β-cell survival and function, even if these are incubated for several weeks in presence of high glucose concentration [24].

The demonstration that FN3K phosphorylates glycated hemoglobin in intact cells causing its partial deglycation was followed by experiments aimed at identifying the fructosamines residues removed from hemoglobin in intact erythrocytes, as a result of FN3K action. In vitro studies indicate that several fructosamines bound to lysines are excellent substrates, whereas others are only poorly phosphorylated [25]. Thus, the fructosamines bound to Lys139α, located near the C-terminus of the α subunits, and Lys16α, located on a loop of the α subunits, are good substrates. On the contrary, fructose bound to Lys61α, whose side chain is partially bound to a heme, is only very slowly phosphorylated. Moreover, the N-terminal glycated valine is a poor substrate, consistent with free fructosevaline being a much poorer substrate than free fructoselysine [26].

Genetics: results from presented studies

Few works have reported genetic variants of *FN3K*. First, Delpierre and collaborators reported an association between FN3K enzymatic activity in red cells and some polymorphisms in the *FN3K* gene, in a Belgian cohort of 31 type 1 diabetic subjects (T1DM) and 26 controls [27]. They found that two single nucleotide polymorphisms (SNPs), besides other gene variants, the CC of the c.900C/G (rs1056534) in exon 6 and the GG of the c.–385A/G (rs3859206) in the promoter region, were associated with reduced enzymatic activity measured in erythrocytes. However, they failed to detect a correlation between *FN3K* SNPs and HbA_{1c} levels [27]. Then, the group of Mohás analyzed a large cohort of type 2 diabetic (T2DM) subjects (859 T2DM and 265 controls) for the presence of the polymorphism c.900C/G (rs1056534) of the *FN3K* gene [28]. They found that the C allele of rs1056534 was coupled with lower HbA_{1c} concentration and with a later onset of type 2 diabetes. However, no association between this variant and diabetic complications, such as nephropathy, neuropathy or retinopathy, were found in their investigation.

In 2014, a group of 314 T2DM subjects was screened for 19 SNPs in six candidate genes encoding for enzymes of metabolic pathways, in order to verify if the genetic variability in such genes could influence the progression of diabetic nephropathy. An association of the polymorphism in exon 6 (rs1056534) of the *FN3K* gene with the progression of diabetic nephropathy and cardiovascular morbidity and mortality was indeed reported [29]. Recently, Škrha and co-workers, in a cohort of 129 T1DM, 340 T2DM and 126 controls, evaluated the association of *FN3K* and *GLO1* polymorphisms with parameters of endothelial dysfunction and soluble receptor for AGEs (sRAGE) [30]. In 126 subjects (50 T1DM, 52 T2DM and 24 healthy individuals), a significant association of *FN3K* rs1056534 and rs3848403 SNPs with sRAGE concentration in patients with diabetes was proven.

Our research group have also analyzed a Caucasian cohort of 70 diabetic patients, 35 T1DM and 35 T2DM and 33 controls, for the coding part of the *FN3K* gene, identifying two new mutations and additional variants within the gene. No significant association was found between certain SNPs and diabetic conditions. However, we noted too that the genotype containing c.900 CC alleles (rs1056534) seemed to be related with low concentration of HbA_{1c} [31].

We have completed the molecular characterization of the *FN3K* gene by analyzing its promoter and we have evaluated the presence of the two polymorphisms, the c.–385A/G (rs3859206) and the c.–232A/T (rs2256339), known to be associated with FN3K enzymatic activity in erythrocytes [27]. Two additional new variants (c.–421C/T; c.–429delATCGGAG) have been found in one patient with T1DM. The statistical analysis performed on our cohort indicates that the Hardy-Weinberg Equilibrium (HWE) was respected in all single groups for both polymorphisms, except for rs2256339 in T1DM patients ($p=0.027$). The genotypes and allele frequencies distributions of the two polymorphisms in the promoter region of the *FN3K* gene are reported in Table 1. The genotypes and allele frequencies were not different among T1DM, T2DM and controls (χ^2-test, $p>0.05$) for all studied polymorphisms. Nevertheless, the sample size we have investigated was enough to reach a power test of 0.8 for two tails test considering an effect size=0.35 and a significance level of $\alpha=0.05$. Interestingly,

Table 1: Genotype and allele frequencies of variants in the FN3K promoter.

Promoter region variants		T1DM[a] (n=35)	T2DM[a] (n=35)	Controls[a] (n=33)
c.–385 A/G	AA	0.20	0.23	0.33
(rs3859206)	AG	0.60	0.54	0.55
	GG	0.20	0.23	0.12
	G-allele	0.50 (0.38–0.62)	0.50 (40.5–55.5)	0.39 (0.28–0.51)
c.–232 A/T	AA	0.17	0.20	0.27
(rs2256339)	AT	0.69	0.63	0.49
	TT	0.14	0.17	0.24
	T-allele	0.49 (0.37–0.60)	0.49 (0.37–0.60)	0.48 (0.36–0.61)

The values for genotypes are frequencies and for the rare alleles, frequencies (95% confidential interval). T1DM, type 1 diabetes; T2DM, type 2 diabetes; controls, healthy subjects. rs, RefSNP ID: http://www.ncbi.nlm.nih.gov/snp. [a]See [31] for clinical description.

by summing up the results of the promoter region to those of our previous investigation [31] we noted that the T2DM subjects carrying the CC genotype at c.900 (rs1056534) and with low concentration of HbA_{1c} also presented the GG genotype for the c.–385A/G (rs3859206).

FN3K and GWAS

In the last few years, genome-wide association studies (GWAS) have proven to be successful in identifying genetic association with complex traits [32]. Two examples of this approach involving *FN3K* have been reported in the recent literature.

First, Soranzo and collaborators [33] studied the association of genetic factors affecting expression, turnover and abnormal glycation of hemoglobin with HbA_{1c} levels in up 46,368 non-diabetic subjects of European ancestry descendent. They identified 10 loci (*FN3K*, *HFE*, *TMPRSS6*, *ATP11A/TUBGCP3*, *ANK1*, *SPTA1*, *GCK*, *G6PC2/ABCB11*, *MTNR1B* and *HK1*) associated with HbA_{1c} at genome-wide level of significance. They assessed that common variants at these loci likely influence HbA_{1c} levels via erythrocytes biology conferring a small but detectable reclassification of diabetes diagnosed by HbA_{1c} [33].

Second, is another meta-analysis of data from 16 cohorts comprising 32,602 non-diabetic individuals of East Asian ancestry. They identified nine loci harboring variants associated with HbA_{1c} levels in East Asian populations: four novel variants at *TMEMT9*, *HBS1L/MYB*, *MYO9B* and *CYBA*, as well as five ones at loci previously identified (*CDKAL1*, *G6PC2/ABCB11*, *GCK*, *ANK1*, and *FN3K*). They demonstrated that common genetic variants associated with HbA_{1c} levels in populations of European ancestry (*G6PC2/ABCB11*, *GCK*, *ANK1* and *FN3K*) have similar effects on HbA_{1c} levels in East Asians [34].

Conclusions

In the last few decades the notion of diabetes has widened, ascertaining that many different overlapping mechanisms can lead to the development of the pathology and that these mechanisms could be influenced by genetic, epigenetic and environmental factors [35, 36].

Since the early 20th century, the diagnosis of diabetes has been based on the measurement of glucose concentrations in the blood [37]. During the past 25 years the measurement of HbA_{1c} has been interpreted as a routine useful integrated measure of glycemic control. However, several studies have highlighted the limits of its use in patients with underlying disorders, including diseases changing erythrocytes turnover (hemolytic anemias, chronic malaria, major blood loss or blood transfusions), as well as genetic hereditary anemias and iron storage disorders that may influence the variability of HbA_{1c} in populations [38]. Furthermore, it has been shown that HbA_{1c} values may not be constant among individuals despite the presence of similar blood glucose or fructosamine concentration [39].

Non-enzymatic glycation has been strongly related to hyperglycemia conditions, and therefore to chronic complications associated with diabetes and renal failure. Thus, the identification of an enzyme, FN3K, as a part of a protein repair system opposing to the consequences of hyperglycemia, is of great interest.

In this report we have shown how the variability in FN3K activity has been associated with some polymorphisms in the *FN3K* gene [27]. Moreover, *FN3K* SNPs associated significantly with typical aspects of diabetes have been described [28–30]. All this evidence reinforces the hypothesis that the combination of particular variants in the promoter and exon regions could turn into enhanced or reduced expression of other enzymes or regulator

factors involved into deglycation. Indeed, probably also other molecular mechanisms impacting gene expression and activity of enzymes in deglycation process may have to be taken into account [39].

The role of FN3K in glycation of biological proteins seems to be more complex than evident at a first glimpse. The variability of FN3K activity may provide another key of explanation in those circumstances in whom HbA_{1c} does not perfectly correlate with the mean glucose level [40]. It has been shown that HbA_{1c} values may not be constant among individuals, despite the presence of similar blood glucose or fructosamine concentration [41]. It would be interesting to expand these studies and to correlate the FN3K activity with the glycation gap and the development of diabetic complications. Larger studies are necessary for a better understanding of the possible effect of *FN3K* genetic variants on the progression of the disease and its possible clinical utility in the management of diabetic patients. In particular, we need to differentiate between T1DM and T2DM, as these are two distinct populations with a well known different predisposition to development of chronic complication, and therefore possibly different genetic backgrounds of key factors involved in the glycation process. Finally, as suggested by Tanhäuserová et al. [29], if association of individual SNPs to progression of chronic complications of diabetes is weak, the combination of multiple SNPs (either related to oxidation pathways) could result in better prediction of such complications.

Furthermore, a lot of work needs to be done to elucidate the consequences of defects in deglycation, particularly in humans. As diabetes is not straightforward dependent on metabolic control, probably, there are also other molecular mechanisms impacting gene expression and activity of enzymes involved in deglycation systems. Therefore, the constitution of "genetic maps", using multiple candidate genes in glycation, oxidation and inflammation processes, is strongly needed for a better and more comprehensive evaluation of genetic predisposition to microvascular and macrovascular damage in diabetes.

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