A splice variant of *GNB3* and peripheral polyneuropathy in type 1 diabetes

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Abstract. Abnormalities in G protein-mediated signal transduction could be involved in the pathogenesis of diabetic polyneuropathy (DPN). Here we test whether the *GNB3* C825T variant confers susceptibility to DPN in type 1 diabetes (T1D) mellitus. The C825T marker of *GNB3* was genotyped in genomic DNA from blood isolated from a total of 213 Russian T1D patients 100 of whom had DPN. Compared to carriers of the wild-type genotype C/C, diabetic subjects with genotypes T/T had significantly increased risk to develop DPN (Odds Ratio (OR) of 4.4 ($p = 0.001$). The adjustment for confounders (age, sex, body mass index, cigarette smoking, and level of reduced glutathione) resulted in increase of the OR value up to 4.72 ($p = 8.9 \times 10^{-3}$). The further adjustment for hypertension abolished the association between the *GNB3* C825T variant and DPN (OR = 1.95, $p = 0.18$). Non-complicated subjects homozygous for T/T showed decreased levels of reduced glutathione (T/T: 69 ± 19 vs. C/T: 74 ± 19 vs. C/C: 77 ± 17 $\mu\text{mol/l}$, $p = 0.009$). Compared to other *GNB3* variants, carriers of the T/T genotype had elevated systolic blood pressure (SBP) in complicated (T/T: 115.8 ± 9.1 vs. C/T: 113.3 ± 8.2 vs. C/C: 109.5 ± 8.7 mm/Hg, $p = 0.036$) and non-complicated T1D patients (T/T: 118.1 ± 8.4 vs. C/T: 116.9 ± 7.9 vs. C/C: 112.1 ± 7.2 mm/Hg, $p = 0.02$). However, the significance of association between the C825T polymorphism was lost after adjustment for confounding risk factors. In conclusion, the 825T allele of *GNB3* is likely to accelerate the development of DPN through primary effects to SBP and hypertension in subgroups of diabetic patients with impaired neurovascular function and advanced oxidative stress.

Keywords: *GNB3*, C825T polymorphism, peripheral polyneuropathy, type 1 diabetes, susceptibility

1. Introduction

Heterotrimeric guanine nucleotide-binding proteins (G proteins) are expressed in all human cells. G proteins play pivotal roles in intracellular signal transduction and act on important regulatory components for cellular responses to receptor activation. They are large molecules consisting of α , β and γ subunits [1]. The *GNB3* gene encoding the $G_{\beta 3}$ subunit of heterotrimeric G proteins is located on chromosome 12p13 [2]. The alternative splicing of *GNB3* is associated with the

C825T (g.4423 C > T, S275S, rs6489738) polymorphism resulting in the loss of 41 amino acids. The truncated $G_{\beta 3S}$ subunit remains functional since it is able to form complexes with various G_{α} and G_{γ} proteins and stimulate intracellular signal transduction processes [3]. Furthermore, the mutated G proteins exhibited enhanced reactivity in signal transduction within cells with the 825T allele [4].

Studies on animal models of diabetic neuropathy suggest that abnormalities in signal transduction mediated by G proteins could be involved in the pathogenesis of this common complication of diabetes. In diabetic rats, enhanced influx of Ca^{2+} into sensory neurons associated with the progressive loss of neural fibers was observed [5,6]. Ca^{2+} channel blockers such as nimodipine improved experimental peripheral neuropathy [7]. Since $G_{\beta\gamma}$ dimers composed of $G_{\beta-}$ and

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G γ -proteins play a critical role in modulating activity of neuronal Ca²⁺ channels by direct binding to the pore-forming α_1 subunit of the channel, elevation of Ca²⁺ influx in diabetic peripheral neurons could result from the impaired regulation of Ca²⁺ channels by G proteins [8,9]. In neural tissue, G proteins represent essential regulatory components in the transmembrane coupling system of many receptors involved in neurotransmission [10]. Alterations in activity of G proteins are involved in pathophysiology of mood disorders such as depression and schizophrenia [11]. Taken together, these findings strongly suggest that impaired function G proteins could contribute to neurodegenerative conditions characterized by neuronal dysfunction and death.

The *GNB3* C825T polymorphism showed association with a higher risk of several complex diseases such as hypertension, obesity, metabolic syndrome, atherosclerosis and diabetes [12,13]. Here we hypothesized that this marker could confer susceptibility to diabetic polyneuropathy (DPN). In this study, we reported association between the *GNB3* C825T variant and neuropathy in type 1 diabetic (T1D) Russian patients.

2. Subjects, materials and methods

A total of 1,855 T1D patients registered in the Department of Endocrinology and Diabetology of the Russian Academy for Advanced Medical Studies during a half-year period from December, 2006 until May, 2007 were requested to participate in a cross-sectional study of diabetic polyneuropathy. Informed consent was obtained from 1,511 patients. 1,320 diabetic patients (71% of 1,855) were screened for neuropathy. Non-overlapping selection criteria were used to overcome probable predominance and masking effects of non-genetic risk factors. 113 diabetic patients, all of whom had diabetes of long duration (>10 years) and no signs of clinical polyneuropathy, were selected to form a control group. Second-round screening of the remaining patients revealed 146 subjects with short duration of diabetes (<5 years) and neuropathy. Of them, 19 patients were ruled out since they had causes of neuropathy other than diabetes. Of 127 remaining individuals, 27 were then excluded from the study since they had forms of diabetic neuropathy other than DPN. The screening procedure is presented in Fig. 1. Finally, 100 T1D patients with short-term diabetes complicated with DPN and 113 non-complicated controls have been selected for further genotyping.

Fig. 1. Scheme of selection of type 1 diabetic patients with short-term diabetes (duration < 5 years) and peripheral polyneuropathy and non-complicated controls with long (> 10 years) duration of diabetes.

DPN was diagnosed, and all neurological characteristics were assessed as previously described [14]. Clinical characteristics of diabetic patients with DPN and non-complicated controls are presented in Table 1. The patients studied were the residents of Moscow or Moscow region. The study was approved by the Academy Review Board and performed according to the principles of the Second Helsinki declaration. A standardized questionnaire was used to obtain the medical history and demographic information. Oxidized glutathione in erythrocyte hemolysates was quantified as described by Bentler et al. [15]. The *GNB3* C825T polymorphism was genotyped by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) as previously described [16].

All statistics were performed with the SPSS 10.0 package (version 10.0; SPSS Inc., Chicago, IL, USA). Results are given as mean $\pm$ S.D. or percentages. The clinical and laboratory characteristics of the patients were compared with the unpaired Student's t-test or χ^2 test as appropriate. To estimate genetic power of the sample tested, the Genetic Power Calculator

Table 1

Clinical characteristics of Russian type 1 diabetic patients with diabetic polyneuropathy and those, who have no sign of clinical neuropathy. Data are shown as a mean value $\pm$ standard deviation. Data range is presented in parentheses (T1D = type 1 diabetes. HbA_{1C} = glycated hemoglobin. BMI = body mass index. MCV = motor nerve conduction velocity. SCV = sensory nerve conduction velocity. VDT – vibration detection threshold. VPT – vibration perception threshold. TPT = temperature perception threshold. GSH, reduced glutathione)

Characteristic	Neuropathy (n = 100)	No neuropathy (n = 113)	p
Male/female ratio	61/39	43/70	0.0013*
Age, years (range)	25.7 $\pm$ 14.5 (7–35)	27.4 $\pm$ 8.6 (11–35)	0.39
Age at T1D onset, years (range)	22.4 $\pm$ 6.3 (4–29)	12.2 $\pm$ 3.2 (4–22)	0.00029
T1D duration, years (range)	1.5 $\pm$ 1.4 (0.5–3.7)	15.5 $\pm$ 5.7 (8.5–20)	1.7 $\times$ 10 ^{−8}
Systolic blood pressure, mm/Hg (range)	112.5 $\pm$ 8.6 (101–125)	114.1 $\pm$ 7.5 (102–129)	0.61
Diastolic blood pressure, mm/Hg (range)	86.2 $\pm$ 6.9 (77–94)	84.4 $\pm$ 6.6 (73–93)	0.72
HbA _{1C} , % (range)	6.3 $\pm$ 1.5 (4.8–9.2)	7.0 $\pm$ 1.7 (5.1–9.5)	0.55
BMI, kg/m ² (range)	22.1 $\pm$ 5.5 (15.9–29.2)	23.2 $\pm$ 4.7 (16.7–28.8)	0.81
MCV, m/s (range)	33.4 $\pm$ 2.7 (29–38.7)	46.4 $\pm$ 2.9 (42.6–49.9)	0.0011
SCV, m/s (range)	35.4 $\pm$ 3.2 (30.1–41.5)	46.7 $\pm$ 3.2 (43–50.3)	0.0031
VDT (range)	25 $\pm$ 0.5 (22–26)	14.5 $\pm$ 4.2 (9–20.5)	0.00035
VPT, V (range)	25.3 $\pm$ 4.6 (18.9–33.8)	12.2 $\pm$ 3.8 (7.7–19.7)	0.0002
TPT, °C (range)	7.5 $\pm$ 2.1 (3.5–11.7)	2.5 $\pm$ 1.1 (1.2–4.1)	0.0068
GSH, μ mol/l (range)	53 $\pm$ 16 (30–77)	72 $\pm$ 19 (39–98)	0.00085
Smokers/nonsmokers	41/59	31/82	0.052*
Hypertension, yes/no	28/62	19/94	0.048*
Diabetic retinopathy, yes/no	18/82	14/99	0.34*
Diabetic nephropathy, yes/no	22/78	20/93	0.54*

* Yates' χ^2 test; other data are compared with the unpaired Student's t-test.

was used [17]. Qualitative data were analyzed using the χ^2 test. To assess the extent to which the various genotypes were associated with DPN, Odds Ratios (ORs) Odds Ratios (ORs) were estimated by logistic-regression analysis. To provide separate ORs for each genotype, dummy variables were used, with wild-type genotype (*C/C*) used as the reference group. The significance of interaction between clinical characteristics and *GNB3 C825T* variant was assessed using two-way ANOVA. Observed relationships were then adjusted for patients' conventional risk factors by analysis of covariance (ANCOVA) using age, gender, body mass index (BMI), smoking status, level of reduced glutathione (GSH), and hypertension as covariates.

3. Results

Comparison of the patients revealed significant differences between all characteristics used to evaluate nerve function (Table 1) thereby suggesting for good suitability of these criteria in diagnosis of neuropathy. In DPN patients, levels of reduced GSH, an important intracellular antioxidant, were significantly lower than those in non-complicating controls. This could therefore reflect the presence of advanced oxidative stress in patients with neuropathy.

Power calculations showed that the sample size studied was enough to reach 80% genetic power at type

I error of 0.05 assuming disease prevalence of 0.01, frequency of a risk marker allele *b* of 0.41, and ORs for heterozygous *B/b* and homozygous *b/b* of 1.55 and 2.9 respectively. In both groups of patients, observed genotype frequencies of the *GNB3 C825T* polymorphism obeyed the Hardy-Weinberg equilibrium (data not shown).

The *C825T* polymorphism showed significant association with DPN (Yates' $\chi^2 = 12.72$, $p = 1.73 \times 10^{-3}$). The presence of each copy of the predisposing allele *T825* increased DPN risk by approximately 2-fold. Compared to the carriers homozygous for the wild-type allele *825C*, subjects heterozygous for *C/T* showed a more than 2-fold higher risk for DPN (Table 2). The greatest risk was conferred by homozygosity for the *825T* variant, which was associated with a more than 4-fold increased probability of disease.

Using the ANOVA test, the genotype *T/T* showed association with lower levels of reduced glutathione compared to other genotypes (*CC* vs. *CT* vs. *TT* = 77 $\pm$ 17 vs. 74 $\pm$ 19 vs. 69 $\pm$ 19 μ mol/l, $p = 0.009$) (Table 3). The genotype *T/T* was also significantly associated with increased systolic blood pressure in both DPN patients (*CC* vs. *CT* vs. *TT* = 109.5 $\pm$ 8.7 vs. 113.3 $\pm$ 8.2 vs. 115.8 $\pm$ 9.1 mm/Hg, $p = 0.036$) and individuals with no neuropathy (*CC* vs. *CT* vs. *TT* = 112.1 $\pm$ 7.2 vs. 116.9 $\pm$ 7.9 vs. 118.1 $\pm$ 8.4 mm/Hg, $p = 0.02$).

Table 2

Association between the *GNB3* C825T polymorphism and diabetic polyneuropathy. In type 1 diabetic patients with and without neuropathy, allele and genotype frequencies of the 825T marker were compared using Yates' χ^2 test (OR = Odds Ratio. 95% CI = 95% Confidence Interval)

Allele/genotype	Neuropathy, n(%)	No neuropathy, n(%)	<i>p</i>	OR	95% CI
<i>C</i>	118 (59.0)	176 (77.9)		1.00	
<i>T</i>	82 (41.0)	50 (22.1)	< 0.0001	2.44	1.6–3.73
<i>CC</i>	40 (40.0)	72 (63.7)		1.00	
<i>CT</i>	38 (38.0)	32 (28.3)	0.021	2.13	1.16–3.93
<i>TT</i>	22 (22.0)	9 (8.0)	0.001	4.4	1.85–10.47

We considered the glutathione level, which could reflect the severity of oxidative stress, as well as age, gender, smoking, hypertension, and BMI as potential confounders that could influence interactions between the C825T polymorphism of *GNB3* and other variances [18]. Adjustment for these confounding risk factors did not revealed any significance between *GNB3* gene variants and clinical characteristics of the patients. Furthermore, after adjustment for covariances, the significance observed between the C825T marker and systolic blood pressure was abolished (Table 3). The lack of significance after ACNOVA analysis suggests that the C825T variant of *GNB3* could not be considered as independent risk factor for DPN, and its association with DPN is mediated by other factors.

In multiple regression analysis, adjustment for age, gender, BMI, GSH level, and smoking status resulted in even more rising OR (up to 4.72, $p = 8.9 \times 10^{-3}$) for homozygotes *T/T* vs. *C/C*. However, including hypertension into the multiple regression model greatly decreased the OR value up to 1.95 ($p = 0.18$). In addition, the *GNB3* marker itself showed the borderline significance for association with hypertension in both complicated and non-complicated individuals ($p = 0.066$ and 0.051 , respectively). These results suggest that hypertension is likely to be one of those factors that mediate association between the *GNB3* C825T variant and DPN risk.

4. Discussion

We found association between the 825T variant of *GNB3* and higher risk of polyneuropathy in Russian T1D patients. However, the allele *T* of *GNB3* is likely to confer susceptibility to DPN via a primary effect on systolic blood pressure and hypertension. This is consistent with results of numerous studies reporting the contribution of this genetic variant of *GNB3* to the variance of systolic blood pressure and essential hypertension [19–21]. The association of *GNB3* C825T with blood pressure could suggest that this marker might be

implicated in DPN pathogenesis through the impaired neurovascular function.

There are several potential mechanisms by which the splice variant of *GNB3* could influence the neurovascular dysfunction. In vascular smooth muscle, diabetic hyperglycemia is shown to alter expression of G proteins and G protein-mediated signaling [22], which could result in the activation of Ca^{2+} channels, increased diacylglycerol formation and subsequent activation of membranous protein kinase C (PKC) [23]. Pathologic activation of PKC has pleiotropic deleterious effects on the endothelial function including PKC-dependent activation of NADH/NADPH oxidase associated with accelerated production of reactive oxygen species and enhanced oxidative stress [24]. Increased activity of G protein-mediated signaling in the vasculature and microvascular endothelium is also coupled with increased blood volume, alterations in plasma sodium and potassium concentrations and vascular remodeling [25]. This could lead to the inhibition of Na^+/K^+ -ATPase, depletion of intracellular ATP pool, changes in permeability of neuronal microvessels and hypoxia [26]. Taken together, these disturbances could promote rapid progression from the endothelial dysfunction and vascular insufficiency to progressive nerve fiber damage and loss in peripheral tissues of diabetic carriers of the predisposing *GNB3* 825T variant.

Rapid development of neuropathy in the studied group of patients with short-term diabetes is likely to be a consequence of enhanced oxidative stress. Decreased levels of GSH in diabetic individuals carrying the predisposing *GNB3* 825T variant suggest for the presence of advanced oxidative stress in these patients. Oxidative stress is an established independent risk factor for DPN [27]. *GNB3* could contribute to glucose-induced oxidative stress through the impairment of G-protein-dependent signal transduction pathways mediated by metabotropic glutamate receptors that leads to the pathologic activation of PKC and disturbs both biosynthesis and homeostasis of glutathione, an important cellular antioxidant [28].

Table 3

Association of the *C825T* polymorphism of *GNB3* with clinical characteristics in patients with diabetic polyneuropathy and non-complicated type 1 diabetic subjects. Clinical characteristics in carriers of different genotypes were compared using the two-way ANOVA test and then adjusted for conventional risk factors (gender, age, smoking status, BMI, hypertension, and glutathione level) using the ANCOVA test (GSH = reduced glutathione. BMI = body mass index. T1D = type 1 diabetes. HbA_{1C} = glycated hemoglobin. MCV = motor nerve conduction velocity. SCV = sensory nerve conduction velocity. VDT = vibration detection threshold. VPT = vibration perception threshold. TPT = temperature perception threshold).

Characteristics	Neuropathy (<i>n</i> = 100)				No neuropathy (<i>n</i> = 113)			
	<i>C/C</i> (<i>n</i> = 40)	<i>C/T</i> (<i>n</i> = 38)	<i>T/T</i> (<i>n</i> = 22)	<i>P</i> (ANOVA)	<i>C/C</i> (<i>n</i> = 71)	<i>C/T</i> (<i>n</i> = 32)	<i>T/T</i> (<i>n</i> = 10)	<i>P</i> (ANCOVA)
Male/female ratio	24/16	24/14	13/9	0.93	26/45	12/20	5/5	0.76
Age, years	24.5 ± 13.5	26.7 ± 15.2	25.9 ± 14.2	0.72	27.7 ± 8.4	27.0 ± 8.9	27.2 ± 8.7	ns
Smokers/nonsmokers	16/24	16/22	9/13	0.99	16/55	11/21	4/6	0.28
Hypertension (yes/no)	7/33	11/27	10/12	0.066	8/63	7/25	4/6	0.051
GSH, $\mu\text{mol/l}$	54 ± 16	52 ± 16	50 ± 17	0.23	77 ± 17	74 ± 19	69 ± 19	0.009
BMI, kg/m^2	21.5 ± 5.3	21.9 ± 5.4	23.3 ± 5.9	0.44	22.9 ± 4.4	23.9 ± 5.1	23.5 ± 5.0	0.81
Age at T1D onset, years	21.9 ± 6.1	23.4 ± 6.9	22.7 ± 6.2	0.67	11.9 ± 3.5	12.7 ± 2.7	12.4 ± 2.9	0.76
T1D duration, years	1.2 ± 1.2	1.6 ± 1.5	1.6 ± 1.4	0.93	15.5 ± 5.8	16.1 ± 5.6	14.3 ± 5.5	0.47
Systolic blood pressure, mm/Hg	109.5 ± 8.7	113.3 ± 8.2	115.8 ± 9.1	0.036	112.1 ± 7.2	116.9 ± 7.9	118.1 ± 8.4	0.02
Diastolic blood pressure, mm/Hg	84.9 ± 7.5	86.7 ± 6.7	88.2 ± 6.6	0.73	82.0 ± 6.8	85.4 ± 6.1	86.9 ± 6.4	0.6
HbA _{1C} , %	6.3 ± 1.8	6.6 ± 1.4	6.1 ± 1.4	0.82	7.2 ± 1.5	6.6 ± 1.9	7.0 ± 1.9	0.77
MCV, m/s	33.7 ± 2.5	34.5 ± 2.2	32.8 ± 2.9	0.79	45.7 ± 2.8	46.9 ± 0.1	47.9 ± 3.0	0.68
SCV, m/s	34.8 ± 3.3	36.3 ± 3.2	35.0 ± 2.2	0.66	47.0 ± 3.2	45.8 ± 3.5	46.2 ± 2.9	0.89
VDT	24.5 ± 0.7	24.7 ± 0.4	25.4 ± 0.4	0.73	14.9 ± 4.5	13.7 ± 4.0	14.3 ± 3.6	0.58
VPT, V	25.9 ± 4.1	24.9 ± 4.9	25.3 ± 4.6	0.85	11.9 ± 3.6	12.5 ± 4.3	12.5 ± 3.8	0.77
TPT, °C	7.5 ± 2.3	7.5 ± 2.0	7.6 ± 1.9	0.96	2.4 ± 1.1	2.8 ± 1.1	2.6 ± 1.0	0.80

In contrast to our results, two earlier studies failed to show any significant association between the *GNB3* C825T marker and DPN in Russian T1D patients residing in St. Petersburg [29] and Japanese type 2 diabetic subjects [30]. The discrepancy could result from the differences in selection of patients and ethnic backgrounds. In both studies, the DPN patients had prolonged duration of neuropathy (>10 years) that in turn could negate the association between the *GNB3* C825T variant and this diabetic complication.

In conclusion, the C825T polymorphism of *GNB3* contributes to rapid progression of DPN in subgroups of T1D patients with advanced oxidation stress. The *GNB3* T825 variant is associated with higher risk of DPN through the impairment of neurovascular function and primary effect on systolic blood pressure and hypertension.

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