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Angiogenin gene polymorphism

*A risk factor for diabetic peripheral neuropathy in the northern Chinese Han population?**

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Research Highlights

(1) We investigated genetic associations between angiogenin gene polymorphisms and diabetic peripheral neuropathy in the Han population of northern China.
(2) Using a case-control design, we compared the genotype and allele frequencies of the angiogenin coding region single nucleotide polymorphism rs11701 between a diabetic neuropathy group and controls. No significant associations were found.
(3) Angiogenin may not be associated with genetic susceptibility to diabetic peripheral neuropathy in the northern Han Chinese population.

Abstract

Angiogenin is associated with the pathogenesis of diabetic peripheral neuropathy. Here, we quenced the coding region of the angiogenin gene in genomic DNA from 207 patients with type 2 diabetes mellitus (129 diabetic peripheral neuropathy patients and 78 diabetic non-neuropathy patients) and 268 healthy controls. All subjects were from the Han population of northern China. No mutations were found. We then compared the genotype and allele frequencies of the angiogenin synonymous single nucleotide polymorphism rs11701 between the diabetic peripheral neuropathy patients and controls, and between the diabetic neuropathy and non-neuropathy patients, using a case-control design. We detected no statistically significant genetic associations. Angiogenin may not be associated with genetic susceptibility to diabetic peripheral neuropathy in the Han population of northern China.

Key Words

neural regeneration; angiogenin; single nucleotide polymorphism; type 2 diabetes mellitus; diabetic peripheral neuropathy; angiogenesis; diabetic microvascular complications; genetic susceptibility; risk factor; peripheral nerve injury; grants-supported paper; neuroregeneration

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Conflicts of interest: None declared.

Ethical approval: The project was approved by the Ethics Committee of Beijing Peking University Third Hospital, China.

INTRODUCTION

Diabetic peripheral neuropathy, which occurs in up to 50% of diabetic patients[1-7], is a serious chronic microangiopathic complication of diabetes mellitus. There is much controversy about the pathophysiological mechanisms of diabetic peripheral neuropathy[8]. However, angiogenesis may play an important role[9-12]. Angiogenesis is a complex process regulated by stimulatory and inhibitory angiogenic growth factors. The expression of various angiogenic growth factors is reduced in diabetes mellitus[13-19].

Angiogenin, an angiogenic factor, is associ-

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ated with diabetic peripheral neuropathy. Angiogenin is an important but still poorly investigated angiogenic growth factor that has a great influence on new vessel formation[9, 20]. When introduced into BALB/c mice in a serum-induced angiogenesis test, the serum angiogenin of diabetic patients caused a significant decrease in new vessel formation[20]. Siebert *et al* [21-22] investigated the relationship between diabetic microvascular complications and angiogenin, and found that serum angiogenin levels were significantly lower in patients with type 2 diabetes mellitus and those with diabetic microvascular complications, leading to impaired new vessel formation.

Interestingly, mutations in human angiogenin[23-24], a member of the ribonuclease A superfamily, have been recently reported in patients with amyotrophic lateral sclerosis[25-28]. These studies indicate that angiogenin is expressed at high levels in cells of the developing nervous system such as motor neurons, dorsal root ganglia, and axons. Subramanian and colleagues[28] reported that angiogenin, which has a neuroprotective function, is an axonal growth promoter. Additionally, diabetic peripheral neuropathy has been postulated to occur by diverse pathogenic mechanisms: microvascular disease with impaired blood flow and ischemia in diabetic nerves have both been implicated. We hypothesized that angiogenin plays an important role in diabetic peripheral neuropathy.

The relationship between genetic variants in angiogenin and diabetic peripheral neuropathy has not yet been reported. Therefore, determination of the factors involved in physiological and pathological angiogenesis in diabetic peripheral neuropathy is of great importance to understand its pathogenesis and to design effective treatments.

In this study, we attempted to identify mutations in the angiogenin gene in diabetic peripheral neuropathy and to investigate the genetic association of three angiogenin single nucleotide polymorphisms with diabetic peripheral neuropathy. To do this, we sequenced the coding region of this gene in Chinese diabetic peripheral neuropathy and diabetic non-neuropathy cohorts, and in healthy controls.

RESULTS

Quantitative analysis and clinical information of subjects

A total of 207 type 2 diabetic patients and 268 controls were consecutively enrolled into the study. The 207 patients comprised 129 diabetic peripheral neuropathy patients and 78 diabetic non-neuropathy patients. Nobody was excluded or dropped out. The patients' clinical characteristics are shown in Table 1. The diabetic peripheral neuropathy and diabetic non-neuropathy groups were well matched with the controls regarding the age, gender, body mass index, blood pressure, creatine, and low-density lipoprotein cholesterol level ($P > 0.05$).

Sequence analysis of the angiogenin gene

A 736 bp fragment of the angiogenin gene, containing the entire coding region and 5′-flanking region, was PCR amplified and sequenced from all the patients and controls. No mutations were found. The single nucleotide polymorphism rs11701 (c.T442G, p.G86G) was present in all three groups (Figure 1). However, the two other common single nucleotide polymorphisms, rs17560 (c.A362G, p.K60E) and rs2228653 (c.A475T, p.T97T), were not polymorphic in our population.

The rs11701 genotype and allele frequencies are not associated with diabetic peripheral neuropathy

The genotype frequencies of the angiogenin single nucleotide polymorphism rs11701 were in accordance with Hardy-Weinberg equilibrium in both the diabetic peripheral neuropathy group and the controls ($P = 1.00$). The 442T/G genotype frequency was higher in the diabetic peripheral neuropathy group than in the controls, but the difference was not statistically significant (3.1% *vs*. 2.6%, respectively; $P = 0.753$). Similarly, the 442G allele frequency was non-significantly

higher in diabetic peripheral neuropathy than in controls (P = 0.754). The 442G/G genotype was not present in either group (Table 2).

When we compared the diabetic peripheral neuropathy group with the diabetic non-neuropathy group, we found that the 442T/G genotype frequency was higher in the latter group, but the difference was not statistically significant (P = 0.530). The 442G allele frequency was also non-significantly higher in the diabetic non-neuropathy group than in the diabetic peripheral neuropathy group (P = 0.529). The 442G/G genotype was not detected (Table 3).

Table 1 Baseline characteristics and standard laboratory variables in all included subjects

Item	Control (n = 268)	Diabetic non-neuropathy patient (n = 78)	Diabetic peripheral neuropathy patient (n = 129)
Sex (n, M/F)	122/146	34/44	54/75
Age (year)	58.89±14.13	60.59±11.50	61.53±12.67
Body mass index (kg/m^2)	24.59±3.87	24.14±3.59	24.26±3.80
Blood pressure (mmHg)	90.37±11.59	94.39±12.24	94.01±11.44
Glycosylated hemoglobin (%)	4.57±6.31	9.33±2.18[a]	9.55±2.61[a]
Total cholesterol (mmol/L)	4.55±2.49	4.89±1.39[a]	5.00±1.19[a]
Low-density lipoprotein cholesterol (mmol/L)	2.98±0.92	3.0 3±0.68	2.96±0.88

[a]P < 0.05, *vs*. controls. All measurement values are expressed as mean ± SD; one-way analysis of variance and chi-square tests were performed to test statistical differences between the mean values among different groups for clinical parameters. M: Male; F: female.

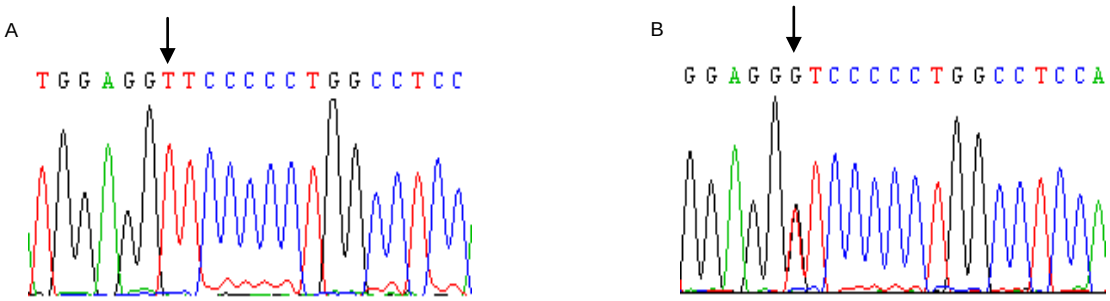

Figure 1 Chromatograms showing the angiogenin single nucleotide polymorphism rs11701.

(A) Wild type (T442T); (B) heterozygous type (T442TG). Arrows indicate the site of the polymorphism.

Table 2 Distribution [n (%)] of rs11701 genotypes and alleles in diabetic peripheral neuropathy patients and controls

Genotype	Control (n = 268)	Diabetic peripheral neuropathy patient (n = 129)	P	OR (95%CI)
442T/T	261(97.4)	125(96.9)	—	—
442T/G	7(2.6)	4(3.1)	0.753[a]	1.193(0.343–4.151)
442G/G	0	0	—	—
442T	529(98.7)	254(98.4)	—	—
442G	7(1.3)	4(1.6)	0.754[a]	1.190(0.345–4.102)

[a]: The overall power was calculated as the average power across the angiogenin single nucleotide polymorphisms that were genotyped. Chi-squared tests were used to assess inter-group significance. The *ORs* were obtained by comparison with the wild-type 442T/T genotype or 442T allele. *OR*: Odds ratio; *CI*: confidence interval.

Table 3 Distribution [n(%)] of rs11701 genotypes and alleles in diabetic non-neuropathy and diabetic peripheral neuropathy patients

Genotype	Diabetic non-neuropathy patient (n = 78)	Diabetic peripheral neuropathy patient (n = 129)	P	OR (95%CI)
442T/T	75(96.1)	125(96.9)	—	—
442T/G	3(3.8)	4(3.1)	0.530	1.094(0.571–2.096)
442G/G	0	0	—	—
442T	153(98.1)	254(98.4)	—	—
442G	3(1.9)	4(1.6)	0.529	1.092(0.572–2.084)

The overall power was calculated as the average power across the angiogenin single nucleotide polymorphisms that were genotyped. Chi-squared tests were used to assess inter-group significance. The *ORs* were obtained by comparison with the wild-type 442T/T genotype or 442T allele. *OR*: Odds ratio; *CI*: confidence interval.

DISCUSSION

Angiogenin is a multifunctional angiogenic molecule that is involved in several steps of angiogenesis, including cell invasion, proliferation, and tube formation. Other angiogenic proteins, such as vascular endothelial growth factor, basic fibroblast growth factor 2, and endothelial growth factor, have also been implicated in vascular formation induced by endogenous angiogenin[29-32]. Therefore, angiogenin plays an important role in regulating the expression of cytokines in angiogenesis[29]. Serum angiogenin levels can highlight the induction of new vessel formation in patients with diabetic microvascular complications[16-21].

Diabetic peripheral neuropathy has been postulated to occur by diverse pathogenic mechanisms, and microvascular disease with impaired blood flow and ischemia in diabetic nerves have been implicated in its pathogenesis. We hypothesized that angiogenin has an important function in diabetic peripheral neuropathy[28]. Diabetic neuropathy is attributed to a variety of pathogenetical mechanisms[8, 33]. Vascular injury is only one of them. Additionally, multiple actors concur in causing vascular remodeling, and angiogenin is only one stimulator for the angiogenesis[34-36]. Moreover, gene problems could be investigated by different layers, such as gene structure and function disorder, protein structure, function alteration and multiple gene expression change.

Angiogenin was discovered as a potent angiogenic factor. Investigating the angiogenin gene polymorphism following diabetic neuropathy is important for both understanding the pathophysiologic mechanisms of diabetic microvascular neuropathy and exploring new methods for the therapy of diabetic microvascular neuropathy. But there is few study recently described to our knowledge.

We sequenced the angiogenin gene coding region and genotyped its three most common single nucleotide polymorphisms in 129 patients with diabetic peripheral neuropathy, 78 diabetic non-neuropathy patients, and 268 healthy controls. The three common single nucleotide polymorphisms in the exon region are rs11701 (T442G, G86G), rs17560 (A362G, K60E) and rs2228653 (A475T, T97T). Among them, rs11701 is the marker of chromosome 14q11.2 and rs11701 could affect angiogenin gene and ribonuclease 4 gene splicing, therefore, single nucleotide polymorphism rs11701 is a functionally important region[37-39]. No mutations were found. Two single nucleotide polymorphisms were not polymorphic in our populations; the third, rs11701, was not associated with diabetic peripheral neuropathy. rs11701 could affect angiogenin gene splicing and might therefore be functionally important[37-39]. Using a case-control design, our findings show that the angiogenin gene polymorphisms in the extron region may be not associated with the pathogenesis of diabetic peripheral neuropathy. And vascular injury induced by angiogenin gene mutation may be not the key factor in the pathogenesis of diabetic peripheral neuropathy, *i.e.*, our findings suggest that angiogenin does not play a role in diabetic peripheral neuropathy in the Chinese Han population.

Our understanding of the biological properties of angiogenin is still incomplete, and this imposes limitations on our ability to identify the molecular mechanisms by which angiogenin mutations are involved in diabetic peripheral neuropathy. In addition, there are many other factors that work together in vascular remodeling[34-36]. Moreover, there are other modes of aberrant protein function such as changes to gene/protein structure, gene/protein expression levels, and epigenetic changes. Thus we cannot rule out a role for angiogenin in the pathogenesis of diabetic peripheral neuropathy in the Chinese population. Results from this study are preliminary because the sample size in this study is not large. Other studies with more patients are needed to confirm these data. Further studies with larger samples sizes are necessary.

SUBJECTS AND METHODS

Design

A case-control genetic association study.

Time and setting

Experiments were conducted at the Department of Neurology, Peking University Third Hospital, China from October to December, 2011.

Subjects

A total of 207 consecutive patients with type 2 diabetes mellitus were recruited from the Department of Endocrinology, Peking University Third Hospital, having attended for a routine examination. Diabetes mellitus was diagnosed according to the Criteria for Diagnosis and Classification of Diabetes Mellitus[40].

Among the 207 diabetic patients, 129 were diagnosed with diabetic peripheral neuropathy. The onset and progression were assessed using modified Dyck criteria[41]

in accordance with the Report from the Diabetic Neuropathy Expert Panel Meeting, Toronto, 2011.

Inclusion criteria: The presence and staging of peripheral neuropathy were evaluated with the Neurological Symptom Score[42], Neurological Disability Score[43], Autonomic Function Testing[44-46], and Quantitative Sensory Examination[47-48]. Testing was performed on each participant by the same experienced physician. The Neurological Symptom Score[42] is based on the patient's reported symptoms, which comprise sensory, motor, and autonomic subcategories. A score > 1 indicates abnormality. The Neurological Disability Score[43] has subcategories of sensory, motor, and reflex function. A score ≥ 6 indicates abnormality. Autonomic Function Testing is calculated with an acetylcholine sweatspot test[44] and autonomic cardiovascular reflex tests[45-46]. The Quantitative Sensory Examination[47-48] consists of thermal and vibration sensitivity tests of the big toe using an NTE-2 Thermal Sensitivity Tester and a Vibration II Sensitivity Tester, respectively (Medoc Ltd., Jerusalem, Israel). The result is classed as abnormal if both the thermal and vibration threshold scores are abnormal.

Exclusion criteria for controls: Individuals meeting any of the following criteria were excluded: diabetes mellitus, impaired glucose tolerance, cerebrovascular disease, heart disease, or any other condition known to cause neuropathy or peripheral vascular disease.

A total of 268 age- and gender-matched non-diabetic controls were obtained from the Physical Examination Center, having attended for a routine examination.

All participants were the Han population from northern China. They were informed of the project's details and risks prior to the experiment, according to the *Administrative Regulations for Medical Institutions* formulated by the State Council of China[49]. All subjects signed informed consent form before the study.

Methods

Sequence analysis

Genomic DNA was extracted from elbow vein peripheral blood leukocytes using the phenol-chloroform method[50]. The GenBank reference sequence for angiogenin (accession number NM_001145.4) was used. Primers were designed using ExonPrimer software (http://ihg.gsf.de/ihg/ExonPrimer.html) to amplify the entire angiogenin coding region plus intron-exon boundaries in a 736 bp fragment. The forward primer sequence was 5′-CGG TTG GAG CTA GAG GTT GT-3′ and the reverse primer sequence was 5′-AAT GGA AGG CAA GGA CAG C-3′.

The PCR mixture contained 1 μg of DNA template, 0.33 μmol/L of each primer, 266 μmol/L dNTPs, 10.8 μL pure water, and 1.5 units of Taq polymerase (Shanghai Sangon Biological Engineering Technology & Services Co., Ltd., Shanghai, China) in a final volume of 15 μL. The cycling conditions were 95°C pre-denaturation for 6 minutes, 35 cycles of 95°C denaturation for 30 seconds, 59°C annealing for 30 seconds, and 72°C extension for 30 seconds, then a final extension at 72°C for 10 minutes in a thermal cycler (DNA Engine, MJ Research, Watertown, MA, USA).

PCR products were sequenced directly in both directions using a BigDye Terminator Cycle sequencing kit 3.1 (Applied Biosystems, Foster City, CA, USA) and analyzed on an ABI3730XL sequencer (Applied Biosystems).

Statistical analysis

Descriptive data are represented by non-normally distributed variables and are presented as mean ± SD for continuous variables. Independent samples two-tailed *t*-tests and one-way analysis of variance were performed to determine the significance of the differences in mean values between each group. The chi-square test was used to examine Hardy-Weinberg equilibrium. *P* values were calculated using the Fisher probability test in a 2 × 2 table, because the theoretical frequency in one grid was less than 5. $P < 0.05$ was considered statistically significant. All analyses were conducted using SPSS 11.5 software (SPSS, Chicago, IL, USA).

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