·Basic Research·

Genotyping analysis of a polymorphic G -954C of *NOS2A* in diabetic retinopathy with cystoid macular edema

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Abstract

• AIM: To analyze the genotype of the allele distribution of a polymorphic G-954C within the 5' upstream promoter region of the nitric oxide synthetase 2A gene (*NOS2A*) in samples of diabetic retinopathy in patients with cystoid macular edema in the mainland of China.

• METHODS: Eighty-nine patients with diabetic retinopathy and cystoid macular edema and 90 healthy controls were enrolled in this study. Nest polymerase chain reaction (PCR) was performed, and restriction endonuclease digestion and gene fragments sequence were examined to detect the genotype of *NOS2A*G-954C.

• RESULTS: The genotypes of the sample population of 89 cases and 90 healthy controls were all detected as GG.

• CONCLUSION: The distribution of G-954C of *NOS2A* polymorphism are at a lower frequency in China, with little relevancy to the frequency of diabetic retinopathy combined with cystoid macular edema.

• KEYWORDS: cystoid macular edema; diabetic retinopathy; *NOS2A*promoter; polymorphism

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INTRODUCTION

In diabetes, the earliest detectable abnormality in the retinal circulation is the increase in blood flow. Pathological changes soon follow: selective loss of retinal pericytes, basement membrane thickening, and subsequent endothelial cell loss and closure of the small capillaries. With the pathological process getting on, macular edema, retina hemorrhage would occur, which might be incompatible with normal vision. Nitric oxide (NO), a potent biological second messenger, is involved in the regulation of vascular tone, wound repair, defense mechanisms and other processes. It is synthesized by the action of nitric oxide synthase (NOS) through the 5-electron oxidation of the terminal guanidino-N2 of the amino acid *L*-arginine[1]. With the effect on vascular smooth muscle as a major function, NO is synthesized by the vascular endothelium which lies close to the vascular smooth muscle, and is recognized as the pathway through which the endothelin-mediated control of blood flow is exercised.

Endothelium-mediated vasodilatory responses in blood vessels are aberrant in response to agonist stimulation in diabetic subjects[2-4]. *In vitro* and *in vivo* studies have shown that the synthesis and release of vasoconstrictors by the vascular endothelium are increased in the diabetic state [5,6]. Evidence suggests that endothelium-mediated vasodilation is defective and reduced in diabetes along with an increase in vasoconstrictor activity. It is now recognized that aberrations in retinal blood flow in early diabetes are also related to vascular endothelial dysfunction [7, 8]. The retinal circulation, which is devoid of any extrinsic innervation, is dependent entirely on endothelium-mediated autoregulation [9]; thus, endothelial dysfunction in diabetes is likely to have a major effect on the circulation within the retina.

In addition to environmental factors, results of the Diabetes Control and Complications Trials [10] indicated that genetic factors may also affect the development of onset of diabetic retinopathy [11]. The risk of severe diabetic retinopathy in the siblings of affected individuals is increased, with a strong tendency of familial clustering of this complication. Studies on twins [12] and various ethnic populations [13] also demonstrated the genetic influence in diabetic retinopathy.

Three members of the NO synthase gene family have been

identified: neuronal NOS gene (*NOS1*), inducible NOS gene (*NOS2A*) and endothelial NOS gene (*NOS3*), all of which could play a role in the diabetic retinopathy. Under normal conditions, *NOS2A* is not expressed in the retinal vasculature. Exposure to high ambient glucose may influence NO release via increased *NOS2A* expression and reduced constitutive *NOS3* expression in cultured retinal vascular endothelial cells [14]. The inducible NOS isoform (*NOS2*) represents the high-output pathway for NO production and is regulated primarily at the transcriptional level by the action of inflammatory cytokines and toxins [15]. This may trigger the cystoid macular edema. Previous work has demonstrated that 14-repeat allele of pentanucleotide (CCTTT)(n) repeat located in an S1 hypersensitive region - 2.5 kb upstream of the human *NOS2A* gene transcription start site was significantly associated with the absence of diabetic retinopathy [16]. Furthermore, in a previous report, a polymorphism in the *NOS2A* gene 954nt upstream of the transcription start site was distributed unevenly among people with severe and mild malaria [17]. *NOS2A* G-954C carriers was hypothesized as binding with a higher affinity to the mutant site than to the wild-type sequence, and a higher base level of NOS activity can be found [18]. In order to study if the G-954C of *NOS2A* mutation affect the onset of diabetic retinopathy as well as 14-repeat allele of pentanucleotide (CCTTT) (n) repeat, the genotypes of G-954C of *NOS2A* from healthy control and patients with cystoid macular edema and diabetic retinopathy in the mainland of Chinese were detected in the present article.

MATERIALS AND METHODS

Clinical Assessment Ethical approval was obtained at the First People's Hospital of Yunnan Province, China for this research. Key inclusion criteria are: individuals with diabetes mellitus were recruited from regional metabolic and ophthalmic clinics after they were given informed consent; fundi were examined using slit-lamp biomicroscope and fluorescence angiography for the diagnosis of retinopathy and/or maculopathy combined with macular edema. Key exclusion criteria are: history of chronic or recurrent severe inflammatory eye disease such as scleritis or uveitis; history of ocular trauma or intraocular surgery within the past 6 months; history of infection or inflammation within the past 3 months; history of clinically significant or progressive retinal disease such as retinal degeneration and retinal detachment; patients with a significant history and/or active alcohol or drug abuse (significant is defined as that which in the opinion of the investigator may either put the patient at risk due to participating in the study or may influence the results of the study or the patients' ability to participate in the study).

Routine clinical examinations were scheduled by the Diagnostics Department in our hospital. The recorded history and the findings of the ophthalmologic examination, including best-corrected visual acuity, intraocular pressure, slit-lamp examination, indirect fundoscopy and fluorescence angiography, were evaluated and completed if needed by one of the investigators (the same for all patients).

The following clinical data were collected: age and sex; type, duration and family history of diabetes; presence of vascular disease (angina, hypertension and peripheral vascular disease); renal function (dipstick microalbuminuria and albumin creatinine ratio) and glycemic control. Type 1 diabetes was defined when patients were 30 years old or younger at diagnosis or there was evidence of absolute insulin dependence. All other cases were deemed to be Type 2 diabetes. A sample population of 89 individuals and 90 healthy controls were enrolled from Yunnan Province, China.

Assessment of Host Genetic Polymorphisms Each patient donated a 10mL venous blood sample. Total DNA was extracted from the white blood cell pellet with Bioteke (Bioteke Corporation, Beijing, China). DNA from each patient was used as a template for PCR (ABI 2720) amplification for the detection of polymorphism in the NOS2A gene. Polymorphisms were detected using nest PCR amplification followed by restriction endonuclease digestion. In brief, the sequence of iNOS was loaded from GeneBank (LOCUS, AF017634), the promoters for PCR were first 5'-TAT TTT TCA TTC ATT CAT CCA, and then 5'-ATC CTA CCT GCC ATT CCA CCA. Nested P1: 5'-TGG TAG CAA AGT GTT GGG ACG-3'; P2: 5'-TGG TGG AAT GGC AGG TAG GAT-3'. The primers were created by Takara (Dalian, China). The PCR conditions were used as follows: the long is that an initial 40 seconds denaturation step at 95℃ (5 minutes), 94℃ (30 seconds), followed by 52℃ (30 seconds), 72℃ (60 seconds), and 72℃ (5 minutes), 35 cycles; the short is that 95℃ (5 minutes), 94℃ (30 seconds), followed by 52℃ (30 seconds), 72℃ (45 seconds), and 72℃ (5 minutes), 30 cycles. The generated fragment was subjected to a 12-hour digestion with the endonuclease Bsa1 (New England, Beverly,MA,USA) at 50℃,and subsequently analyzed on 30g/L agarose gel after 20 minutes (65℃). To confirm the sequence of Bsa1-restriction fragments, the

following fragments (boldface represent the polymorphic position-954 in the NOS2 promoter), 5'- TTG AGT TCG AGA CCA GCA TGG ACA ACA TGG TG 3' (wild type) and 5'- TTG AGT TCG ACA CCA GCA TGG ACA ACA TGG TG 3' (NOS2 G - 954 C), were analyzed. The wide genotype of GG were cut into two fragments: one was 231bp, the other was 330bp. The heterocyclic genotype of GC was cut into three fragments: 231bp, 330bp and 561bp. The mutation genotype of CC was remained as one fragment of 561bp. The sequences of the Bsa1-restriction fragments were detected by ABI 3130 Sequencer (Hitachi High-technologies Corporation, Hitachi, Japan).

Statistical Analysis Frequency distributions of characteristics of study participants were examined according to case-control status.

RESULTS

The age and gender were matched between cases and control subjects. The genotype of the sample population of 89 individuals and 90 healthy controls were detected as GG. In other words, we did not find any *NOS2A* G-954C carries in the 179 Chinese people, neither with diabetic retinopathy nor healthy control. The results are shown in Table 1, Figures 1 and 2.

This result is in accord with previous researches. The *NOS2A* G-954C carries geographic distribution is a relatively high frequency in sub-Saharan Africa and lower frequency in Asia and Mediterranean basin [18].

DISCUSSION

There were some evidences indicating that the mutation genotype NOS2G-954C polymophism was associated with elevated NOS activity and the production of NO increased. This phenomenon was postulated as modifying binding of constitutive acting DNA binding protein to the polymorphic site [18]. This result led us to hypothesize that the NOS2G-954C mutation is taking part in the process of diabetic retinopathy via the constitutively high baseline level of NOS activity.

Diabetic maculopathy is the leading cause of visual loss in diabetic patients. The pathogenesis is yet not fully understood. Malfunction of the blood-retinal barrier plays a central role in the disease and leads to retinal edema and secondary photoreceptor dysfunction. Diabetic vascular leakage and macular edema are regulated by a distinct combination of direct paracellular transport, alterations in endothelial intercellular junctions and endothelial cell death. The distribution and relevance of these three factors of diabetic

Table 1 The genotype of *NOS2A* – 954

	Mean age	Male	Female	Genotype		
				GG	GC	CC
Diabetic retinopathy	56.43±9.34	49	40	89	0	0
Control	57.89±7.32	48	42	90	0	0

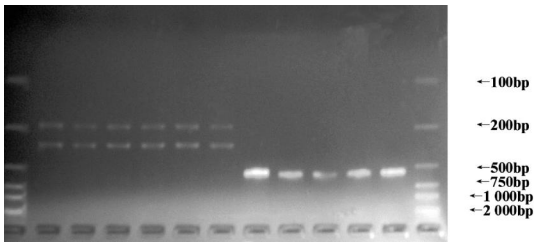

Figure 1 Electriophoretic mobility shift assay DNA Marker: DL2000. The left is the result of Bsa1-restriction fragments. The right is the production of polymerase chain reaction (PCR)

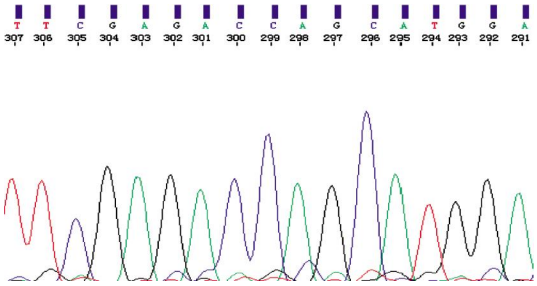

Figure 2 The sequence of all the Bsa1-restriction fragments (NOS2A G-954:302 G)

maculopathy varies over the course of the disease. Cumulative endothelial cell death will become more relevant after prolonged diabetic conditions. One of the mimic diabetic retinopathies is the abnormal tone of retinal vessel. In brief, there are abnormal arteriolar tone occuring as microaneurism and venous congestion. This kind of abnormal vessels tone should come from abnormal vessels regulation. The retina blood flow regulation is characterized as auto-regulation. It is defined as the ability of the blood vessels to keep blood flow constant under varied perfusion pressure in order to match it to tissue oxygen and metabolic requirements. The failure of auto-regulation is an important and often early feature of diabetic retinopathy. Since human retina vessels lack extrinsic innervation, retinal vessel calibre and local blood flow are normally regulated by non-nervous mechanisms intrinsic to the retina. There is now a considerable body of evidences suggesting that retinal pericytes are the main regulators of vascular tone in the retinal capillaries because they contain components of contractile proteins similar to vascular smooth muscle cells and they also possess ET-1 receptors. Furthermore, ET-1 has been shown not only to cause vasoconstriction of retinal vessels but also to have mitogenic effects on retinal

pericytes. Hence, alterations in the pericyte-ET interaction may have a role causing early hemodynamic and histopathological abnormalities found in diabetic retinopathy [19]. On one hand, NO may balance the vasoconstriction role of endothelin. It is widely recognized to be a quite important intercellular messenger in the cardiovascular and nervous systems or immunological reactions, including that in the eye. It contributes to physiologically regulating ocular hemodynamics and cell viability and protects vascular endothelial cells and nerve cells or fibers against pathogenic factors. Ocular blood flow is regulated by NO derived from the endothelium and efferent nitrergic neurons. Endothelial dysfunction impairs ocular hemodynamics by reducing the bioavailability of NO and increasing the production of reactive oxygen species (ROS). On the other hand, NO formed by inducible NOS (iNOS) expressed under influences of inflammatory mediators evokes neurodegeneration and cell apoptosis, leading to serious ocular diseases. Although there were several evidences making us to doubt the mutation of inducible NOS gene taking part in the onset of diabetic retinopathy with macular cystoid edema, unfortunately, in present work, the distribution of G-954C of NOS2A polymorphism looks like at a lower frequency in China, and it semms irrelevant to the frequency of diabetic retinopathy with macular cystoid edema to some degree, at least in Chinese population.

The role of "genetic influences" in diabetic retinopathy has been difficult to define due to differences in patient recruitment methods, patient selection criteria, risk factors, variation in ethnicity and clinical differences in evaluating retinopathy status. There have been a number of association studies of diabetic retinopathy, including those implicating involvement of the major histocompatibility complex (MHC, HLA-DQB1* 0201/0302) [20]. The signal transduction components of the NO- regulating pathway in the vasculature may provide a starting point for understanding the complex genetic background of the pathological processes in diabetic retinopathy.

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