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·Original Article·

Cerebral vasculopathy in a Chinese family with neurofibromatosis type I mutation

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ABSTRACT

Neurofibromatosis type I (NF1) is a hereditary, autosomal dominant, neurocutaneous syndrome that is attributed to *NF1* gene mutation. NF1 has been associated with scoliosis, macrocephaly, pseudoarthrosis, short stature, mental retardation, and malignancies. NF1-associated vasculopathy is an uncommon and easily-overlooked presentation. Examination of a Chinese family affected by NF1 combined with cerebral vessel stenosis and/or abnormality suggested a possible relationship between *NF1* and vessel stenosis. To determine which *NF1* gene mutation is associated with vascular lesions, particularly cerebral vessel stenosis, we examined one rare family with combined cerebral vessel lesions or maldevelopment. Vascular lesions were detected using transcranial Doppler sonography and digital subtraction angiography in family members. Next, denaturing high-performance liquid chromatography and sequencing were used to screen for *NF1* gene mutations. The results revealed a nonsense mutation, c.541C>T, in the *NF1* gene. This mutation truncated the NF1 protein by 2659 amino-acid residues at the C-terminus and co-segregated with all of the patients, but was not present in unaffected individuals in the family. Exceptionally, three novel mutations were identified in unaffected family members, but these did not affect the product of the *NF1* gene. Thus the nonsense mutation, c.541C>T, located in the *NF1* gene could constitute one genetic factor for cerebral vessel lesions.

Keywords: neurofibromatosis type I; cerebral vessel stenosis; stroke; mutation

INTRODUCTION

Neurofibromatosis type 1 (NF1) is an autosomal-dominant disease caused by a single gene mutation and affects all ethnic groups[1]. The gene for NF1, *NF1*, was cloned on chromosome 17q11.2 and comprises 60 exons spanning 350 kb of genomic DNA[2]. The *NF1* gene has a large molecular size, variable transcripts, and heterogeneous mutation types. To date, >1270 mutations have been associated with restricted recurrence (Human Gene Mutation Database; http://www.hgmd.org/). NF1 is the most commonly inherited neurological disorder, affecting 1 in 3500 births[1]. Clinically, it is characterized by café-au-lait spots, skin freckling, small or plexiform neurofibromas, and Lisch nodules. Other manifestations, such as scoliosis, macrocephaly, pseudoarthrosis, short stature, mental subnormality, and malignancies are present in a minority of patients[3], among whom, NF1-associated vasculopathy is

an uncommon and easily-overlooked presentation that can have serious complications and contribute to mortality at younger ages[4]. Characteristic NF1 vascular lesions have been described in the entire vascular tree[5]. Many studies of the *NF1*$^{-/+}$ mouse model have also suggested that *NF1* is intimately assocated with vasculopathy[6-8]. Here, we present a Chinese family with NF1 in which 10 members (21 total) suffered from stroke, fatal cerebral vessel stenosis, and vessel lesions, including cerebral vessel and/or vertebral artery (VA) abnormalities.

MATERIALS AND METHODS

NF1 Family with Combined Vascular Lesions or Other Abnormalities

The study was approved by the Xuan Wu Hospital of Capital Medical University, Beijing, and the subjects gave informed consent. All NF1 patients in this study satisfied the National Institutes of Health diagnostic criteria, and clinical details are available[9]. Patient IV-4 was the proband (Fig. 1, Table 1): a 24-year-old man with normal intelligence who visited a doctor 8 years ago because of abrupt hypertension and a large neurofibroma on his head. His blood pressure was 180/100 mmHg during the medical examination. In the latest examination, severe headache without an obvious causative factor with nausea and vomiting while looking at dim objects brought him to the hospital. His blood pressure was 210/135 mmHg. Renal vascular hypertension and NF1 were the primary diagnoses.

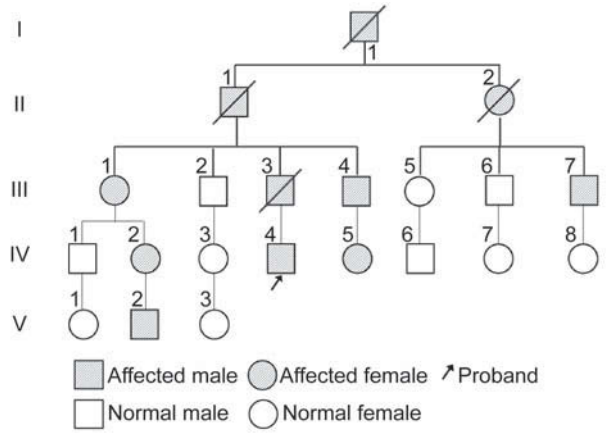

Fig. 1. Pedigree of a family affected by NF1 coincident with cerebral arterial stenosis. IV-4 is the proband. DNA samples from all surviving NF1 patients (except for III-1) and 7 normal members (III-2, 55/M; III-5, 52/F; III-6, 50/M; IV-1, 29/M; IV-3, 28/F; IV-7, 24/F; IV-8, 22/F) were screened by DHPLC to search for *NF1* gene mutations.

There were 11 NF1 patients in the proband's family, four of whom had died (Fig. 1). Interestingly, three of the deaths were due to stroke. Moreover, in these three NF1 patients, the ages at death were 54 years for the proband's grandfather, only 40 for the proband's father, and 47 for II-2 (a sister of the proband's father) (Fig. 1). On all of the NF1 patients in five generations, multiple alutaceous (leathery) patches or brown café-au-lait spots (CLSs) appeared during childhood. After childhood, the dermal symptoms worsened, and the incidence of vascular lesions increased. Patient IV-4 had a dermal neurofibroma (surgically removed) and many 10–20 mm round or oval alutacous patches with sharp borders on the chest, back, and abdomen (Fig. 2A). Patient III-4 was a 39-year-old man who had multiple unequal-sized neurofibromas on his skin. Patient III-7 was a 45-year-old man who, in addition to multiple unequal-sized neurofibromas on his skin, had an enormous neurofibroma on his abdomen. Both patients IV-2 and IV-5 were women (31 and 15 years old, respectively) who had multiple CLSs. In addition, patient IV-5 had a large CLS on her forearm. Patient V-2 was a six-year-old child who had CLSs and freckles in the axillary fossa. Except for patient III-7, every NF1 patient had vascular lesions or abnormalities to different extents (Table 1). The physical examination of the other members of the family revealed none of the major clinical features of NF1, and the results of vascular ultrasound for them were normal.

DNA samples from the surviving NF1 patients (except for III-1) and 7 normal family members were used for PCR and denaturing high-performance liquid chromatography (DHPLC) analysis to search for *NF1* gene mutations.

Vascular Detection

Routine transcranial Doppler sonography and ultrasonography in the carotid territory were carried out for every member in our study[10, 11]. Digital subtraction angiography (DSA) was used for some patients with cerebral vessel stenosis[12].

Oligonucleotide Primers, PCR Optimization, and PCR Amplification

Primer sequences for PCR amplification of all 60 exons of the *NF1* gene were as described elsewhere[13-16]. To

Table 1. Results of screening the entire coding region of the *NF1* gene

NO.	Subject No.	Age(years) /Sex	Cerebral vessel and other lesions	NF1	Exon	Genomic mutation	Codon substitution	Amino-acid substitution	Predicted protein product
1	IV-4	24/M	ACOA; renal angiostenosis; renal hypertension; renal tumor; stenosis in L-MCA	+	5	c.541C>T	CAG→TAG	Q181X	Truncation
2	IV-5	15/F	VSD; stenosis at origin of celiac artery (50-69%); variation in bilateral VA; circuitous in double stenosis	+	5	c.541C>T	CAG→TAG	Q181X	Truncation
3	IV-2	31/F	Severe stenosis in bilateral ICA, MCA, PCA, ACA and BA; segmentate stenosis in distal end of L-ICA; variation in L-VA	+	5	c.541C>T	CAG→TAG	Q181X	Truncation
4	III-4	39/M	Variation in bilateral VA	+	5	c.541C>T	CAG→TAG	Q181X	Truncation
5	V-2	6/M	Circuitous in bilateral ICA and VA	+	5	c.541C>T	CAG→TAG	Q181X	Truncation
6	III-7	45/M	Congenital absence of R-kidney	+	5	c.541C>T	CAG→TAG	Q181X	Truncation
7	III-5	52/F	-	-	31	c.4144G>A	GTA→ATA	V1382I	Normal size
8	III-6	50/M	-	-	38	c.5306C>G	CGA→CCA	R1769P	Normal size
9	IV-3	28/F	-	-	6	c.638T>G	ATA→AGA	I213R	Normal size

ACA, anterior cerebral artery; ACOA, anterior communicating artery; BA, basilar artery; ICA, internal carotid artery; MCA, middle cerebral artery; VA, vertebral artery; VSD, ventricular septal defect; PCA, posterior cerebral artery; L, left; R, right.

design specific amplimers, annealing temperatures for each amplicon were individually optimized by performing the amplification over a range of temperatures from 53°C to 68°C, as recommended by Hybaid (Ashford, Middlesex, UK).

Genomic DNAs isolated from peripheral blood cells were PCR amplified in 96-well microtiter plates with an oil-free system in 25-μL reaction volumes, each containing 50 ng genomic DNA, 5-10 pmol of each primer, 0.2 mmol/L dNTPs, 1 U Taq DNA polymerase (Qiagene), and 1× buffer. The PCR cycling was as described by Han[16]. The working procedure comprised an initial denaturation at 94°C for 4 min, followed by 35 cycles of denaturation at 94°C for 30 s, annealing at a specific temperature (Supplementary Table 1) for each fragment for 30 s, extension at 72°C for 30 s, and a final extension at 72°C for 10 min. Amplification of an appropriately-sized PCR product was confirmed on 2% agarose gels. An additional denaturation and re-annealing step was required after standard PCR amplification for heteroduplex formation prior to DHPLC[17]. Samples were denatured at 95°C for 5 min and then allowed to re-anneal for 30 min.

DHPLC Analysis

DHPLC was performed on a WAVE DNA fragment analysis system using a DNAsep column (Transgenomic)[18]. Full details are available from the authors. In brief, the successful resolution of heteroduplexes from homoduplexes requires an elution gradient at a partially-denaturing temperature. At this temperature, only heteroduplexes are destabilized by the mismatched bases and are therefore slightly more melted than the homoduplexes, resulting in an earlier elution than the homoduplexes[16]. This special resolution temperature (Tr) can be predicted by the DHPLCMelt software (http://insertion.stanford.edu/melt.html). Re-annealed DNA duplexes (7 μL) were injected onto the column and eluted with a linear acetonitrile gradient at a flow rate of 0.9 mL/min, with a mobile phase consisting of a mixture of buffers A (0.1 mol/L TEAA and 1 mmol/L EDTA) and B (25% acetonitrile in 0.1 mol/L TEAA). When more than one Tr for a particular fragment was recommended by the DHPLCMelt software, each recommended Tr was applied to determine the optimum sensitivity. Depending on the melting domains predicted by the software, the use of one, two, or three Trs was required to allow the successful

analysis of individual PCR fragments[16].

Sequencing Analysis

PCR products displaying a heterozygous pattern were purified and sequenced with an ABI Prism 371 Genetic Analyzer (PE Applied Biosystems).

RESULTS

Vascular Lesions and Abnormalities in NF1 Patients

The vascular lesions and abnormalities found in the NF1 family are presented in Table 1. Almost all of the NF1 patients had an abnormal distribution of the VA. DSA images of cerebral vessel stenosis are presented in Fig. 2. The results suggested that *NF1* mutations in these NF1 patients may be related to vascular development.

Prospective Screening for *NF1* Mutations by DHPLC

All 60 exons of the *NF1* gene were screened by optimized DHPLC analysis of 13 samples (6 NF1 patients and 7 normal members of the family). An identical mutation, c.541C>T, was found in all six patients (Table 1, Fig. 3). Furthermore, this distinct mutation (Q181X) in all patients suggested a relationship between the symptoms and the mutation. Q181X has only been reported in a screen of 169 unrelated NF1 patients by Griffiths[19] but has not been reported in any clinical study of this *NF1* site. In addition, 3 new missense mutations (c.4144G>A, c.5306C>G and c.638T>G) were detected in 3 normal members of the family (Table 1, Fig. 4). The significance of these mutations needs further study.

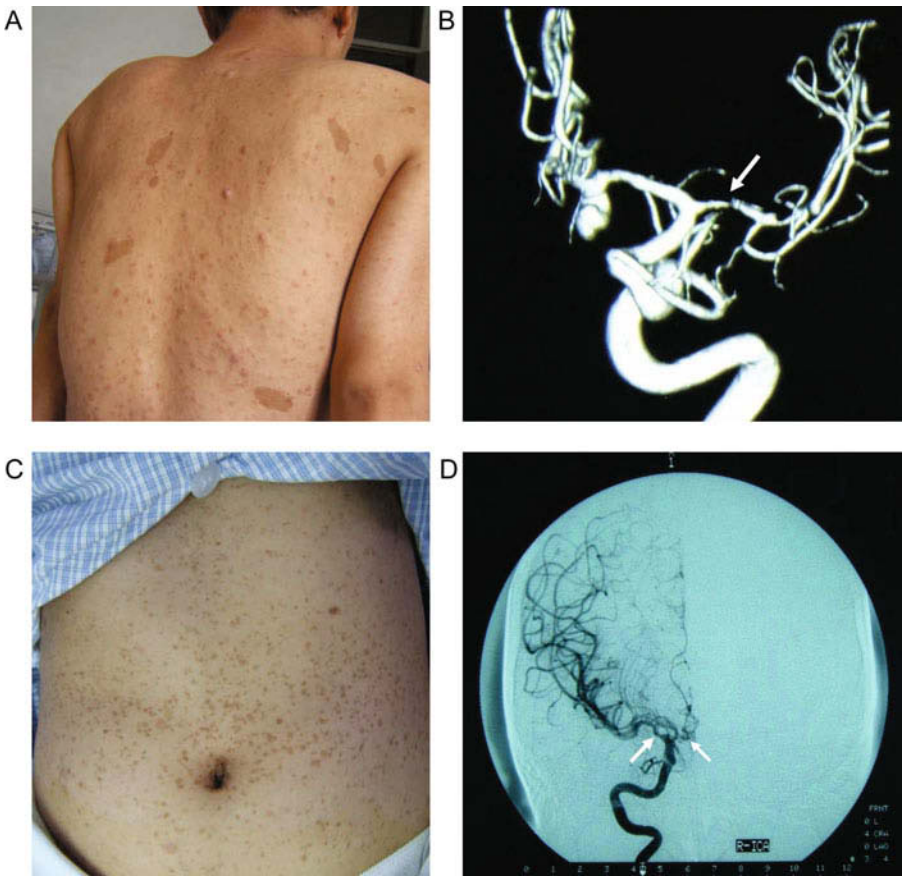

Fig. 2. Dermal manifestations in NF1 patients and the results of DSA. A and B show the results from the proband, IV-4. A: Dermal café-au-lait spots and neurofibroma; B: DSA image; arrow indicates region of cerebral vessel stenosis. C and D show the results from IV-2. C: Dermal café-au-lait spots; D: Arrows indicate regions of cerebral vessel stenosis.

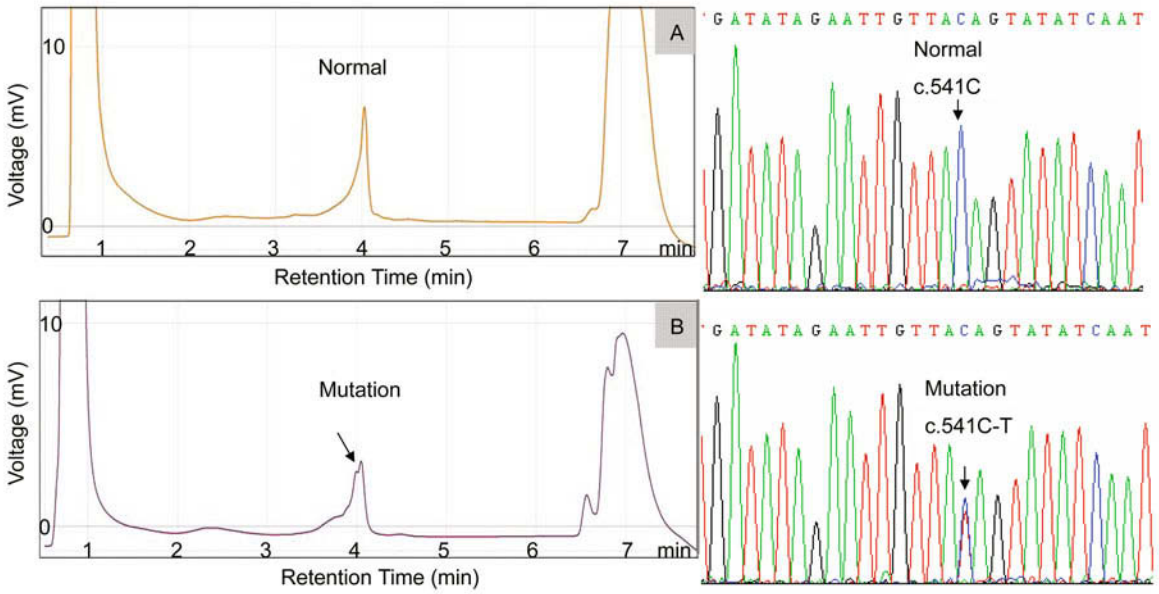

Fig. 3. DHPLC chromatograms identifying an *NF1* mutation in exon 5. A: DHPLC results for the wild-type *NF1* gene; the corresponding sequencing result is shown at the right. B: DHPLC results (arrow indicates variant elution profile for the *NF1* gene from NF1; sequencing result on right (arrow indicates variant base site: c.541C-T).

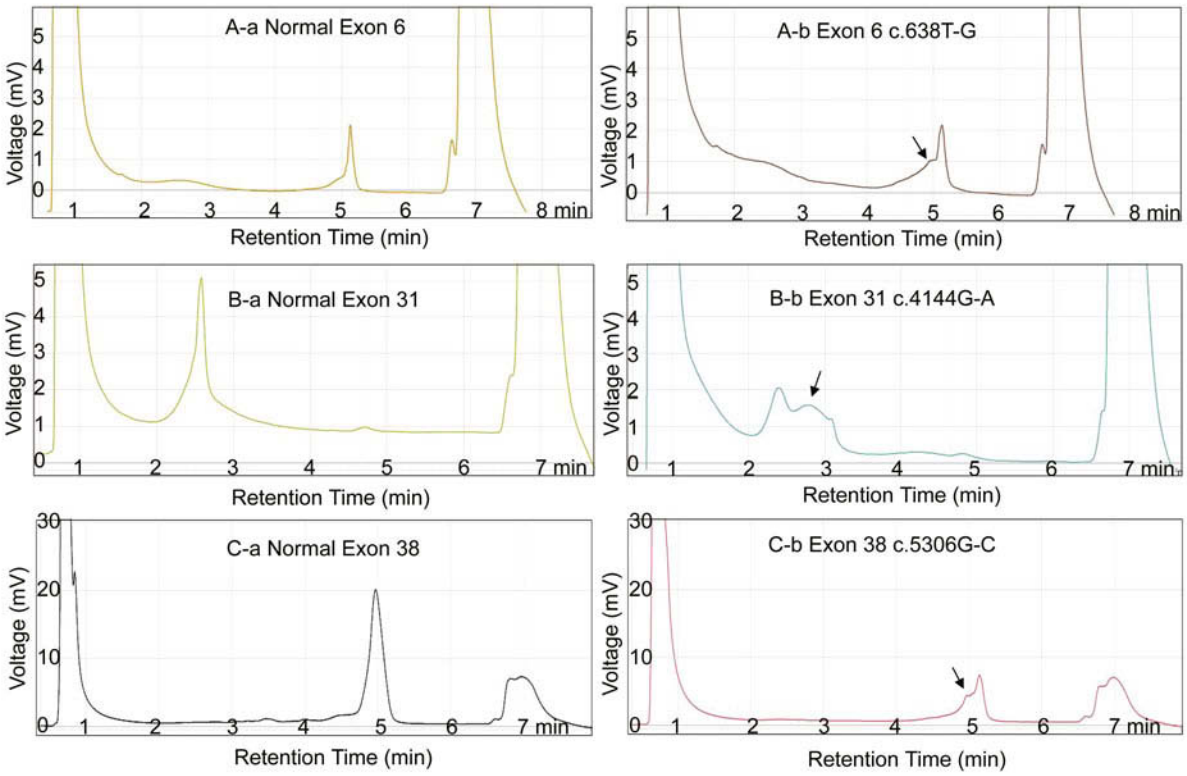

Fig. 4. DHPLC for *NF1* mutations identified in exons 6, 31, and 38 (arrows indicate variant elution profiles). *NF1* mutations in exons 6, 31 and 38 occurred singly in three non-NF1 members of the family [A-b (IV-3), B-b (III-5) and C-b (III-6)]).

DISCUSSION

NF1 and Vasculopathy

NF1 is probably the single most common dominant genetic disorder[20]. Neurological deficits that affect the central nervous system can result from cerebrovascular disease, tumors, and malformations, and can occur secondary to deformities of the skull and spine and from the pressure of neurofibromas on the spinal cord[21].

Neurofibromin, the product of the *NF1* gene, is also expressed in the endothelial and smooth-muscle cells of blood vessels, suggesting that abnormal function might cause vasculopathy by impairing the response of these cells to growth suppressor signals[5, 20, 22]. The underlying pathophysiology of vasculopathy in NF1 is incompletely understood, and defective neurofibromin function in the cellular components of the blood vessel wall may play a role[5, 22].

In this report, the patients demonstrated the classical skin manifestations of NF1 and an autosomal-dominant family history, which fulfilled the NIH consensus criteria[9]. The major physiological events in this Chinese family were multiple intracranial stenosis and an abnormal or circuitous VA, but examination also disclosed other arteriostenosis, aneurysm, kidney tumor, and dys-allelotaxis. Because these patients did not have any risk factors for stroke, such as diabetes mellitus, hypertension, and hyperlipidemia, *NF1*-associated cerebral vasculopathy was suspected.

NF1 Mutation and Cerebrovascular Stenosis and Abnormality

Thus far, whether there is a close link between the specific mutation and the vasculopathy remains unknown[5, 22]. It was important to this study that the participants were relatively centralized NF1 patients with cerebrovascular stenosis. Thus, the Chinese family in our study allowed us to determine whether there was a specific mutation related to the vasculopathy. The use of DHPLC for mutation detection represents a significant advance in the molecular diagnosis of NF1[16]. The common mutation (Q181X, Fig. 3) in all of the NF1 patients with cerebral vasculopathy suggested that the nonsense mutation which results in a truncated protein is the genetic factor for the manifestations of vasculopathy associated with NF1. Although this mutation (Q181X) has been reported by Griffiths *et al.* (http://www.hgmd.org/)[19], no further clinical data related to the mutation has been presented.

It is known that NF1 is caused by *NF1* gene mutation, but the relationship between the mutation and the corresponding symptoms, such as the 3 missense mutations in normal members of the NF1 family (Table 1, Fig. 4), remains unclear.

Mutation of *NF1* as a Risk Factor for Ischemic Stroke

Stroke is the third most common cause of death and the most common cause of disability in developed countries. About 80% of strokes are ischemic[23]. Non-modifiable risk factors (age, African and Asian race, male sex) and acquired risk factors (hypertension, cigarette smoking, diabetes, atrial fibrillation, and obesity) account for much of the risk of ischemic stroke[24], yet, stroke risk remains insufficiently explained by these factors[23, 25]. In our report, the stroke deaths were all in patients 40–55 years old. Thus, not only may pediatric stroke be associated with *NF1*[25], but also middle-aged stroke without risk factors for vascular disease may be caused by *NF1* mutation. Continuing the theme of gene-environment interaction, some findings suggest that chronic inflammation is an important contributor to *NF1* mutation-associated arteriopathy[6, 7]. Although the trigger for converting from a state of asymptomatic vascular dysfunction and inflammation to the development of arteriopathy remains clinically unclear, in stroke patients without obvious causative factors, *NF1* mutation may be one genetic factor that needs to be considered.

In conclusion, in this study, a Chinese NF1 family, who presented with a high incidence of cerebral vasculopathy compared to the normal members of the family, was analyzed. All of the NF1 patients had a common nonsense mutation (Q181X) of the *NF1* gene. These results suggest that this gene mutation may be one genetic factor for cerebral vasculopathy without any evident risk factors for stroke.

SUPPLEMENTAL DATA

Supplemental data include one table and can be found online at http://www.neurosci.cn/epData.asp?id=135.

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