RESEARCH ARTICLE

Isolation, characterization and genetic analysis of canine *GATA4* gene in a family of Doberman Pinschers with an atrial septal defect

SHIN-AEH LEE[1], SEUNG-GON LEE[1], HYEONG-SUN MOON[1], LOPETI LAVULO[2], KYOUNG-OH CHO[3] and CHANGBAIG HYUN[1,*]

[1]*Section of Small Animal Internal Medicine, School of Veterinary Medicine, Kangwon National University, Chuncheon, 200 701 Korea*
[2]*School of Veterinary Sciences, University of Melbourne, Parkville, VIC 3052, Australia*
[3]*Institute of Veterinary Medicine, College of Veterinary Medicine, Chonnam National University, Kwangju, 501 152 Korea*

Abstract

GATA4 is expressed early in the developing heart where it plays a key role in regulating the expression of genes encoding myocardial contractile proteins. Gene mutations in the human *GATA4* have been implicated in various congenital heart defects (CHD), including atrial septal defect (ASD). Although ASD is the third most common CHD in humans, it is generally rare in dogs and cats. There is also no obvious predilection for ASD in dogs and cats, based on sex or breed. However, among dogs, the incidence rate of ASD is relatively high in Samoyeds and Doberman Pinschers, where its inheritance and genetic aetiology are not well understood. In this study, we identified and investigated the genetic aetiology of an ASD affected family in a pure breed dog population. Although the *GATA4* gene was screened, we did not find any mutations that would result in the alteration of the coding sequence and hence, the predicted *GATA4* structure and function. Although the aetiology of ASD is multifactorial, our findings indicate that *GATA4* may not be responsible for the ASD in the dogs used in this study. However, this does not eliminate *GATA4* as a candidate for ASD in other dog breeds.

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Introduction

The GATA-binding proteins are comprised of a family of transcription factors containing a unique 'GATA' motif, a DNA-binding domain consisting of one or two zinc finger motifs (Arceci *et al*. 1993). These proteins control gene expression and differentiation in a variety of cells. *GATA4*, *GATA5* and *GATA6* are expressed in the developing and the adult vertebrate heart (Laitinen *et al*. 2000). Although *GATA5* expression is restricted to the endocardium, *GATA4* and *GATA6* are expressed in the myocardium. During foetal development, *GATA4* is expressed in the yolk sac endoderm and in cells that are involved in heart formation. In particular, *GATA4* is a key regulator of the expression of genes encoding myocardial contractile proteins, such as troponin C and cardiac alpha-myosin heavy chain (MYH6) (Molkentin *et al*. 1994; Huang *et al*. 1995).

It has been suggested that the combinatorial interaction among *GATA* families and other cofactors may differentially control various stages of cardiomorphogenesis (Durocher *et al*. 1997; Crispino *et al*. 2001; Cirillo *et al*. 2002). GATA4 is an established cofactor of NKX2-5, a key player in the myocardial developmental regulatory hierarchy in vertebrates, where they specifically cooperate both *in vitro* and *in vivo* to activate atrial natriuretic factor (ANF) and other cardiac promoters (Durocher *et al*. 1997). *GATA4* has also been found to interact with TBX5 and TBX20, which raises the possibility that GATA4, NKX2-5, TBX5 and TBX20 function in a complex to regulate a subset of genes required for cardiac septal formation (Garg *et al*. 2003; Stennard *et al*. 2003).

*For correspondence. E-mail: hyun5188@kangwon.ac.kr.

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GATA4-deficient mice have severe additional developmental problems that are unrelated to the heart (Plageman and Yutzey 2004). However, *GATA4* null mice have cardiac bifida due to the failure of the heart field to fuse at the midline. The chromosomal deletion (del8p23.1) has been implicated in some CHDs (Pehlivan *et al*. 1999). In humans, hemizygosity for *GATA4* was seen in four patients with congenital heart disease, but not in a patient without known cardiac anomalies (Pehlivan *et al*. 1999). The autosomal dominant *GATA4 G296S* mutation, has been identified in a family with various CHD (Garg *et al*. 2003). In this family, all affected individuals had ASD and eight individuals had additional forms of CHD, such as ventricular septal defect (VSD), atrioventricular septal defect, pulmonary valve thickening or insufficiency of the cardiac valves. However, none had cardiac conduction or other organ defects. So far, four additional novel mutations have been found in various human CHD (Garg *et al*. 2003; Hyun *et al*. 2004). One study showed that *GATA4* mutations may account for up to 7% of ASD cases. Additionally, the *GATA4 S377G* homozygous mutation appeared to be the cause for 7% of ASD and 21% of patent foramen ovale (PFO) cases with stroke via a Mendelian-recessive mechanism (Hyun *et al*. 2004).

ASDs results from a continued postnatal communication between the two atria that occurs because of a hole (foramen ovale) in the interatrial septum that failed to close at birth. Although small ASDs do not cause any significant clinical abnormality, moderate to large defects result in left-right shunting (L-R shunting) accompanied by substantial haemodynamic consequences, such as dilatation of the atria, right ventricle and pulmonary artery (Brickner *et al*. 2000).

In humans, ASD is the third most common CHD where it is two to three times more common compared to other CHDs. The most common type of ASD is, ostium secundum defect (up to 75%), is often observed with other cardiac defects, such as mitral valve prolapse (whereas primum ASD is often observed with mitral valve regurgitation). Most ASDs are sporadic but often observed as part of syndromic diseases, such as Down syndrome, pentalogy of Fallot, Holt-Oram syndrome, Noonan syndrome and 8p23 deletion syndrome (Allanson 1987; Korenberg *et al*. 1992; Basson *et al*. 1997; Schott *et al*. 1998).

To date, three genes (*NKX2-5, TBX5*, *GATA4*) and one genetic locus (*6p21*) have been identified as having key roles in the cardiac morphogenesis regulatory hierarchy in humans (Basson *et al*. 1997; Schott *et al*. 1998; Garg *et al*. 2003). Most mutations relating to these genes were found to accumulate in functionaly important domain such as the homeobox domain in *NKX2-5*, T-box domain in *TBX5*, and the C-*trans*-activation domain and the zinc finger in *GATA4*. The most familial ASDs have an autosomal dominant inheritance pattern (Hyun *et al*. 2004). Autosomal recessive inheritance has also been reported in *GATA4* from a *S377G* mutation. A gene mutation in *TBX5* is responsible for the human Holt-Oram syndrome, which is characterized by cardiac and hand malformations (Basson *et al*. 1997). Cardiac defects seen in Holt-Oram are primum and secundum ASD, muscular VSD and hypoplastic left heart syndrome (HLHS) (Basson *et al*. 1997).

Compared to humans, the frequency of ASD cases in lower mammals is only 0.2 per 1000 animals examined (Kittleson and Kienle 1998). Despite the smaller number of cases, it is intriguing to note that most cases diagnosed were secundum and primum ASDs, which are common in dogs and cats, respectively (Kittleson and Kienle 1998). Certain dog breeds, such as Doberman Pinschers, Boxers and Samoyeds, are predisposed to ASDs (Hyun and Park 2006). Generally, most ASD cases in dogs exhibited left-right atrial shunting which is occasionally responsible for the observed pulmonary hypertension (Kittleson and Kienle 1998). Interestingly, similar findings have also been observed in humans (Hyun and Park 2006). However, major medical advances in human CHD studies have not translated into similar approaches in animal studies where only a single genetic locus responsible for canine Epstein anomalies has been identified (Andelfinger *et al*. 2003).

Since *GATA4* is expressed in the developing heart and is involved in regulating cardiac gene expression, and has found to be responsible for human CHD including ASD, *GATA4* is a good candidate gene for CHD in dogs. Therefore, the comparison of the known human *GATA4* information to any canine *GATA4* information should advance our understanding of the genetic aetiology of CHD, especially atrial and ventricular septal defects, in dogs. In this study, we isolated and characterized the canine *GATA4* gene. Furthermore, we screened whether any mutation on *GATA4* may be responsible for the observed ASDs in a Doberman Pinscher family.

Materials and methods

Phenotypic evaluation

All dogs used in this study were phenotypically evaluated by cardiac auscultation, thoracic radiography, echocardiography (two dimensional, M-mode and color Doppler), after routine clinical examination.

Isolation of the canine GATA4 gene

The heart tissue was taken from a pound dog (6 months old female Kelpie cross). Total RNA was extracted from the heart tissue and treated with DNAase I, according to the manufacturer's instructions (SV RNA extraction kit, Promega, USA). Corresponding cDNA was synthesised by reverse transcription from the total RNA sample (SuperScriptIII® RT-Kit, Carlsbad, USA). A primer set was designed from the conserved region of the 5′ and 3′ end sequence of the human *GATA4* mRNA sequences (table 1). PCR amplification was carried out using 1 μl cDNA, 500 μM dNTP, 0.5 μM of each primer, 1× PCR buffer, G/C rich buffer (Roche, Basel, Switzerland) (PCR buffer 3, GC-rich PCR system, Basel, Switzerland), 1.5 mM magnesium chloride and 0.5 U of *Taq*

Table 1. Primer sequences and PCR protocols used in this study.

Primer name	Primer sequence	PCR annealing temperature (^{o}C)
GATA-cDNA_F	5′ - tttaattgtccttcttctgtctcc - 3′	54
GATA-cDNA_F	5′ - atcccctaaccgaactgtcaactt - 3′	
GATA-Ex1F	5′ - acccgtacacgatcatcactattt - 3′	54
GATA-Ex1R	5′ - agcttctgcgcccacccgtctgt - 3′	
GATA-Ex2F	5′ - ttgcaaggaaaaggtgttatt - 3′	57
GATA-Ex2R	5′ - tcccctgacttgtgctctcctact - 3′	
GATA-Ex3F	5′ - cagcacttgccttctatttg - 3′	59
GATA-Ex3R	5′ - ctgccctccccctgcttgtgt - 3′	
GATA-Ex4F	5′ - aattgcttagatgtttccttcac - 3′	59
GATA-Ex4R	5′ - ggctggcccacctaatg - 3′	
GATA-Ex5F	5′ - aggaccccgcttcactc - 3′	59
GATA-Ex5R	5′ - gcctgccactcgctcctt - 3′	
GATA-Ex6F	5′ - ctccagcccagacctccatcct - 3′	59
GATA-Ex6R	5′ - agcctcatccctgccgtcctc - 3′	
GATA-probe-F	5′ - ccaaccccgccgcccgacacc - 3′	59
GATA-probe-R	5′ - aggcattgcacactggctcac - 3′	

polymerase (GC-rich PCR system, Basel, Switzerland). The PCR-amplification protocol involved initial denaturation at 94°C for 3 min, followed by 35 cycles (94°C, 15 s; 54°C, 30 s; 72°C, 1 min) in an automated thermal cycler (PCR Tetrad, MJ Research, Wattam, USA).

After PCR amplification, 1 μl of PCR amplicon was ligated into pGEM-T easy® vector (Promega, Madison, USA) followed by transformation into *E. coli* competent cells (XL-Blue Gold, Stratagene, La Jolla, USA). After an overnight culture, plasmid DNA was purified using a commercial mini-prep kit (SV mini prep kit®, Promega, Madison, USA). The plasmid DNA containing the canine *GATA4* PCR amplicon was sequenced with SP6 and T7 primers using capillary electrophoresis (ABI 3000, Sciex, Ontario, USA) with a dye terminator (version 3.1, BigDyeTM, Applied Biosystems, Foster City, USA). The obtained sequence data was then analysed using the software package DNA star (Lasergene, Madison, USA).

Expression of canine GATA4

To investigate the *GATA4* gene expression pattern in adult tissues, equal amounts of total RNA from each tissue (Doberman Pinscher dog; Seegene, Seoul, Korea) were electrophoresed in 1.2% agarose in the presence of 2.2 M formamide with subsequent blotting onto Hybond-N membrane (GE Healthcare, Pittsburg, USA). High stringency hybridization was done as described by Sambrook *et al*. (1989). The canine *GATA4* probe (table 1) was prepared using a [$\alpha - ^{32}$p] dCTP random labelling method. The housekeeping gene, *GAPDH*, was used as an equal loading control.

Mutation screening of canine GATA4 in a family of Doberman Pinchers having ASD

Six sets of mutation screening primers were designed from the intron boundary of each exon of the canine *GATA4* gene in the canine *GATA4* genomic clone (GenBank No. NW_876279) (table 1). All exons of *GATA4* gene were amplified by polymerase chain reaction (RT-PCR) from 50 ng of genomic DNA extracted from blood, purified with PCR Cleanup Plates (Millipore, Billerica, USA) and sequenced using Big Dye Terminator v3.1 kit (Applied Biosystems, Foster City, USA) and ABI PRISM® 3700 DNA Analyzer (AMC Bioscience, Toroed, Norway). PCR amplification was carried out using two protocols: (i) for *GATA4* cDNA and exon 1 of *GATA4* DNA, using 1 μl DNA, 500 μM dNTP, 0.5 μM of each primer, 1× PCR buffer, G/C rich buffer (Roche, Basel, Switzerland), 1.5 mM magnesium chloride and 0.5 U of *Taq* polymerase (GC-rich PCR system, Basel Switzerland); (ii) for exon 2–6 of *GATA4* DNA, using 1 μl DNA, 500 μM dNTP, 0.5 μM of each primer, 1× PCR buffer, 1.5 mM magnesium chloride and 0.5 U of *Taq* polymerase (NeoThem^{TM} DNA polymerase, Basel, Switzerland). The PCR-amplification protocol involved initial denaturation at 94°C for 3 min, followed by 35 cycles (94°C, 15 s; specific annealing temperature, 30 s; 72°C, 1 min in an automated thermal cycler) (PCR Tetrad, MJ research, Walttam, USA). To produce *GATA4* cDNA probe, *GATA*-probe-F and *GATA*-probe-R primer were used with a 1/10 diluted dCTP containing dNTP solution.

Results and discussion

In this study, we identified one Doberman Pinscher family in which two dogs had ASD and four dogs had intact atrial septa (figure 1). In one affected dog (A2; three years of age, female), a moderate systolic murmur was heard over the left heart base, subsequent with moderate right side heart enlargement on a thoracic radiography. In echocardiographic studies, this dog had a moderate size ASD with severe interatrial communications confirmed by

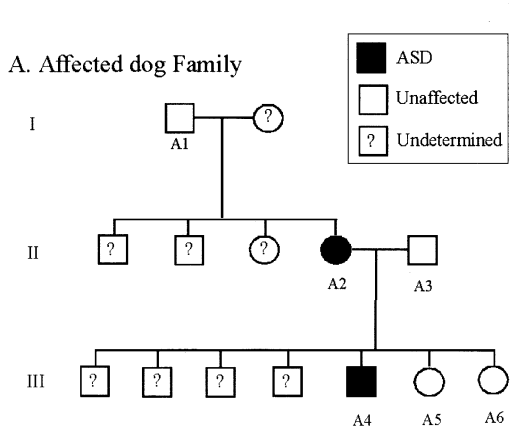

Figure 1. Affected Doberman Pinscher family used in this study. square, male; circle, female; filled, affected for ASD; clear, unaffected for ASD; question mark, not determined.

colour-Doppler echocardiography (figure 2). In another affected dog (A4, one year old, male) that was an offspring of A2, a similar systolic murmur was heard over the left heart base. Significantly right side heart enlargement was not detected, although pulmonary vasculature was mildly engorged. In color-Doppler echocardiography, the moderate interatrial communication was clearly observed. No cardiac abnormalities including interatrial communication, were observed in the other dogs.

For genetic analysis, we successfully isolated a full length of coding mRNA sequence of canine *GATA4* (GenBank Access No. DQ666280; figure 3). Using the deduced amino acid sequence, we analysed sequence homology with the other *GATA4* sequences and found that it was highly conserved across species (figure 4). In sequence analysis, canine *GATA4* is quite close to human *GATA4* in mRNA sequence as well as amino acid sequence (figure 3).

To evaluate the gene expression pattern in different tissues, we did Northern blot analysis. A strong hybridization to the *GATA4* probe was observed in RNA from the heart, ovary, testes and liver, while a weak hybridization was observed in RNA from lung, small intestine and large intestine. No hybridization signal was detected in skeletal muscle, spleen, kidney, eye and brain (figure 5).

In mutation screening of *GATA4* gene of this affected Doberman Pinscher family, no mutation causing amino acid changes have been found between affected and unaffected dogs in our study population. Furthermore, there were no nucleotide differences between Doberman Pinschers and other dog breeds.

GATA4 is an important transcription factor due to its critical role in early cardiomorphogenesis. Recent studies have found gene mutations in *GATA4* linked to various human CHDs, including ASD. Atrial septal dysmorphogenesis including ASD and its mild variant, PFO, is very common in humans whereas it is less frequent, but not uncommon in dogs. However, it is not clear why the prevalence rate of septal dysmorphogenesis in dogs (and cats), is much lower than in the human population. It may be because the lower prevalence rate is attributed to the species

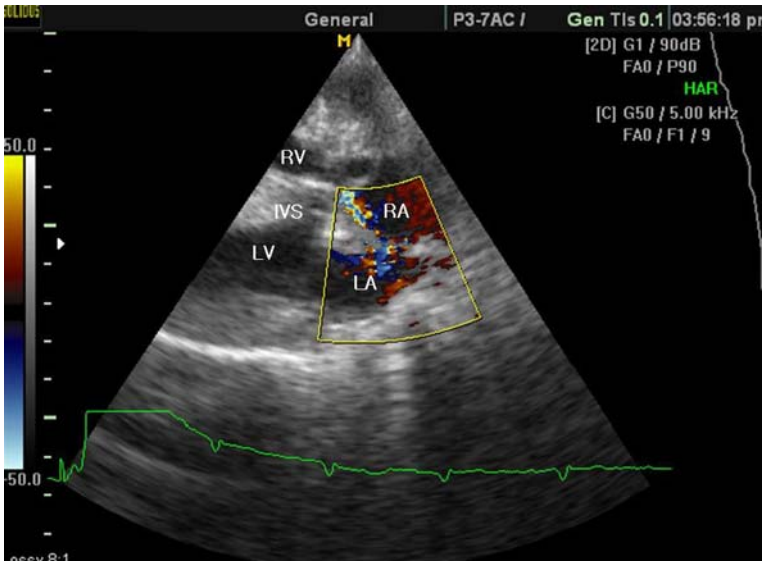

Figure 2. Colour-Doppler echocardiography of an affected dog (A2). In the colour-Doppler echocardiogram, red and blue colors indicate blood flows inside heart. In dogs having intact atrial septum, there is no blood flow between right and left atria. In this dog, there is blood flow between right and left atria, because of interatrial communication (atrial septal defect). RV, right ventricle; IVS, interventricular septum; LV, left ventricle; RA, right atrium; LA, left atrium.

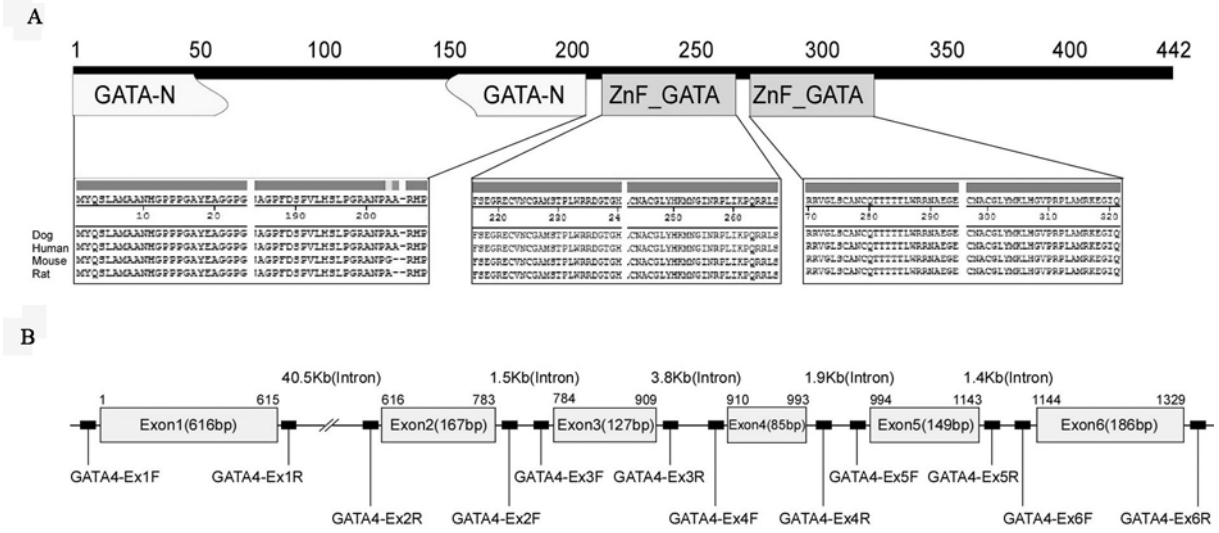

Figure 3. Predicted structure (A) and conserved domains (B) of canine *GATA4*.

Figure 4. The multiple alignment of GATA4 protein sequences from human (NP.002043); dog (ABG75570); mouse (NP.032118) and rat (NP.653331). This alignment revealed that GATA protein is highly conserved across species.

anatomical variation during heart development. However it is more plausible that it is due to lack of further investigation into early cardiac death in pups and kittens. Therefore, it is possible that the actual prevalence rate of CSD (cardiac septal defect) in dogs (and cats), could be higher than currently documented.

Despite major advances in identifying genetic aetiologies in human CHDs, parallel investigations into canine CHDs are extremely limited. In this study, we chose *GATA4* as candidate, and then isolated and characterized the canine *GATA4*, based on its involvement in human CHD, especially ASD. *GATA4* is highly conserved across species,

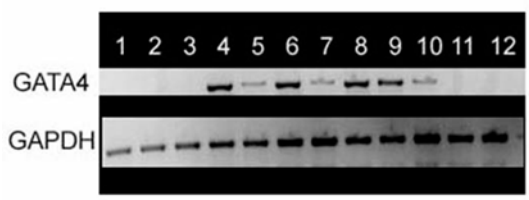

Figure 5. The *GATA4* RNA expression pattern in different tissues from dogs. Lane 1, skeletal muscle; lane 2, brain; lane 3, eye; lane 4, heart; lane 5, lung; lane 6, ovary; lane 7, small intestine; lane 8, testis; lane 9, liver; lane 10, large intestine; lane 11, spleen; lane 12, kidney.

especially in two DNA binding zinc fingers. Known mutations of human *GATA4* are located at the nuclear localization zone (NLZ) and the potential C-terminus *trans*-activation domain (C-TAD). Since *GATA4* plays critical roles in early cardiomorphogenesis by regulating down stream cardiac genes, any mutation in the TAD may affect the activation of a myriad of cardiac gene expressions.

Similar tissue expression patterns of *GATA4* mRNA have been previously reported, although *GATA4* gene expression is higher in the murine liver and weaker in the canine large intestine. It is unclear whether these different expression levels are only due to species differences, or if these differences will provide an important clue for the different roles of the GATA4 protein in GATA4-mediated signaling pathways. Further studies are therefore, warranted.

ASD is a common congenital heart defect in humans that is also influenced by several factors such as drugs, alcohol and viral infection (e.g. Rubella). The reported heritability (h^2) of ASD is 0.6, which implies a strong genetic aetiology for this disease (Brickner *et al*. 2000). Known gene mutations affecting human ASD are all inherited as autosomal dominants, except *GATA4 S377G* (autosomal recessive) (Schott *et al*. 1998; Hyun and Park 2006). Several pure dog breeds, including Doberman Pinschers, have a predilection for ASD, although the reported prevalence rate in this dog breed is much lower than in the human population (Kittleson and Kienle 1998). Our results indicate that in dogs, *GATA4* is not directly linked to the observed ASD in the affected pure breed Doberman Pinscher family we studied, although the possibility of a mutation in the noncoding sequences in this affected family should be formally ruled out. Since human ASD is caused by multiple genetic aetiologies (mutation in *NKX2.5*, *TBX5*, *GATA4*), the possibility remains that a *GATA4* mutation is responsible for ASD in other dog breeds. Furthermore, human *GATA4* mutations are also responsible for other type of CHDs including VSD, tetralogy of Fallot, which suggests that canine *GATA4* mutations could be responsible for other types of CHDs, because *GATA4* is important for endocardial cushion formation which later form the cardiac valve and septum (Arceci *et al*. 1993; Crispino *et al*. 2001; Garg *et al*. 2003). Further mutation screening studies in other ASD dog families and in different CHD phenotypes are warranted. Therefore, the isolation and characterization of the canine *GATA4* gene will help to advance our understanding of the genetic aetiologies of canine CHDs.

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