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AGPAT6 polymorphism and its association with milk traits of dairy goats

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ABSTRACT. As one of the eight members in the 1-acylglycerol-3-phosphate-O-acyltransferase (AGPATs) family, *AGPAT6* is a crucial enzyme for the biosynthesis of glycerolipids and triacylglycerol in eukaryotes, as well as catalyzing the conversion from lysophosphatidic acid to phosphatidic acid. *AGPAT6* can be considered as a candidate gene for regulating milk composition. DNA sequencing and PCR-RFLP methods were applied to detect genetic variation in the *AGPAT6* gene in 549 Chinese dairy goats. Four polymorphisms (NC_007328.3:g.152G>C, 8124G>A, 9263C>G, 16436G>A) were detected in 5'UTR, intron 2, exon 4, and 3'UTR, respectively. For the *Kpn*I locus, the frequencies of the *AGPAT6*-G allele were 0.955 and 0.936 for SN (Xinong Sannen) and GZ (Guanzhong) dairy goat breeds, respectively. In the PCR-RFLP analysis for *Kpn*I, *Eco*RII, *Nco*I, and *Bgl*I, the frequencies of the G allele of *AGPAT6* were 0.955 and 0.936, 0.694 and 0.819, 0.206 and 0.254, 0.729 and 0.623 for SN and GZ dairy goat breeds, respectively. The 9263C>G mutation revealed a synonymous genetic code of Thr (threonine). Associations between the

four mutations and milk traits were analyzed in two dairy goat breeds. At the 9263C>G locus, genotype GG and CG individuals showed significantly better milk performance than genotype CC individuals ($P < 0.05$). Therefore, the G allele is suggested to be a molecular marker for milk production in dairy goats.

Key words: Dairy goat; *AGPAT6* gene; Polymorphism; PCR-RFLP; Milk traits

INTRODUCTION

The 1-acylglycerol-3-phosphate-O-acyltransferase (AGPAT) enzyme is crucial for the synthesis of glycerolipids as well as triacylglycerol biosynthesis in eukaryotes (Ye et al., 2005). It catalyzes the conversion of lysophosphatidic acid (LPA) to phosphatidic acid (PA) (Aguado and Campbell, 1998; Coleman and Lee, 2004; Agarwal et al., 2006, 2007; Sukumaran et al., 2009). So far, eight members of the *AGPAT* family in human have been described, which are *AGPAT1*, *2*, *3*, *4*, *5*, *6*, *7*, and *8* (Ye et al., 2005), and all of them possess LPAAT (lysophosphatidic acid acyltransferase) motifs (Nagle et al., 2008). *AGPAT6* is one of the eight *AGPAT* isoforms identified through sequence homology (Nagle et al., 2008). It is a novel gene similar in sequence to glycerolipid acyltransferases (Agarwal et al., 2007). Although *AGPAT* family members share certain amino acid sequence similarities, *AGPAT6*'s closest family member is another novel gene, *AGPAT8*, and both of them are conserved from plants, nematodes, and flies to mammals (Beigneux et al., 2006). *AGPAT6* is highly expressed in mammary epithelium of breast tissue (Beigneux et al., 2006). It has been implicated in triglyceride synthesis in mammary glands and has been recognized as microsomal glycerol-3-phosphate acyltransferase (GPAT), which catalyzes the glycerolipid biosynthesis pathway (Beigneux et al., 2006).

Based on the regulatory roles of the *AGPAT6* gene in glycerolipid biosynthesis catalysis and milk fat production, the *AGPAT6* gene could be considered as a potential gene for milk composition. Therefore, the objectives of this study are to detect SNPs of the *AGPAT6* gene in dairy goats and explore their possible association with milk traits in two Chinese dairy goat breeds.

MATERIAL AND METHODS

DNA samples

Five hundred and forty-nine blood samples were obtained from two Chinese dairy goat breeds including Xinong Sannen (SN, N = 268) and Guanzhong (GZ, N = 281). Genomic DNA was extracted from blood samples following standard procedures (Sambrook and Russell, 2001) and stored at -80°C. The records of milk components including milk fat (%), protein (%) and milk yield (kg) from all animals (1 year old) were collected for statistical analysis.

Primer design and polymerase chain reaction (PCR) amplification

Based on GenBank sequence No. NC_007328.3, 10 pairs of PCR primers (Table 1)

were designed to amplify 5'UTR, exon 1-11, intron 2, partial exon 12, and 3'UTR of the goat *AGPAT6* gene. The 25 µL volume contained: 20 ng genomic DNA, 0.5 µM of each primer, 1X buffer (including 1.5 mM $MgCl_2$), 200 µM dNTPs, and 0.625 U *Taq* DNA polymerase (MBI). The cycling protocol was 5 min at 95°C, 34 cycles of 94°C for 30 s, annealing (°C) for 30 s as indicated in Table 1 and 72°C for 35 s, with a final extension at 72°C for 10 min.

Table 1. Primer information for PCR amplification of the goat *AGPAT6* gene.

Loci	Primers (5'-3')	Length (bp)	Tm (°C)	Note
P1	F: GAGCGGAACAGAGCCCAGAG R: GGCGGGGACGATGATGAA	386	58	7-392 (Exon 1, partial 5'UTR)
P1-*Kpn*I	F: GCGGAACAGAGCCCAGAGCG R: GTTCGCATCCCTCGATGGCCGGTAC	175	63	2-176 (5'UTR)
P2	F: ACCCTATGTGTTGCTGTTCC R: GATGGTCTCCATTCCTTTCC	372	60	7873-8244 (Exon 2, Intron 2)
P3	F: TCTCCCAGCAGACCACCT R: CGAACCCGCATGAGTAGG	315	60	8193-8507 (Exon 3)
P4	F: ATCTGGCATTTTCACACATT R: CTGACTCCATCTAAGAGCCT	241	53	9135-9375 (Exon 4)
P5	F: GCTTACCGTAGACACTAATAAT R: CCACTCGGCTCACAGCACCCAG	635	55	9889-10523 (Exon 5-6)
P6	F: TCTTTGTAGGGTTTTCAGTT R: CTCTATATTCCACATTTCGG	419	49	10811-11229 (Exon 7)
P7	F: TTCATACCGCACCCAGCATC R: AGTTCCAGGACCAGCCCAAA	271	61	11740-12010 (Exon 8)
P8	F: GCTGTCAGAGGGCTTTTACTATTC R: CCGAGCTGCTGGGTTTATG	324	55	12193-12516 (Exon 9)
P9	F: CACCCCGCCCTGTCGCCC R: AACCGCCTCAGAAATCCC	371	61	15509-15879 (Exon 10-11)
P10	F: TACAGGAACGAACCCCAAAC R: TGGGGCACCGTCTGTCTGAG	336	59	16298-16633 (Exon 12, 3'UTR)

The underlined base shows mismatches changed for creating restriction sites. Tm = annealing temperature.

After amplifying the goat genomic DNA pool (1 µL from every individual) (Sham et al., 2002), PCR products were sequenced directly by ABI PRIZM 377 DNA sequencer (Perkin-Elmer) and then analyzed with the BioXM software (version 2.6).

PCR restriction fragment length polymorphism (PCR-RFLP)

The PCR-RFLP technique was used to detect the polymorphism of *AGPAT6*. Aliquots of 10 µL PCR products of the *AGPAT6* gene were digested with 10 U restriction endonuclease (MBI fermentas) for 9 h at 37°C following supplier instructions. The digested products were detected by electrophoresis on 2.5% agarose gel stained with ethidium bromide.

Statistical analysis

Statistical analysis was performed on the basis of records of milk traits in SN and GZ breeds. Genotypic and allelic frequencies of polymorphism sites on the *AGPAT6* gene were analyzed by the chi-square test for the two Chinese dairy goat breeds using the SPSS software (version 16.0). Gene homozygosity (H_O), heterozygosity (H_E), effective allele numbers (N_E), and polymorphism information content (PIC) were evaluated by Nei's methods (Nei and Roychoudhury, 1974). The association between genotypes and milk performance was analyzed

through the general linear model (GLM) procedure of SPSS according to the following model:

$$Y_{ijkl} = \mu + B_i + A_j + G_k + (BG)_{ik} + E_{ijkl}$$

where Y_{ijkl} is the trait measured on each of the ijklth animal; μ is the overall mean; B_i is the type of the ith breed; A_j is the type of the jth age; G_k is the type of the kth genotype; $(BG)_{ik}$ is the interaction between the ith breed and the kth genotype, and E_{ijkl} is the random error (Lan et al., 2007).

RESULTS

Variations of the goat *AGPAT6* gene were detected by DNA sequencing and PCR-RFLP methods. Four mutations (NC_007328.3:g.152G>C, 8124G>A, 9263C>G, 16436G>A) were detected in 5'UTR (P1-*Kpn*I), intron 2 (P2), exon 4 (P4), and 3'UTR (P10), respectively. No polymorphism was detected in the remaining loci (P1, P3, P5-P9) of the goat *AGPAT6* gene.

The g.9263C>G mutation resulted in a synonymous genetic code of threonine, in detail, ACC (Thr) > ACG (Thr) at position 195 aa of *AGPAT6* (457 aa).

A new pair of primers for the P1-*Kpn*I (Table 1) was designed to introduce a *Kpn*I endonuclease restriction site (GGTACC) for the detection of the 152G>C mutation, hence 175-bp PCR products digested with *Kpn*I showed two bands (150 and 25 bp) for the C allele and one band (175 bp) for the G allele, and indicated GG, GC and CC genotypes (Figure 1A,B).

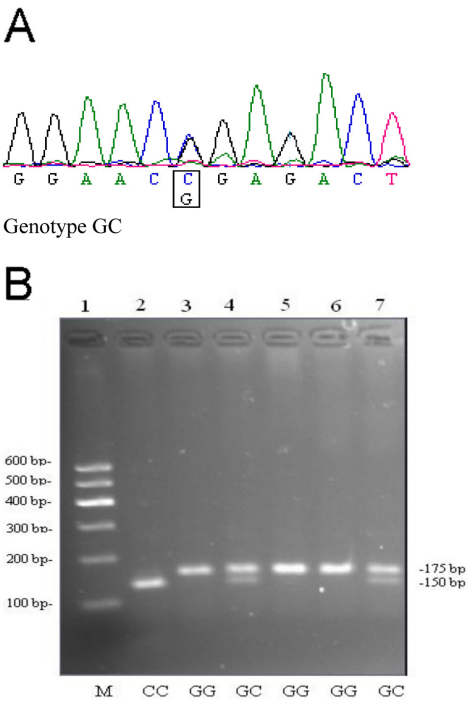

Figure 1. A. Sequencing maps at position of NC_007328.3:g.152G>C in the goat *AGPAT6* gene. **B.** Electrophoresis patterns of *Kpn*I forced PCR-RFLP analysis of the goat *AGPAT6* gene. GG = 175 bp; GC = 175 + 150 + 25 bp; CC = 150 + 25 bp. *Lane M* = marker I (600, 500, 400, 300, 200, and 100 bp).

The 8124G>A mutation holded an *Eco*RII endonuclease restriction site (CCWGG), and the digestion of the 372-bp PCR products showed only one fragment (372 bp) for the G allele and two fragments (253 and 119 bp) for the A allele, and indicated GG, GA and AA genotypes (Figure 2A,B).

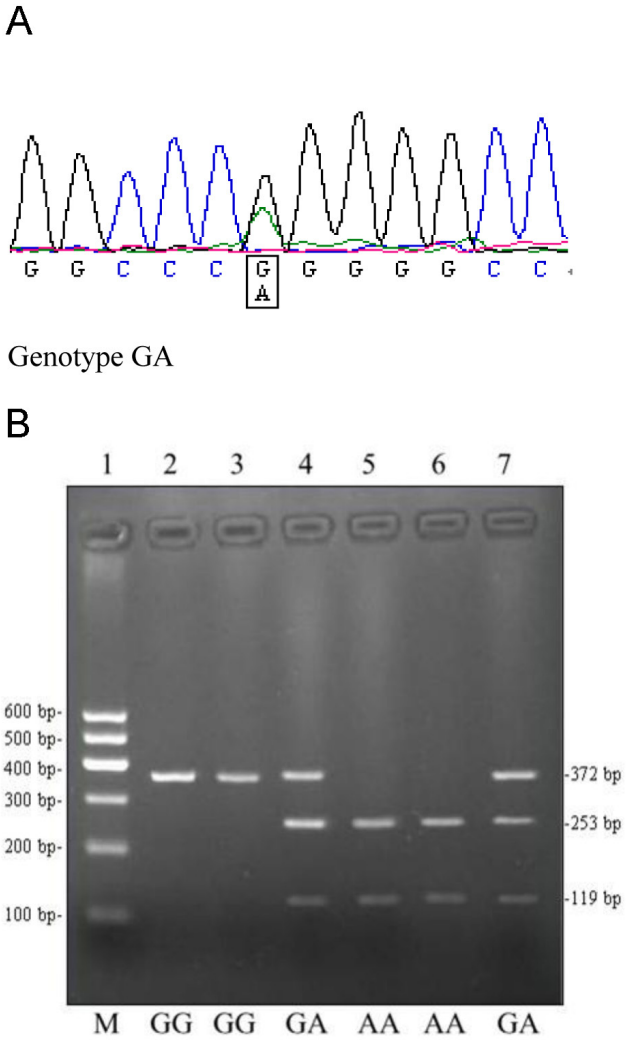

Figure 2. A. Sequencing maps at position NC_007328.3:g.8124G>A in the goat *AGPAT6* gene. **B.** Electrophoresis patterns of *Eco*RII PCR-RFLP analysis of the goat *AGPAT6* gene. GG = 372 bp; GA = 372 + 253 + 119 bp; AA = 253 + 119 bp). *Lane M* = marker I (600, 500, 400, 300, 200, and 100 bp).

The 9263C>G mutation added an *Nco*I endonuclease restriction site (CCATGG), and digesting by *Nco*I, 241-bp PCR products showed two fragments (130 and 111 bp) for the G allele and only one fragment (241 bp) for the C allele, and indicated CC, GC and GG genotypes (Figure 3A,B).

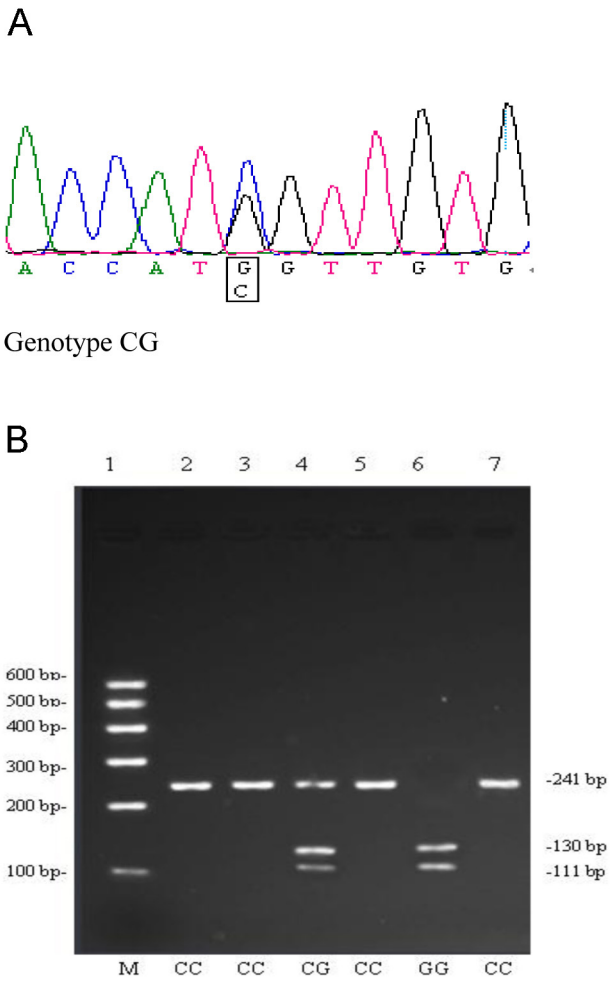

Figure 3. A. Sequencing maps at position NC_007328.3:g.9263C>G in the goat *AGPAT6* gene. **B.** Electrophoresis patterns of *Nco*I PCR-RFLP analysis of the goat *AGPAT6* gene. CC = 241 bp; CG = 241 + 130 + 111 bp; GG = 130 + 111 bp. *Lane M* = marker I (600, 500, 400, 300, 200, and 100 bp).

Moreover, the 16436G>A mutation holds a *Bgl*I endonuclease restriction site (GCCNNNNNGGC), and a 336-bp PCR product digestion with *Bgl*I demonstrated two fragments (195 and 141 bp) for the A allele and only one fragment (336 bp) for the G allele, and indicated GG, GA and AA genotypes (Figure 4A,B).

The frequencies of genotype and allele were calculated in the two Chinese goat breeds (Table 2). In the PCR-RFLP analysis with *Kpn*I, *Eco*RII, *Nco*I, and *Bgl*I, the frequencies of the G allele were 0.955 and 0.936, 0.694 and 0.819, 0.207 and 0.254, 0.729 and 0.623 for the two breeds, respectively. H_O, H_E, N_E, and PIC were evaluated (Table 3). At the 152G>C locus, two breeds showed low polymorphism (PIC <0.250). At the other three loci, the two breeds possessed moderate genetic diversity (0.250 < PIC < 0.500).

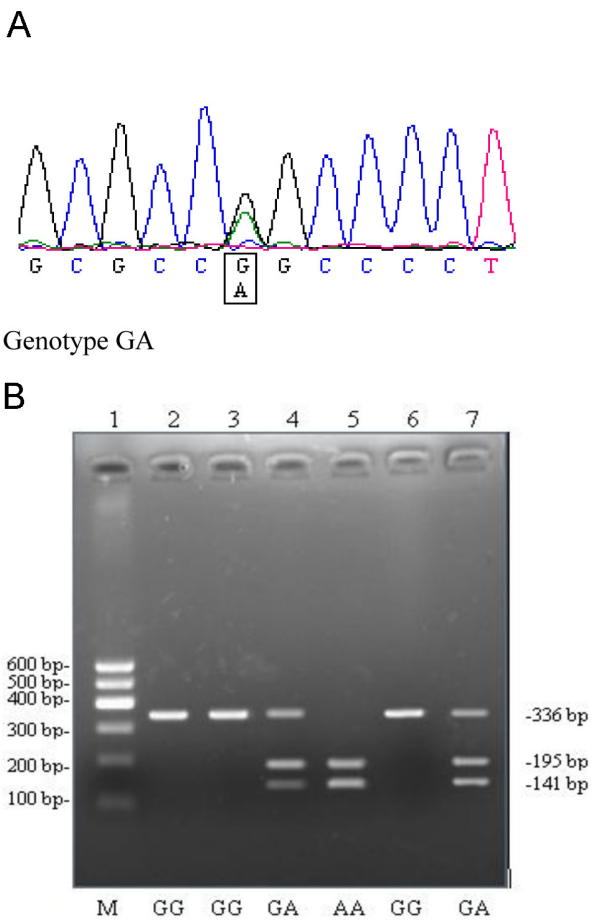

Figure 4. A. Sequencing maps at position NC_007328.3:g.16436G>A in the goat *AGPAT6* gene. **B.** Electrophoresis patterns of *Bgl*I PCR-RFLP analysis of the goat *AGPAT6* gene. GG = 336 bp; GA = 336 + 195 + 141 bp; AA = 195 + 141 bp. *Lane M* = marker I (600, 500, 400, 300, 200, and 100 bp).

Table 2. Genotypic and allelic frequencies of *AGPAT6* in two goat breeds.

Loci	Breeds	Genotype frequencies				Allelic frequencies	
*Kpn*I		GG	GC	CC	Total	G	C
	SN	0.922 (247)	0.067 (18)	0.011 (3)	268	0.955	0.045
	GZ	0.890 (250)	0.093 (26)	0.018 (5)	281	0.936	0.064
*Eco*RII		GG	GA	AA	Total	G	A
	SN	0.519 (139)	0.351 (94)	0.131 (35)	268	0.694	0.306
	GZ	0.665 (187)	0.306 (86)	0.029 (8)	281	0.819	0.181
*Nco*I		CC	CG	GG	Total	C	G
	SN	0.694 (186)	0.198 (53)	0.108 (29)	268	0.793	0.207
	GZ	0.623 (175)	0.246 (69)	0.132 (37)	281	0.746	0.254
*Bgl*I		GG	GA	AA	Total	G	A
	SN	0.593 (159)	0.272 (73)	0.134 (36)	268	0.729	0.271
	GZ	0.384 (108)	0.477 (134)	0.139 (39)	281	0.623	0.377

SN = Xinong Sannen; GZ = Guanzhong.

Table 3. H_O, H_E, N_E, and PIC at four polymorphic loci of the goat *AGPAT6* gene.

Loci	Breeds	H_O	H_E	N_E	PIC
*Kpn*I	SN	0.9145	0.0855	1.0935	0.0819
	GZ	0.8801	0.1199	1.1362	0.1127
*Eco*RII	SN	0.5753	0.4247	1.7382	0.3345
	GZ	0.7029	0.2971	1.4227	0.2530
*Nco*I	SN	0.6732	0.3268	1.4855	0.2734
	GZ	0.6214	0.3786	1.6093	0.3069
*Bgl*I	SN	0.6053	0.3947	1.6520	0.3168
	GZ	0.5301	0.4699	1.8863	0.3595

H_O = gene homozygosity; H_E = gene heterozygosity; N_E = effective allele number; PIC = polymorphism information content; SN = Xinong Sannem; GZ = Guanzhong.

In this research, we also revealed an association between the polymorphisms of the *AGPAT6* gene and milk traits of two dairy goat breeds. The statistical results showed that, in the *Nco*I locus, the individuals with genotypes GG and CG have significantly higher milk fat, protein content and milk yield ($P < 0.05$ or $P < 0.01$, respectively; Table 4) than genotype CC individuals. No other statistically significant differences were observed between the genotypes of three polymorphic loci (P1-*Kpn*I, *Eco*RII, *Bgl*I) and milk traits in two dairy goat breeds ($P > 0.05$; data not shown).

Table 4. Association of the *Nco*I locus with goat milk traits.

Loci	Milk traits	Genotypes		
		CC	CG	GG
*Nco*I	Milk fat (%)	3.92 ± 0.09^{A}	4.41 ± 0.15^{B}	4.55 ± 0.20^{B}
	Protein (%)	3.43 ± 0.05^{a}	3.64 ± 0.08^{b}	3.69 ± 0.11^{b}
	Milk yield (kg)	587.44 ± 6.97^{a}	621.68 ± 12.97^{b}	630.17 ± 17.75^{b}

Least squares means in a column with no common superscripts differ significantly; lower-case characters (a, b) represent significance at $P < 0.05$; capital characters (A, B) represent significance at $P < 0.01$.

DISCUSSION

In human, overexpression of the *AGPAT6* gene showed higher levels of lysophosphatidic acid, phosphatidic acid and GPAT in cells (Chen et al., 2008; Takeuchi and Reue, 2009). Furthermore, substrate specificity studies suggested that *AGPAT6* was active against both saturated and unsaturated long-chain fatty acyl-CoAs (Chen et al., 2008). Histological research revealed that milk produced by *AGPAT6*-deficient mice was markedly depleted in diacylglycerols and triacylglycerols (Beigneux et al., 2006). Besides, a mouse model deficient in *AGPAT6* exhibited a 25% reduction in body weight and resistance to both diet-induced and genetically induced obesity, which further showed its unique role in determining triglyceride content that could not be compensated for by other members of the *AGPAT* family (Vergnes et al., 2006), while in bovine, it is reported that *AGPAT6*, together with other genes, coordinately regulate the channeling of fatty acids toward copious milk fat synthesis (Bionaz and Loor, 2008). So, *AGPAT6* is crucial for the components and yield of milk. In this study, for *Nco*I loci, individuals with GG and CG genotypes had higher milk components (milk fat, protein) and

milk yield than those of the CC genotype. Although it is a silent mutation, recently there have been some reports about the relationship between the silent mutation and the function of the gene (Kimchi-Sarfaty et al., 2007; Komar, 2007). That is, the protein product with the same acid sequence but different gene sequence had different structural and functional properties. It would be interest to discover how the mechanism for the silent mutation functions. Maybe it changes the character of the protein or it relates with some crucial QTL or gene, which is related to the milk traits.

In conclusion, four novel mutations (NC_007328.3:g.152G>C, 8124G>A, 9263C>G, 16436G>A) were found in the goat *AGPAT6* gene. The 9263C>G mutation was significantly associated with milk traits. This study may contribute to evaluating it as a genetic marker for dairy goat breeding strategies.

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