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New polymorphisms of *PAPPA* and *PAPPA2* genes and their associations with egg production traits in Chinese Dagu chickens

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ABSTRACT

Pregnancy-associated plasma protein A, pappalysin1 (*PAPPA*) and pappalysin2 (*PAPPA2*) genes were implicated in regulation of hen ovarian follicular development and growth. Four novel single nucleotide polymorphisms (SNPs) were identified using PCR-single strand conformation polymorphism (PCR-SSCP) and DNA sequencing. Among them, A/G transition at position 172864 and T/C mutation at position 172952 in 3′-untranslated region (UTR) of *PAPPA* named SNP A172864G and T172952C, respectively. A/G transition at position 77421 and T/C at position 77455 in 3′-UTR of *PAPPA2* gene named as SNP A77421G and T77455C, respectively. For SNP A172864G and T172952C (*PAPPA*), 360 Dagu hens were classified as AA, AB and BB genotypes based on PCR-SSCP patterns, and BB genotype correlated significantly ($P<0.05$) with higher hen-housed egg production (HHEP) at 30 and 57 weeks (wks) of age and higher egg weight (EW) at 43 wks of age. Consequently, these SNPs identified will be potential genetic markers to improve egg productivity in chicken breeding.

Key words: Chinese Dagu hens, Egg production trait, *PAPPA2*, *PAPPA*, Polymorphism.

INTRODUCTION

Chicken *PAPPA* gene containing 22 exons was mapped to chromosome 17, encoding the protein pregnancy associated plasma protein A, pappalysin1 (*PAPPA*) that is composed of 1627 amino acids. *PAPPA* was originally found be secreted by the placenta groups, later study confirmed its more broad expression spectrum (Conover *et al*., 2012). *PAPPA* has been identified as insulin-like growth factor (IGF) binding protein 4 (IGFBP4), protease in ovaries of human and domestic animals (Conover *et al*., 2001; Mazerbourg *et al*., 2001). The ovarian *PAPPA* is expressed in granulosa cells and is confirmed as a marker of follicle selection in follicular hierarchal development (Conover *et al*., 2001; Hourvitz *et al*., 2002). Chicken *PAPPA2* gene contains 23 exons and located on the chicken chromosome 8, codes for protein *PAPPA2* composed of 1496 amino acids. The *PAPPA2*, a protease of IGFBP5 was initially identified as a circulating protein of placental origin (Overgaard *et al*., 2001), receiving increasing attention for its roles in ovarian follicular development. The recent studies have shown that the single nucleotide polymorphisms (SNPs) in the gene *PAPPA2* was associated with the reproductive phenotypes in cattle (Luna-Nevarez *et al*., 2011; Wickramasinghe *et al*., 2011). Hence, it was presumed that variations in *PAPPA* and *PAPPA2* genes might have a potential influence on egg production traits in chickens. Nevertheless, the polymorphisms in the genomic DNA sequences of *PAPPA* and *PAPPA2* genes remain poorly reported in chicken.

The objective of this work was to investigate the novel SNPs in the genomic DNA of *PAPPA* and *PAPPA2* in a population of Chinese Dagu chickens using the PCR-SSCP approach and sequencing analysis. Then, associations between the newly identified genotypes of the two genes and the egg production traits were explored in the Dagu hens. We hope that it would provide a valuable, possible molecular marker for elevating egg production traits in local chicken breeding practices.

MATERIALS AND METHODS

Chickens and trait collection: The Chinese Dagu chickens used in this work were provided by College of Animal Science and Technology of Jilin Agricultural University. As previously reported by our group (Qin *et al*., 2015; Tyasi *et al*., 2017), the eggs for hatching were randomly selected from the Dagu chickens, of which 360 hens were hatched and raised in layered batteries under the same rearing conditions. These hens were free access to water and feed according to the nutrient requirements of local Chinese Dagu hens (NY/T 33-2004, China). The hens were reared in individual cages under constantly maintained conditions at 16 wks of age. All of the chickens were exposed to the 16L: 8D photoperiod, with lights on at 5:00 am. After the start of laying, eggs were collected and recorded daily, with egg weights determined on one day in a week. Body weight was recorded following feed and water restrictions at 30 and 43 wks of age, with the individual laying performance

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calculated. Egg production traits examined in this study included hen-housed egg production (egg laying number) at 30, 43, 57 and 66 wks of age, egg weight and body weight at 30 and 43 wks of age. The experiment was performed in accordance with laws of the People's Republic of China regarding animal protection.

PCR amplification: At 300 days of age, peripheral blood samples were collected from wing vein of the hens. Genomic DNA was extracted using a standard phenol-chloroform method and the DNA quality was examined by 1per cent agarose gel electrophoresis and ultraviolet-spectrophotometer assay. Primers for PCR were designed in accordance to the genomic DNA sequences of the *PAPPA* (accession No. NC_006104) and *PAPPA2* (accession No. NC_006095). All the primers are listed in Table 1. PCR reactions were performed in a total volume of 50 µl, containing 25 µl of 2×Taq Master Mix, 100 nM of each primer, 50 ng template DNA. The PCR conditions included: 94°C for 2 min, followed by 30 cycles at 94°C for 30 s for denaturing, 57.3°C (56°C) for 30 s for annealing (see Table 1), 72°C for 30 s for extension, and a final extension at 72°C for 2 min.

Purification and identification of PCR products: PCR products were depurated with Wizard prep PCR purification system (Promega, Madison, WI, USA). The amplified products were cloned into the Promegap GEM-T easy vector for sequencing, according to the methods published by Sambrook and Russell (2001). For each sampled bird, two independent PCR reactions were conducted, with sequences analyzed using BLAST

(https://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM= blastn & PAGE_TYPE= Blast Search & LINK _LOC = blasthom) to confirm the expected chicken *PAPPA* and *PAPPA2* gene sequences. The acquired sequences were then aligned using the DNAMAN software (version 6.0) to identify nucleotide variations.

Genotyping by PCR-SSCP and reconstruction of combined genotypes: To determine for *PAPPA* and *PAPPA2* gene polymorphism, the PCR products were further analyzed by using the single-strand conformation polymorphism (SSCP) assay as previously described (Qin *et al*., 2015). Briefly, Each 10 µl PCR product was mixed with 6 µl of loading dye (0.025% bromophenol blue, 0.025% xylene cyanol, 98% deionized formamide, 2% glycerin and 10 mM EDTA). Denatured at 99°C for 10 min beforehand, the samples were cooled rapidly on ice and then loaded on 10% polymerized gels (acrylamide: bisacrylamide, 39:1) of size 16 cm × 18 cm. Electrophoresis was carried out at 110 V for 5 h at room temperature in 1× TBE buffer. After silver stain, the gels were detected under upper white by gel photography system (GeneSnap from SynGene). To avoid false positive/ negative results due to artificial manipulation in the experiment, each sample was confirmed by repeated amplifications and detections. Combined genotypes were reconstructed according to the genotyping data obtained from all 360 individuals with the PHASE program (Stephens *et al*., 2001).

Polymorphism evaluation: Genotype and allelic frequencies at each SNP site were estimated, with each polymorphism evaluated for Hardy-Weinberg equilibrium using a Pearson's goodness-of-fit chi-square test (degree of freedom = 1). Gene homozygosity (Ho), heterozygosity (He), effective number of alleles (Ne) and polymorphism information content (PIC) were statistically analyzed using the POPGENE v. 1.32 software (Yeh *et al*., 1997).

Marker-trait association analysis: General linear model (GLM) procedure of SPSS 18.0 software was utilized for marker-trait association analysis of single polymorphisms and haplotypes in the current study. Linear mixed effects model was used as follows:

$$Y_{ijk} = m + L_i + G_j + F_k + e_{ijk}$$

Where Y_{ijk} is the phenotypic value of the target trait, such as egg laying number, m is the population mean, L_i is the fixed effect of the line, G_j is the fixed effect of the SNP genotype or haplotype, F_k is the random effect of the family and e_{ijk} are the residuals. Type III sum of squares was used in each test. Values were considered significant at $P < 0.05$ and presented as least square means ± standard errors (SE).

Predicted SNP genotype effects: For the SNP (s) that showed significant association with the egg-laying traits, differences between the means of each genotype and allelic frequencies were used to estimate additive effects (Falconer *et al*., 1996). The percentage of additive genetic variance ($\%V_j$) explained by the SNPs was determined using the following formula:

$$\%V_j = 100 p_j q_j \alpha_j \text{^} 2 / V_g$$

Where p and q are the allele frequencies for the jth SNP estimated across the entire population; α_j is the estimated additive effect of the jth SNP (allele substitution effect) on the trait under analysis; and V_g is the restricted maximum likelihood (REML) estimate of the (poly-) genetic variance for the trait.

Table 1: Primer information for amplification of the chicken *PAPPA* and *PAPPA2* fragments.

Genes	Sequences of primers (5'-3')	Product length (Location)	Annealing temperature (°C)
PAPPA	F:TCTCCCATCCACATCACC	205bp	57.3
	R:CCACAGCAGCAAGTAGCA	(172770-172974nt)	
PAPPA2	F: TGGGCAAGCCAGAATAAC	215bp	56.0
	R:GACACGGCGATAAGGAAA	(77389-77603nt)	

RESULTS AND DISCUSSION

Analysis of the nucleotide sequence amplified and genotyping by PCR-SSCP analysis: As shown in Fig 1, two targeted fragments were magnified from the Chinese Dagu hens, in which a 205 bp PCR amplicon for *PAPPA* and a 215 bp fragment for *PAPPA2* gene were obtained, respectively. In all of the examined birds, no more than two allelic sequences were observed. To explore more effective ways of breeding, many SNP detection methods were established and widely used in this field, such as the method of PCP-SSCP analysis, this genotyping method has been extensively applied in animal breeding (Zhang *et al.*, 2012; Qin *et al*., 2015).

PCR-SSCP analysis results of the current study showed that there were three genotypes (AA, AB and BB) in 3′-UTR of *PAPPA* gene (Fig 2) and two genotypes (TC and CC) in 3′-UTR of *PAPPA2* gene (Fig 3). Five combined genotypes (AACC, ABTC, BBTC, AATC and ABCC) were identified among the 360 hens sampled. The genotype present at the highest frequency was the ABCC (0.255), with the AATC type as the next most frequent (0.242), followed by AACC (0.217) and ABTC (0.203) the last one is the BBTC type (0.083).

Polymorphism of the target sequences: Two single nucleotide polymorphisms (SNPs) were identified corresponding to the PCR-SSCP banding patterns of the *PAPPA* and *PAPPA2* genes following sequence alignment. In *PAPPA* gene, a A/G and T/C a transition at position 172864 nt and 172952 nt in the 3′- UTR (Fig 2b), named SNP A172864G and T172952C. In the *PAPPA2* gene fragment, a A/G and a T/C transition at position 77421 nt and 77455 nt in the 3′-UTR, named SNP A77421G and T77455C (Fig 3b). In the present study, two novel SNPs in the chicken

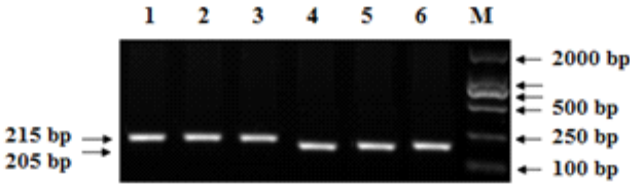

Fig 1: Amplification of the chicken *PAPPA* and *PAPPA2* fragments M, DL 2000 DNA marker (2,000; 1,000; 750; 500; 250 and 100bp, respectively); Lanes 1-3, fragments of *PAPPA2* gene amplified; Lanes 4-6, fragments of *PAPPA* gene amplified.

PAPPA and *PAPPA2* fragments were identified and their association with egg production traits of Chinese Dagu chicken.

Frequencies of genotype and allele at the SNP locus: Genotypic and allelic frequencies (Table 2), at SNP A172864G and T172952C of the *PAPPA* (P1 locus) gene, frequency of the allele A was higher than allele B, with the frequency of genotype AA and AB higher than genotype BB. At SNPA77421G and T77455C of the *PAPPA2* (P2 locus), the frequency of allele C was higher than allele T, with the frequency of genotype TC higher than genotype CC. Table 3 showed that gene homozygosity (*Ho*) was higher than gene heterozygosity (*He*) for the P1 and P2 loci, with effective allele numbers of 1.852 (P1) and 1.564 (P2).

Association of the SNP genotypes with laying performance and the predicted SNP genotype effects: It was demonstrated that, at the P1 locus, genotype BB was significantly associated with the higher HHEP at 30 and 57 wks of age ($P < 0.05$) and with EW at 43 wks (Table 4; $P < 0.05$). At the P2 locus, genotype TC was markedly associated with the higher HHEP at 30, 43, 57 and 66wks of age (Table 4; $P < 0.05$). According to the genotype-based association

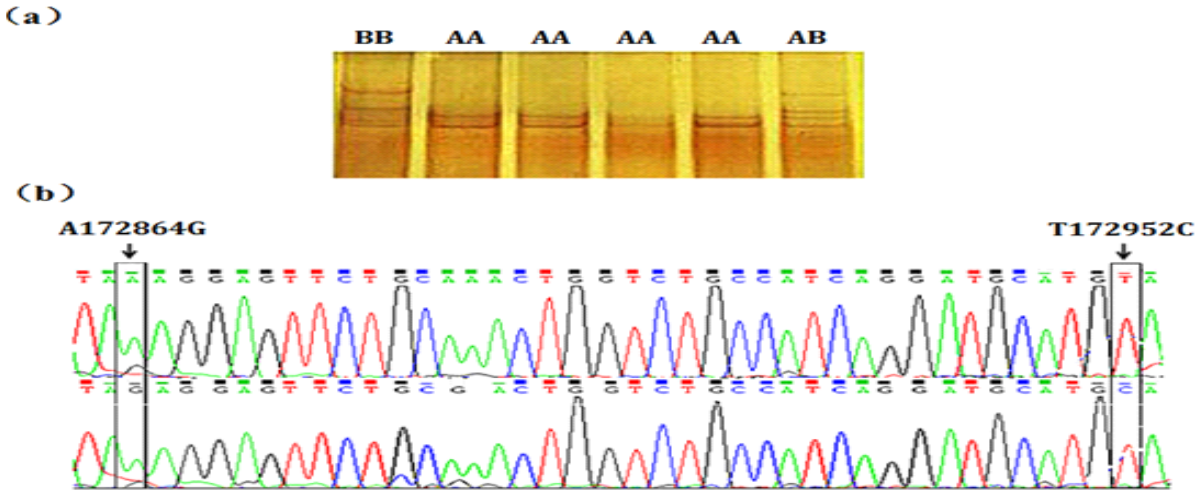

Fig 2: PCR-SSCP band patterns at the sites of A172864G and T172952C in the *PAPPA* fragment of chicken.
(a) The capital letters (AA, AB and BB) on the top indicate the different genotypes at the SNP locus by PCR-SSCP. (b) The A/G and T/C transition at base position 172864 and 172952 in the 3′-UTR of *PAPPA* gene (Accession No. NC_006104) were detected by sequencing and alignment.

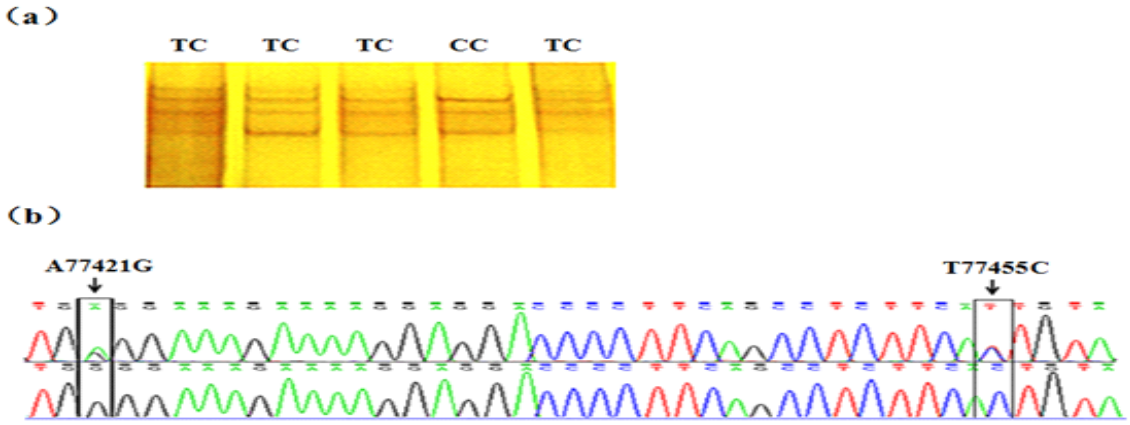

Fig 3: PCR-SSCP band patterns at the sites of SNPA77421G and T77455C in the *PAPPA2* gene of chicken.
The capital letters (TC and CC) on the top indicate the different genotypes at the SNP locus by PCR-SSCP.(b)the A77421G and T77455Ctransition in the 3′- UTR of the chicken *PAPPA2* gene.

Table 2: Genotypic and allelic frequency at the SNP *PAPPA* and *PAPPA2 l*oci in the Dagu chicken population.

SNP	Genotype	No. of chickens	Genotype frequency	Allele	Allele frequency	χ^2
A172864G+T172952C (*PAPPA1*)	AA	165	0.46	A	0.69	1.47
	ABBB	16530	0.460.08	B	0.31	
A77421G+T77455C (*PAPPA2*)						46.27*
	TC	190	0.53	C	0.74	
	CC	170	0.47	T	0.26	

*P<0.05 was accepted to be statistically significant when the data were analyzed using a Pearson's goodness-of-fit chi-square test (degree of freedom = 1).

Table 3: Polymorphism information analysis of the *PAPPA* and *PAPPA2* genes in the Dagu chicken population.

SNP	Gene Homozygosity (*Ho*)	Gene Heterozygosity (*He*)	Effective allele number (*Ne*)	Polymorphism information content (*PIC*)
A172864G + T172952C (*PAPPA*)	0.597	0.403	1.852	0.375
A77421G+T77455C (*PAPPA2*)	0.639	0.361	1.564	0.296

Table 4: Association between the polymorphism in chicken *PAPPA* and *PAPPA2* genes and egg production traits in the local Dagu hens.

	Genotypes				
Egg production	***PAPPA***		***PAPPA2***		
trait	**AA (165) ($\bar{X}$ ±SE)**	**BB (30) ($\bar{X}$ ±SE)**	**AB (165) ($\bar{X}$ ±SE)**	**TC (190) ($\bar{X}$ ±SE)**	**CC (170) ($\bar{X}$ ±SE)**
BW at 30wks (kg)	2.80±0.06	2.97±0.18	2.72±0.06	2.83±0.05	2.74±0.07
BW at 43wk s(kg)	3.01±0.06	3.25±0.33	2.84±0.10	2.97±0.08	2.94±0.11
HHEP at 30wks (No.)	20.93±2.82^b	39.67±5.92^a	20.10±2.13^b	25.61±2.45^a	16.22±2.27^b
HHEP at 43wks (No.)	83.50±7.30	84.00±7.81	79.03±6.80	93.21±5.44^a	71.89±7.98^b
HHEP at 57wks (No.)	120.17±9.5^b	130.33±8.35^a	128.60±7.7^a	133.87±7.89^a	100.04±8.4^b
HHEP at 66wks (No.)	134.13±9.03	132.67±7.69	138.57±8.47	144.66±9.04^a	98.07±9.41^b
EW at 30wks (g)	56.71±0.90	55.80±4.78	56.58±0.70	56.57±0.82	56.09±0.78
EW at 43wks (g)	58.28±0.70	61.00±0.15^a	59.45±0.83	59.45±0.73	58.91±0.84

^{a,b}Means within a row for each gene lacking a common superscript differ (P< 0.05). The No. represents the total number of eggs produced at the corresponding age. The digits in the parenthesis indicate the number of hen individuals in each group, n = Sample size. BW = body weight; HHEP = hen-housed egg production (total egg laying number); EW = egg weight. The 30 wks of age is the average age at first egg of the Dagu hens.

Table 5: Percentages of additive genetic variance explained by the SNPs identified in the fragments of chicken *PAPPA* and *PAPPA2*enes.

Egg production trait	Percentage of additive genetic variance explained by the SNPs (%)	
	A172864G + T172952C (*PAPPA*)	A77421G+T77455C (*PAPPA2*)
BW at 30wks (kg)	-	-
BW at 43wk s(kg)	-	-
HHEP at 30wks (No.)	1.030	2.365
HHEP at 43wks (No.)	-	2.012
HHEP at 57wks (No.)	3.676	4.923
HHEP at 66wks (No.)	-	9.164
EW at 30wks (g)	-	-
EW at 43wks (g)	-	-

Table 6: Association between the combined genotypes with egg production traits in the Dagu hens.

Egg production trait	Combined genotypes				
	AACC (78) ($\bar{X}$ ±SE)	ABTC (73) ($\bar{X}$ ±SE)	BBTC (30) ($\bar{X}$ ±SE)	AATC (87) ($\bar{X}$ ±SE)	ABCC (92) ($\bar{X}$ ±SE)
BW at 30wks (kg)	2.71±0.11	2.83±0.20	2.83±0.07	2.82±0.08	2.74±0.08
BW at 43wk s(kg)	3.03±0.09^{a}	3.02±0.33^{a}	2.96±0.10^{a}	2.91±0.17^{ab}	2.86±0.16^{b}
HHEP at 30wks (No.)	19.18±3.80^{a}	25.54±3.20^{b}	27.25±3.86^{b}	18.73±3.26^{a}	18.40±3.51^{a}
HHEP at 43wks (No.)	90.08±5.80	93.930±6.11	99.38±8.95^{a}	92.73±6.96	89.46±9.17
HHEP at 57wks (No.)	116.00±8.22	144.15±8.80^{a}	145.12±8.4^{a}	128.82±9.16	121.09±6.30
HHEP at 66wks (No.)	134.64±9.15	151.92±9.18^{a}	155.94±8.8^{a}	143.56±8.27	138.36±9.50
EW at 30wks (g)	55.45±1.44	55.55±0.95	55.76±1.29	55.86±1.28	57.04±0.91
EW at 43wks (g)	58.56±1.44	60.05±0.25	58.36±0.87	59.19±1.40	58.77±1.46

^{a,b,}Means within a row for each gene lacking a common superscript differ (P< 0.05). The No. represents the total number of eggs produced at the age. The digits in bracket indicate the number of the individuals in the group.

analysis, both of the newly determined BB genotype at the P1 locus and TC genotype at the P2 locus were manifested to be significantly associated with the higher HHEP at 30 and 57 wks of age.

Furthermore, it was found that a large percentage of the additive variance was explained by the SNP for its significant association (P < 0.01) with the traits, respectively (Table 5). Furthermore, in order to analyze the genetic effect of the SNPs on the associated traits including the HHEP at 30 and 57 wks of age, considering that additive variance was one of the most important genetic variance component in the expression of traits related to egg production (Falconer *et al*., 1996; Almasy and Blangero, 2010), the percentage of additive genetic variance explained by the SNPs was evaluated in the current study. The present data indicated that a larger proportion of variance explained by these markers (>1%), especially for the phenotypes including the HHEP at 30 and 57 wks of age by the *PAPPA* and *PAPPA2* loci. As shown in Table 6, the combined genotype BBTC was found to be significantly (P < 0.05) correlated with the highest HHEP at30, 43, 57 and 66 wks of age and the BW at 43 wks , but there were no significant differences (P < 0.05) between the type with the EW at 30 and 43 wks of age . In this wok, five combined genotypes (AACC, AATC, ABCC, ABTC and BBTC) were determined and the association analysis of the genotypes revealed that *PAPPA* and *PAPPA2* polymorphisms are significantly associated with egg production traits in Dagu chickens. Moreover, we found that the combined genotype-based association analysis results were consistent with the significant effect detected by the genotype-based association analysis. Among those combined genotypes, BBTC carry the favorable genotypes (BB and TC), which were remarkably for correlating with the highest HHEP at 43, 57 and 66 wks of age. Hence, it obviously supports the conclusion that the novel variations of *PAPPA* and *PAPPA2* are strongly associated with the egg production traits.

CONCLUSION

In conclusion, our data have demonstrated that the polymorphisms newly determined in *PAPPA* and *PAPPA2* genes were notably associated with hen-housed egg production in the Chinese Dagu chickens. It might serve as a novel genetic marker for early selection and prediction of the advantageous egg production traits in chicken breeding programs.

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