

The relationship between the variants in the 5'-untranslated regions of equine chorionic gonadotropin genes and serum equine chorionic gonadotropin levels

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Submitted Feb 15, 2017; Revised Mar 23, 2017;
Accepted Mar 30, 2017

Objective: An experiment was conducted to study the association between the single nucleotide polymorphisms (SNPs) in 5'-untranslated regions (5'-UTR) of equine chorionic gonadotropin (*eCG*) genes and the serum *eCG* levels.

Methods: SNPs in 5'-UTR of *eCG* genes were screened across 10 horse breeds, including 7 Chinese indigenous breeds and 3 imported breeds using iPLEX chemistry, and the association between the serum *eCG* levels of 174 pregnant Da'an mares and their serum *eCG* levels (determined with ELISA) was analyzed.

Results: Four SNPs were identified in the 5'-UTR of the *eCGα* gene, and one of them was unique in the indigenous breeds. There were 2 SNPs detected at the 5' end of the *eCGβ* subunit gene, and one of them was only found in the Chinese breeds. The SNP g.39948246T>C at the 5'-UTR of *eCGα* was associated significantly with *eCG* levels of 75-day pregnant mare serum ($p < 0.05$) in Da'an mares. Prediction analysis on binding sites of transcription factors showed that the g.39948246T>C mutation causes appearance of the specific binding site of hepatocyte nuclear factor 3 forkhead homolog 2 (HFH-2), which is a transcriptional repressor belonging to the forkhead protein family of transcription factors.

Conclusion: The SNP g.39948246T>C at the 5'-UTR of *eCGα* is associated with *eCG* levels of 75-day pregnant mare serum ($p < 0.05$).

Keywords: Equine Chorionic Gonadotropin (*eCG*); Single Nucleotide Polymorphism (SNP); Transcription Factor

INTRODUCTION

Equine chorionic gonadotropin (*eCG*) is a gonadotropic hormone produced in the chorion of pregnant mares, previously known as pregnant mare's serum gonadotropin, which was found in 40 to 130 days pregnant mare serum, first reported by Cole et al [1]. *eCG* is composed of two dissimilar subunits, alpha and beta subunit consisting of 96 and 149 amino acids, respectively. The alpha subunit (*eCGα*) is the same as its counterparts of all other glycoprotein hormones (equine luteinizing hormone, equine follicle-stimulating hormone, equine thyroid stimulating hormone). The beta subunit (*eCGβ*) is specific for *eCG* and is responsible for receptor binding specificity. The *eCG* activity and specificity is mainly determined by the beta subunit, and the alpha subunit also has a significant effect on biological activity of the hormone [2]. The use of *eCG* in follicular stimulation and superovulation has substantial effects on follicular development and corpus luteum function [3,4]. It stimulates the growth of the dominant follicle [5,6] and promotes the increase of circulating progesterone concentrations in the subsequently estrus cycle [6,7].

Breeds, maternal and paternal effects, and fetal genders are the main genetic factors which

influence the eCG secretion. Previous studies showed that the breed factor has a great influence on the secretion level of eCG. It was reported that the serum eCG concentration of ponies and Mongolia horses was significantly higher than draft horses and Thoroughbreds [8-10]. The mutations of human and cattle CG were identified, which are associated with male infertility, recurrent miscarriage and superovulation traits [11-13]. But the mutations of *eCG* genes and their biofunctions have not been fully studied, and only a single nucleotide polymorphism (SNP) in the 5'-untranslated regions (5'-UTR) of *eCGβ* was identified in previous study [10]. The objective of the present study was to investigate SNPs in *eCG* genes and to evaluate the association between the polymorphisms and serum eCG levels.

MATERIALS AND METHODS

Sample collection and DNA extraction

A total of 417 blood samples were collected, which included 7 Chinese indigenous breeds and 3 imported breeds (Table 1). Da'an (DA), Inner Mongolia (MG), Elunchun (ELC), and Ningqiang (NQ) were distributed in the north of China, and Jinjiang (JJ), Changdu (CD), and Baise (BS) were located in southern regions of China. Generally, the northern horses were higher and stronger than the southern horses. MG and ELC have a closer genetic relationship than others. Genomic DNA was isolated from samples using phenol-chloroform method and preserved at -20°C [14]. The amount of DNA from each sample was determined by measuring A260/A280 value using NanoDrop 2000 (Thermo Scientific, Waltham, MA, USA) and further evaluated by agarose gel electrophoresis. All samples were obtained following the principles approved by the Animal Care and Use Committee of China Agricultural University.

Single nucleotide polymorphism selection and genotyping

To detect SNPs, primers were designed using primer 5.0 software based on the sequences of the 5'UTRs of *eCGα* and *eCGβ* gene (Gene ID: 100034174 and 100054774). The primer pairs (eCGαF:

5'-CCCTTTCAATTTGTATGCC-3', eCGαR: 5'-TATG CAGA AACACGGACAA-3' and eCGβF: 5'-CAATGACTCGCTGAC CTCCTG-3', eCGβR: 5'-GTCTCCATCCTCGGTGCCTC-3') were designed to amplify the 1,818-bp and 1,266-bp fragments of 5'UTRs of *eCGα* and *eCGβ* gene, respectively. Polymerase chain reaction (PCR) amplifications were performed in a 20 μL reaction mixture consisting of the 25 ng DNA, 0.5 μM each primer, 2×Taq PCR MasterMix 10 μL (Tiange, Beijing, China) and ddH₂O 8 μL. All amplifications included an initial denaturing step of 10 min at 95°C, followed by 35 cycles of 30 s at 95°C, 30 s at an optimized annealing temperature (eCGα, 54°C and eCGβ, 62°C) and 90 s at 72°C, with a final extension of 10 min at 72°C. The PCR products were sent to the Beijing Genomics Institute (Beijing, China) for sequencing.

The samples were genotyped by two steps. i) The polymorphisms in the 5'UTRs were screened by sequencing five individuals for each breed. ii) Based on the polymorphic information from the sequencing, all other samples were genotyped using iPLEX chemistry on a matrix-assisted laser desorption/ionization time-of-flight mass spectrometer (Applied Biosystems, Waltham, MA, USA).

Detection of eCG serum levels

Serum samples of 174 Da'an mare horses with ages between 4 to 11 years were collected three times on day 55, 65, and 75 of pregnancy, respectively. The eCG levels in serum were determined by enzyme-linked immunosorbent assay (ELISA) with commercially available kits (DRG, Marburg, Germany), according to the manufacturer's instructions. The optical density (OD) at 450 nm was measured using an ELISA microplate reader (infiniteM200 NanoQuant)(TECAN, Hombrechtikon, Switzerland).

Statistical analysis

Genotypic and allelic frequencies for each SNP, Hardy-Weinberg equilibrium (HWE) χ^2 test for genotype distributions were analyzed using Microsoft Excel (Microsoft Corporation, Redmond, WA, USA). The genotype sample size and eCG serum levels of Da'an horses were analyzed by SPSS-Statistics V17.0 (Chicago, IL, USA). Association analysis between the SNPs and serum eCG levels of Da'an horses was analyzed following the Student-Newman-Keuls (SNK), $y_{ij} = \mu + a_i + e_{ij}$, where y_{ij} indicates observed value; μ , overall mean; a_i , fixed effect of genotype; e_{ij} , random error. The binding sites of transcription factors were predicted using online software (<http://www.phylofoot.org/>) (Center for Genomics and Bioinformatics, Karolinska Institutet, Stockholm, Sweden).

RESULTS

SNPs in 5' UTR of *eCG* genes

After sequencing and alignment, four SNPs in 5'UTR of *eCGα* gene (g.39947046C>G, g.39947259C>T, g.39947735C>G, g.39948246T>C) were identified while two SNPs were found in the

Table 1. The location and breed information of the 417 samples

Breed name	Abbreviation	Sample size	Location
Da'an ¹⁾	DA	174	Jilin, China
Inner Mongolia	MG	30	Inner Mongolia, China
Elunchun	ELC	20	Inner Mongolia, China
Ningqiang	NQ	28	Shanxi, China
Jinjiang	JJ	30	Fujian, China
Changdu	CD	30	Tibet, China
Baise	BS	27	Guangxi, China
Arabian horse	AR	25	Russia
Thoroughbred	TB	30	Ireland
Warm-blood horse	WB	23	Germany
Total	10	417	10

¹⁾ The serum equine chorionic gonadotropin (eCG) levels of the mares in the breed were determined.

5'UTR region of *eCGβ* gene (g.18962466G>T, g.18962516G>A).

The genotypes and allele frequencies of the SNPs in the 5'UTR of *eCG* genes are shown in Table 2, 3, 4. Results of χ^2 test indicated that the SNP g.39947046C>G and g.39947259C>T met the HWE ($p>0.05$) in all breeds, and g.39947735C>G and g.39948246T>C were in HWE only in the indigenous breeds.

Relationships between the SNPs of *eCG* genes and the serum eCG levels

Effects of the 6 polymorphic loci of the *eCG* genes on the serum eCG levels on day 55, 65, and 75 of pregnancy of Da'an horses are shown in Table 5. Statistical analysis revealed that the eCG levels successively decreased with the increase of gestational time. SNP g.39948246T>C was associated with 75-day pregnant serum

eCG levels ($p<0.05$), and there was more than twice as much average eCG concentration of individuals with CC genotype as that of horses harboring TT. Based on the transcription factors prediction, the mutation g.39948246T>C leads to the appearance of binding site of an important transcription factor, HFH-2.

DISCUSSION

The *eCG* genes are located on chromosome 10 in horse [15]. Reproductive physiology of *eCGα* and *eCGβ* subunits was subjected to intensive studies [2,16,17], while mutations in *eCG* genes have not been fully studied yet. Mutations of *eCG* gene may affect serum eCG levels. It was reported that a low level of human chorionic gonadotropin (hCG) in maternal serum during the first

Table 2. Polymorphisms in the 5' UTR of *eCG* genes

Gene	Site	Genotype	Genotype frequency		Allele frequency				Indigenous		Imported	
			Indigenous	Imported	Indigenous	Imported	C	G	C	G	χ^2	p
<i>eCGα</i>	g. 3994-7046	CC	0.56	0.46	C	G	C	G	0.78	0.68	0.39	0.83
		CG	0.39	0.42	0.76	0.24	0.66	0.34	-	-	-	-
		GG	0.05	0.13	-	-	-	-	-	-	-	-
	g. 3994-7259	CC	0.58	0.46	C	T	C	T	0.12	0.94	0.39	0.83
		CT	0.37	0.42	0.76	0.24	0.66	0.34	-	-	-	-
		TT	0.05	0.13	-	-	-	-	-	-	-	-
	g. 3994-7735	CC	0.95	1.00	C	G	C	G	0.11	0.95	-	-
		GC	0.05	0	0.97	0.03	1.00	0	-	-	-	-
		GG	0	0	-	-	-	-	-	-	-	-
g. 3994-8246	TT	0.37	0.98	T	C	T	C	0.43	0.81	33.54	0	
	TC	0.46	0.01	0.60	0.40	0.98	0.02	-	-	-	-	
	CC	0.17	0.01	-	-	-	-	-	-	-	-	
<i>eCGβ</i>	g. 1896-2466	GG	0.92	0.84	G	A	G	A	126.00	0	58.00	0
		GA	0.01	0.03	0.93	0.07	0.85	0.15	-	-	-	-
		AA	0.06	0.14	-	-	-	-	-	-	-	-
	g.1896-2516	GG	0.57	1.00	G	T	G	T	120.00	0	-	-
		GT	0.06	0	0.60	0.40	1.00	0	-	-	-	-
		TT	0.37	0	-	-	-	-	-	-	-	-

eCG, equine chorionic gonadotropin.

Table 3. The allele and genotype frequencies for the polymorphisms of *eCGα* gene in the studied breeds

Breeds ¹⁾	g. 39947046					g. 39947259					g. 39947735					g. 39948246			
	CC	CG	GG	C	G	CC	CT	TT	C	T	CC	GC	C	G	TT	TC	CC	T	C
DA	0.59	0.32	0.09	0.75	0.25	0.59	0.32	0.09	0.75	0.25	0.96	0.04	0.98	0.02	0.51	0.42	0.07	0.72	0.28
CD	0.60	0.30	0.10	0.75	0.25	0.60	0.27	0.13	0.73	0.27	0.97	0.03	0.98	0.02	0.37	0.60	0.03	0.67	0.33
MG	0.46	0.54	0	0.73	0.27	0.50	0.50	0	0.75	0.25	0.86	0.14	0.93	0	0.36	0.50	0.14	0.61	0.39
NQ	0.75	0.21	0.04	0.86	0.14	0.75	0.21	0.04	0.86	0.14	1.00	0	1.00	0	0.36	0.32	0.32	0.52	0.48
BS	0.59	0.37	0.04	0.78	0.22	0.63	0.33	0.01	0.80	0.20	0.96	0.04	0.98	0.02	0.22	0.48	0.30	0.46	0.54
ELC	0.54	0.31	0.15	0.69	0.31	0.54	0.31	0.15	0.69	0.31	1.00	0	1.00	0	1.00	0	0	1.00	0
JJ	0.43	0.57	0	0.72	0.28	0.43	0.57	0	0.72	0.28	0.43	0.57	0.97	0.03	0.27	0.57	0.16	0.55	0.45
AR	0.68	0.28	0.04	0.82	0.18	0.68	0.28	0.04	0.82	0.18	1.00	0	1.00	0	1.00	0	0	1.00	0
TB	0.30	0.53	0.17	0.57	0.43	0.30	0.53	0.17	0.57	0.43	1.00	0	1.00	0	1.00	0	0	1.00	0
WB	0.41	0.41	0.18	0.61	0.39	0.41	0.41	0.18	0.61	0.39	1.00	0	1.00	0	0.91	0.05	0.04	0.93	0.07

eCGα, equine chorionic gonadotropin α.

¹⁾ DA, Da'an; CD, Changdu; MG, Inner Mongolia; NQ, Ningqiang; BS, Baise; ELC, Elunchun; JJ, Jinjiang; AR, Arabian horse; TB, Thoroughbred; WB, Warm-blood horse.

Table 4. The allele and genotype frequencies for the polymorphisms of *eCGβ* gene in the studied breeds

Breeds ¹⁾	g. 18962466					g.18962516				
	GG	GA	AA	G	A	GG	GT	TT	G	T
DA	0.21	0.68	0.11	0.55	0.45	0.84	0	0.16	0.84	0.16
CD	0.90	0.10	0	0.95	0.05	0.47	0.43	0.10	0.68	0.32
MG	0.83	0.13	0.04	0.90	0.10	0.87	0.13	0	0.93	0.07
NQ	0.96	0.04	0	0.98	0.02	0.38	0.54	0.08	0.65	0.35
BS	1.00	0	0	1.00	0	0.44	0.52	0.04	0.70	0.30
ELC	0.85	0.10	0.05	0.90	0.10	0.85	0.15	0	0.93	0.07
JJ	1.00	0	0	1.00	0	0.44	0.44	0.12	0.67	0.33
AR	1.00	0	0	1.00	0	1.00	0	0	1.00	0
TB	0.82	0.18	0	0.91	0.09	1.00	0	0	1.00	0
WB	0.70	0.22	0.08	0.80	0.20	1.00	0	0	1.00	0

eCGβ, equine chorionic gonadotropin β.

¹⁾ DA, Da'an; CD, Changdu; MG, Inner Mongolia; NQ, Ningqiang; BS, Baise; ELC, Elunchun; JJ, Jinjiang; AR, Arabian horse; TB, Thoroughbred; WB, Warm-blood horse.

Table 5. The association between the genotypes of *eCG* genes and the serum *eCG* levels¹⁾

Loci	Genotype/sample size	eCG serum levels (mIU/mL)			p-value		
		55 d	65 d	75 d	55 d	65 d	75 d
g. 39947046C>G	CC/103	19,751.67 ± 1,732.80 ^a	17,561.78 ± 1,794.48 ^a	13,734.95 ± 1,688.01 ^a	0.51	0.68	0.61
	CG/55	21,096.92 ± 2,371.29 ^a	18,937.65 ± 2,455.70 ^a	14,641.79 ± 2,310.00 ^a			
	GG/16	25,167.61 ± 4,396.49 ^a	21,687.66 ± 4,552.99 ^a	18,260.43 ± 4,282.86 ^a			
g. 39947259C>T	CC/103	19,751.67 ± 1,732.80 ^a	17,561.78 ± 1,794.48 ^a	13,734.95 ± 1,688.01 ^a	0.51	0.68	0.61
	CT/55	21,096.92 ± 2,371.29 ^a	18,937.65 ± 2,455.70 ^a	14,641.79 ± 2,310.00 ^a			
	TT/16	25,167.61 ± 4,396.49 ^a	21,687.66 ± 4,552.99 ^a	18,260.43 ± 4,282.86 ^a			
g. 39947735 C>G	CC/167	20,521.04 ± 1,361.01 ^a	18,363.38 ± 1,408.40 ^a	14,453.32 ± 1,325.57 ^a	0.57	0.96	0.95
	CG/7	24,345.94 ± 6,647.68 ^a	18,678.84 ± 6,879.17 ^a	14,065.87 ± 6,474.59 ^a			
g. 39948246T>C	TT/88	20,711.06 ± 1,870.61 ^a	17,786.05 ± 1,930.00 ^a	12,482.65 ± 1,792.49 ^a	0.35	0.25	0.03
	TC/73	19,478.69 ± 2,053.83 ^a	17,642.92 ± 2,119.03 ^a	14,709.69 ± 1,968.05 ^a			
	CC/13	27,147.52 ± 4,866.91 ^a	26,486.98 ± 5,021.43 ^a	26,144.95 ± 4,663.65 ^b			
g. 18962466G>T	GG/36	21,513.81 ± 2,941.74 ^a	20,093.28 ± 3,038.72 ^a	16,414.55 ± 2,858.31 ^a	0.95	0.82	0.74
	GA/119	20,446.06 ± 1,618.01 ^a	17,940.58 ± 1,671.36 ^a	13,878.96 ± 1,572.12 ^a			
	AA/19	20,518.77 ± 4,049.29 ^a	17,849.96 ± 4,182.79 ^a	14,191.85 ± 3,934.45 ^a			
g.18962516G>A	GG/147	20,171.38 ± 1,448.71 ^a	17,930.08 ± 1,498.68 ^a	13,794.89 ± 1,407.40 ^a	0.38	0.45	0.25
	TT/27	23,416.39 ± 3,380.32 ^a	20,804.26 ± 3,496.92 ^a	17,937.65 ± 3,283.92 ^a			

eCG, equine chorionic gonadotropin.

¹⁾ For a column of each cell, three mean values with the same letter (a) superscripted do not differ significantly while those having different letters (a and b) are significantly different at the 0.05 level.

trimester of the pregnancy is a clinically accepted risk factor for miscarriage, and variation in *hCGβ* gene contributes to the susceptibility of recurrent miscarriage [12].

In this study, we detected 6 novel SNPs in *eCGα* and *eCGβ* genes by screening 10 horse breeds. Our results showed that the mutation g.39948246T>C in *eCGα* gene was significantly associated with the serum *eCG* levels, but its biofunction needs to be further studied. In Table 3, the indigenous and imported horse populations showed significant difference on allele frequencies at the mutant sites. And the imported horse breeds had the similar frequencies with each other, so did the indigenous breeds except ELC horses which experienced bottleneck of genetic diversity and presently has a small population. mRNA expression of *eCG* receptor indicated that *eCG* had high expression levels during

55 to 70 days of gestation [18]. As the results show in Table 5, g.39948246T>C was associated significantly with the serum *eCG* levels on day 75 of pregnancy (p<0.05). Due to effect of a gene-substitution, the horses harboring CC have higher *eCG* secretion levels. Therefore, the SNP g.39948246T>C may have the potential to be used as a genetic maker for the selection of high level of serum *eCG*.

Transcription factors play important role on controlling the flow of genetic information from DNA to mRNA [19]. According to the prediction results, at the mutant site of g.39948246T>C of *eCGα*, the substitution of T for C leads to the appearance of the binding site of the transcription factor HFH-2. Previous studies showed that HFH-2 (alias Foxd3) decreased interleukin-10 expression in B cells, and FOXD3/miR-214/MED19 axis sup-

presses tumors growth and metastasis in human colorectal cancer [20,21]. Individuals carrying TT genotype had lower eCG levels than those of GG genotype which may be attributed to the suppression caused by inhibitory effect of HFH-2.

CONCLUSION

In conclusion, we detected 6 SNPs in 5' UTRs of *eCG α* and *eCG β* genes. The SNP g.39948246T>C was significantly associated with eCG secretion levels, and individuals harboring genotype CC had the highest serum eCG level in all of the three genotypes. Thus, this SNP has the potential to be used as a molecular marker on the selection for high serum eCG levels. So the present study contributes new information on understanding functions of the *eCG* genes.

CONFLICT OF INTEREST

We certify that there is no conflict of interest with any financial organization regarding the material discussed in the manuscript.

ACKNOWLEDGMENTS

The present study was financially supported by the Public Science and Technology Research Funds Projects of Agriculture (Grant No. 201003075), the Program for Changjiang Scholars and Innovative Research Team in University (Grant No. IRT_15R62), and the fund from Beijing Key Laboratory for Genetic Improvement of Livestock and Poultry.

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