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Research Article

Molecular Analysis of Alternative Spliced Transcripts of Equine Pleckstrin Homology and RhoGEF Domain Containing G1 (*PLEKHG1*) gene

Jeong-Woong Park[1], Jae-Young Choi[2], Kyoung Hwan Kim[3], Sujung Kim[4], Jae-Rung So[5], Byeong-Woo Kim[6], Byung-Wook Cho[6*]

[1]Center for Industrialization of Agricultural and Livestock Microorganisms (CIALM), 241, Cheomdangwahag-ro, Jeongeup-si, Jeollabuk-do 56212, Republic of Korea

[2]Subtropical Livestock Research Institute, National Institute of Animal Science, RDA, 63242, Korea

[3]Jeju Racing Resources Management Team, Jeju Racecourse Division, Korea Racing Authority, Jeju, 63066, Republic of Korea

[4]The Animal Molecular Genetics and Breeding Center, Jeonbuk National University, 54896, Republic of Korea

[5]Department of Agricultural Convergence Technology, Jeonbuk National University, 54896, Republic of Korea

[6]Department of Animal Science, College of Natural Resources and Life Sciences, Pusan National University, Miryang 50463, Korea.

***Corresponding author:** bwcho@pusan.ac.kr

ABSTRACT

Since horse industry get bigger with horse racing and horse riding, athletic performance become most important trait on horses, but the molecular analysis and regulatory pathway studies remain insufficient for evaluation and prediction of horse athletic abilities. In our previous study, we conducted RNA-sequencing analysis with muscle and blood samples by exercise in Thoroughbreds. Through RNA-sequencing, we identified Pleckstrin Homology and RhoGEF Domain Containing G1 (*PLEKHG1*) gene expressed differentially by alternative spliced isoforms in skeletal muscle during exercise. In this study, we conducted reverse transcriptase polymerase chain reaction (RT-PCR) to validate two isoforms of equine *PLEKHG1* transcripts (*PLEKHG1a*, *PLEKHG1b*), and cloned the transcripts to confirm the sequences. Additionally, we validated expression pattern of *PLEKHG1a* (long form of transcript) and *PLEKHG1b* (short form of transcript) in horse tissue by quantitative RT-PCR (qRT-PCR). Prediction of protein structure of these isoforms revealed two putative phosphorylation sites at the amino acid sequences encoded in exon 7, which is deleted in *PLEKHG1b*. Expression pattern of *PLEKHG1a* and *PLEKHG1b* shows cross expressed pattern as RNA-sequencing data. *PLEKHG1a* increased after exercise whereas *PLEKHG1b* decreased after exercise. Collectively, it is assumed that the expression patterns of *PLEKHG1a* and *PLEKHG1b* transcripts would be involved in regulation of myogenic differentiation and myogenic proliferation through the Ras signaling pathway. Further study should be necessary to uncover biological function(s) and significance of the alternative splicing isoforms in equine skeletal muscle

Key words: Horse, Pleckstrin Homology and RhoGEF Domain Containing G1, Alternative Splicing, Athletic Performance, Muscle, RNA-Sequence

INTRODUCTION

Most important economic traits in horse is the racing abilities and now horse is used for racing and riding. Among various breeds in horses, Thoroughbreds are one of the most famous breeds in racing horses, and they were bred to improve their speed, endurance, and strength. But researches on horse are barely focused on molecular biological researches and most of researches are focused on physical and physiological

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studies (MCCONAGHY et al., 1988; MCILWRAITH et al., 2012).

In our previous study, our team secured blood and muscle tissue samples before and after exercise in six thoroughbreds, and RNA-sequencing was conducted with total 24 samples. As a result of RNA-sequencing, we discovered 32,361 of unigene clusters and 1,305 of differentially expressed gene (DEGs). Among DEGs, we identified 848 unigenes are up-regulated, 285 unigenes are down-regulated in muscle, and 62 unigenes are up-regulated, 80 unigenes are down-regulated in blood. In addition, we discovered 275,044 of novel transcripts from RNA-sequencing. Among them, we discovered alternative spliced form in cordon-bleu WH2 repeat protein-like 1 (*COBLL1*), cytoplasmic dynein 1 light intermediate chain 2 (*DYNC1LI2*), pleckstrin homology domain containing, family member 1 (*PLEKHG1*), and AXL receptor tyrosine kinase (*AXL*) showed reversed expression patterns before and after exercise in skeletal muscle (PARK et al., 2012).

PLEKHG1 encoded a Rho guanine nucleotide exchange factor, *PLEKHG1* protein, and *PLEKHG1* expression in wide range of tissues was reported (GRAY et al., 2018). Despite its ubiquitous expression, the function of *PLEKHG1* is largely unknown. The *PLEKHG1* locus also reported to related with sickle cell anemia (BHATNAGAR et al., 2013), cognitive decline (SHERVA et al., 2014), obesity-related traits (COMUZZIE et al., 2012), alcohol and nicotine codependence (ZUO et al., 2012), panic disorder (OTOWA et al., 2009), and blood pressure (Liang et al., 2017; Franceschini et al., 2013). In addition, the *PLEKHG1* has reported to involved with actin cytoskeletal organization, reorientation of cells and their stress fibers in response to mechanical stress (Abiko et al., 2015). Also, *PLEKHG1* locus has been associated with hypertensive disease in other GWAS (Franceschini et al., 2013). But despite of various studies about *PLEKHG1*, the function of *PLEKHG1* is largely unknown in horse and the specific roles of *PLEKHG1* gene on athletic abilities are still unclear. Therefore, in this study, we focused on molecular biological analysis of *PLEKHG1* and expression analysis which was identified as one of the alternatively spliced genes during exercise. We confirmed the presence of alternative splicing form of *PLEKHG1* transcripts and investigated expression pattern of *PLEKHG1* transcripts in response to exercise. In addition, we conducted comparison analysis of protein structure of each of alternative spliced protein.

MATERIALS AND METHODS

Horse sampling

All procedures were conducted by following the protocol that had been reviewed and approved by the Institutional Animal Care and Use Committee at Pusan National University (protocol numbers: PNU-2013-0417, PNU-2013-0411). Horse tissue sampling and RNA sequencing data were described in our previous study (PARK et al., 2012). The animals performed a combination of different horse gaits which included trotting and cantering through lunging and long-reining (circular bridge lunging) as their form of exercise. Generally, racehorses are subjected to exercise for 17 to 18 min per day, however horses in this study followed a combined 30-min exercise of trotting and cantering. Briefly, the samples were obtained from the blood and skeletal muscle samples before and 30 min after exercise.

Total RNA isolation

Total RNA samples for investigation of *PLEKGH1* transcript expression were collected from three Thoroughbreds. Skeletal muscle tissues were extracted for polymerase chain reaction (PCR) analysis. The various tissues sampled from the horses (50 to 100 mg, or 3 mL in the case of blood) were crushed with a mortar-pestle and mixed with 9 mL of red blood cell (RBC) lysis buffer (Solgent Co. Ltd., Daejeon, Korea) to remove RBC. The cells were then dissolved using 1 mL of TRIzol (Invitrogen, Karlsruhe, Germany), and 200 μL of chloroform was added to remove cells from the organic solvent. The mixture was then shaken for 10 s and left at 4°C for 5 min. Centrifugal separation was carried out at 4° C for 15 min, and then the supernatant was removed to a new test tube and mixed with the same amount of isopropanol. The test tube was left at 4°

C for 15 min to produce RNA pellets. The isopropanol was removed by carrying out centrifugal separation at 4°C for 15 min and the sample was then sterilized with 85% ethanol and dissolved in RNase-free water. The purity of the extracted RNA was confirmed by measuring the absorbance at 230 nm and 260 nm using a spectrophotometer (ND-100, Nano Drop Technologies Inc., Wilmington, DE, USA), and only the extracted RNA with purity (optic density value at 230 nm divided by optic density value at 260 nm) over 1.8 (found via quantitative analysis) was used. The selected RNA was stored at –70°C until the experiment occurred.

Total RNA isolation

In order to synthesize cDNA, 2 μg of RNA, 1 μL of oligo-dT (Invitrogen, Germany), and 1 μL of RNase-free water were added, the mixture was denatured at 80°C for 3 min, and then the cDNA was synthesized using 4 μL of 5× RT buffer, 5 μL of 2 mM deoxynucleotide (dNTP), 0.5 μL of RNase inhibitor (Promega Corporation, Madison, WI, USA), and 1 μL of moloney murine leukemia virus reverse transcriptase (Promega, USA).

Reverse transcription polymerase chain reaction amplification and sequencing

The nucleotide sequences of horse *PLEKHG1* gene from the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov) and the Ensembl Genome Browser (http://www.ensembl.org) were retrieved to design the primers with PRIMER3 software (http://bioinfo.ut.ee/primer3-0.4.0/). The original horse *PLEKHG1* transcripts were amplified by the primer pairs which were designed to detect each of alternative variants, *PLEKHG1a*-forward (5'- TAC AGC CCC CGA AGG GAA CTA-3') and *PLEKHG1a*-reverse (5'- AGA GTC CCT GGC TGA TTT GA-3') for *PLEKHG1a*, and *PLEKHG1b*-foward (5'- TGG CTC TCA GGA ACT ACA GA-3') and *PLEKHG1b*-reverse (5'- AGA GTC CCT GGC TGA TTT GA-3') for *PLEKHG1b*, were used to specifically detect each isoform. The PCR to amplify the target genes on the cDNA was carried out under the following conditions: 1.8 μL dNTP, 2 μL 10× buffer, 0.2 μL Taq, and 12 μL distilled water were added to 2 μL, 50 ng/μL diluted DNA and 5 pmol/μL diluted forward primer and reverse primer. The PCR was carried out in a total volume of 20 μL. The PCR procedure was: denaturation at 94°C for 10 min, and a second denaturation at 94°C for 30 s, followed by annealing at 58°C for 30 s, and extension at 72°C for 30 s. This was repeated for 40 cycles, and then a final extension was performed at 72°C for 10 min. The band was confirmed on UV using a 1.5% SeaKem LE agarose gel (Lonza, Rockland, MD, USA).

Reverse transcription polymerase chain reaction amplification and sequencing

To analyze the expression level of *PLEKHG1* alternative splicing isoforms in muscle before and after exercise, quantitative reverse-transcription polymerase chain reaction (qRT-PCR) was conducted by using the BioRad CFX-96 machine (BioRad, Hercules, CA, USA). Each reaction was executed in a total 25 μL of mixture containing 14 μL of SYBR green master mix, 2 μL of forward primer (5 pmol), 2 μL of reverse primer (5 pmol), 5 μL of distilled water, and 2 μL (50 ng/μL) of cDNA. The PCR conditions were at 94°C for 5 min of pre-denaturation step, 39 cycles of 94°C for 20 s, 56°C for 20 s, and 72°C for 30 s, and followed by 72°C for 10 min as a final step. All measurements were performed in triplicate for all specimens, and the comparative method used was the 2–ΔΔCt method (Livak and Schmittgen, 2001). The relevant expression of the target genes was calculated using glyceraldehyde-3-phosphate dehydrogenase as a normalizer.

Phylogenetic analysis

The amino acid sequences of *PLEKHG1* of various species were obtained NCBI: Amino sequence of *PLEKHG1* was obtained with cow (NP_001192850.1), human (NP_001025055.1), Thoroughbreds (Horse, XP_023488690.1), Przewalski (wild horse, XP_008515218.1), mouse (NP_001028425.1), rat (NP_001177928.1), dog (XP_005615567.1), frog (XP_017949742.1), chicken (XP_025004704.1). Amino acids were aligned using Multiple Sequence Comparison by Log-Expectation (MUSCLE) (http://www.ebi.ac.uk/Tools/msa/muscle/). Phylogenetic analysis was performed using Neighbor-Joining method (SAITOU and Naruya, 2004) with pairwise deletion, 1,000 bootstrap replication, and

Kimura 2 as described previously (CHO et al., 2015). The web-based protein prediction program, PredictProtein (www.predictprotein.org), was used to predict the secondary and tertiary structure (ROST et al., 2004).

RESULTS AND DISCUSSION

The equine *PLEKHG1* gene has 16 exons and 15 introns on the chromosome 31, and 7 transcriptional variants had been predicted in NCBI gene database. Two forms of alternative spliced transcripts from RNA-sequencing were discovered and the genomic structure of horse *PLEKHG1* was shown in Figure 1A. One of the alternative spliced transcripts shown short form by exon deletion. Long form of transcripts (*PLEKHG1a*) has 16 exons and short form (*PLEKHG1b*) has 15 exons. Full lengths of each of transcripts (*PLEKHG1a* and *PLEKHG1b*) are 4,449 and 4,356 and encoded protein of *PLEKHG1a* is 1,421 amino acids and protein of *PLEKHG1b* is 1,390 amino acids with deleted exon 8 occurred by alternative spliced exon (Figure 1B and Figure 1C). To investigated evolutionary relationship of equine *PLEKHG1* gene, we obtained *PLEKGH1* gene sequence of various species (human, cow, dog, horse, wild horse, rat, chicken, frog and mouse) from NCBI database. As a result of phylogenetic analysis with eight vertebrata (Figure 2A), we obtained insight that equine *PLEKHG1* was evolutionarily closer to dog and cow. When we conducted multiple alignment with the amino acid sequence of *PLEKHG1* in various species, Dbl homology (DH) domain (Figure 2B, solid box) and Pleckstrin homology (PH) domain (Figure 2B, dashed box) of *PLEKHG1* showed higher identity, and alternative splicing occurred on exon 8 (Figure 2B, red dashed box).

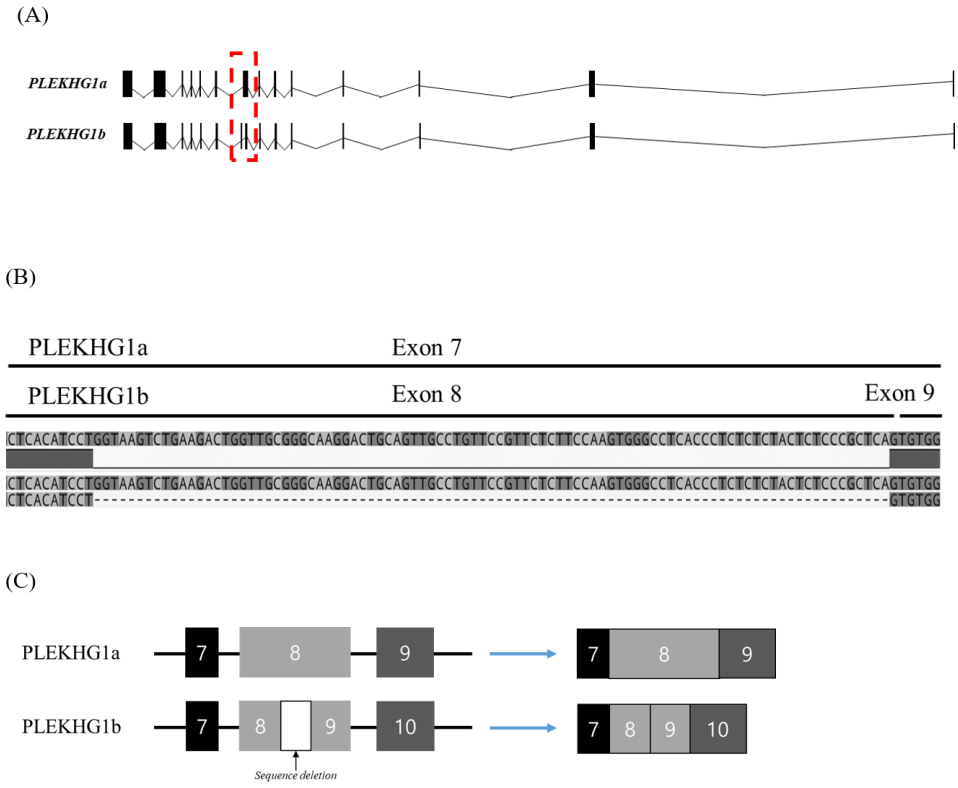


Figure 1. Alternative splicing isoforms of equine *PLEKHG1* (Pleckstrin Homology And RhoGEF Domain Containing G1). (A) Genomic structure of *PLEKHG1* alternative splicing variants. Equine *PLEKHG1* gene has 9 exons and two major alternative splicing forms; *PLEKHG1a* as long form and *PLEKHG1b* as short form. *PLEKHG1a* has deletion in exon 7 by alternative splicing. (B) Sequencing of alternative splicing region. (C) Structure of *PLEKHG1* variants by alternative splicing.

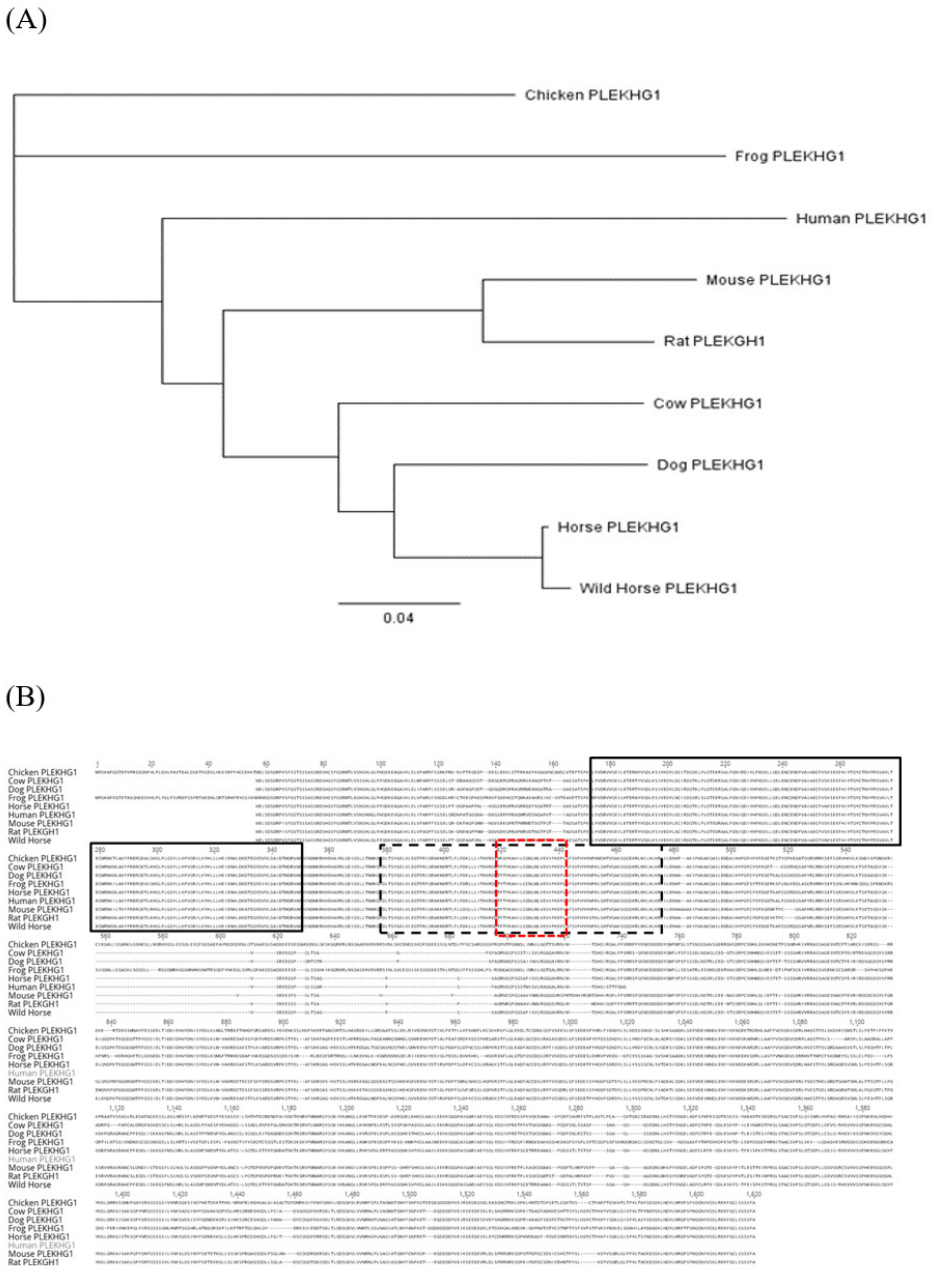
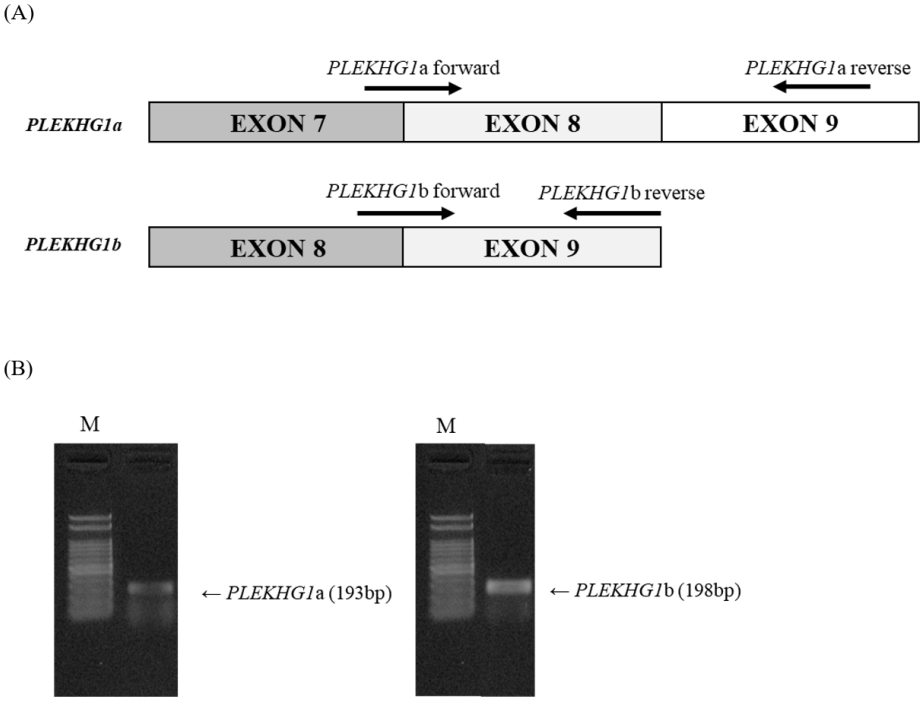


Figure 2. Analysis of amino acid sequences and phylogenetic tree of equine *PLEKHG1* (Pleckstrin Homology And RhoGEF Domain Containing G1) gene among various species. (A) Phylogenetic tree of *PLEKHG1*. The phylogenetic tree was made with the full amino acid sequences of each species by Neighbor-Joining method after aligned by the MUSCLE method in GENEIOUS program. Horse *PLEKHG1* was similar to cow and dog than frog and chicken. (B) Alignments of Dbl homology (DH) domain and Pleckstrin homology (PH) domain of *PLEKHG1* from various species. The sequences were aligned by the MUSCLE method in GENEIOUS program. The DH domain is marked by solid box, The PH domain is marked by dashed box and the sequences deleted by alternative splicing are marked by red solid box.

In our previous study, we identified unique expression pattern of two alternative spliced transcripts in *PLEKHG1* gene. Both of alternative spliced transcripts were found in Ensembl (ENSECAT00000007575 and ENSECAT00000007523). To investigate expression pattern of both of transcripts, we designed specific primer sets (Figure 3A). For *PLEKHG1a* transcript, forward and reverse primers were positioned at exon

7-exon 8 and exon 9, respectively (Figure 3A). Similarly, for *PLEKHG1b* transcript, forward and reverse primer were positioned at exon 8-9 and exon 9, respectively (Figure 3A). To identify two form of alternative spliced transcripts, two transcriptional variants were amplified by RT-PCR with horse skeletal muscle tissue (Figure 3B). Additionally, qRT-PCR were conducted for validate expression pattern of two alternative spliced transcripts in horse skeletal muscle tissue before and after exercise (Figure 3C). As a result, it was confirmed that the expression patterns of *PLEKHG1a* and *PLEKGH1b* are different. The transcript level of *PLEKHG1b* was up-regulated over than 3- fold change after exercise (Figure 3C left panel, $p<0.05$ while *PLEKHG1a* was down-regulated (Figure 3C right panel, $p<0.05$), and it showed matched pattern with RNA-sequencing. According to a recent genome-wide association study, Rho GEFs, including BCR and *PLEKHG1*, are a large, diverse family of proteins defined by their ability to catalyze the exchange of GDP for GTP on small GTPase proteins such as Rho family members. GEFs act as integrators from varied intra- and extracellular sources to promote spatiotemporal activity of Rho GTPases that control signaling pathways regulating cell proliferation and movement (MILLER et al., 2014). Also, Rho family small GTPases is known that activated PI3K (YANG et al., 2012), and it is well known that PI3K is related with muscle differentiation and muscle proliferation in various species (SARKER and LEE, 2004; WILDEN et al., 1998; KORNASIO et al.,2009; MURGIA et al., 2002; STITT et al., 2004). Unfortunately, there have been few independent studies of the *PLEKHG1* gene, but recent studies revealed *PLEKHG1* gene are up-regulated by muscle differentiation (TRIPATHI et al., 2014). Already numerous studies discovered exercise can induced generation of alternative spliced variants (NORRBOM et al., 2011; GUSTAFSSON et al., 2005), and also some studies investigated alternative spliced transcripts showed different expression pattern by some stimuli (ASCENZI et al., 2019). Suggesting the importance of relationship between muscle differentiation, proliferation and muscle regeneration, it is reasonable that regulation of *PLEKHG1* transcripts expression maybe has important role for regulating muscle differentiation, proliferation and wound healing after exercise through Ras/PI3K pathway in skeletal muscle. Given the importance of muscle proliferation and differentiation in exercise, it is of interest that the regulation of each of *PLEKHG1* transcripts expression maybe important for regulating muscle proliferation or switching of muscle differentiation to muscle regeneration, possibly through an Ras/PI3K signaling pathway, in muscle after exercise.



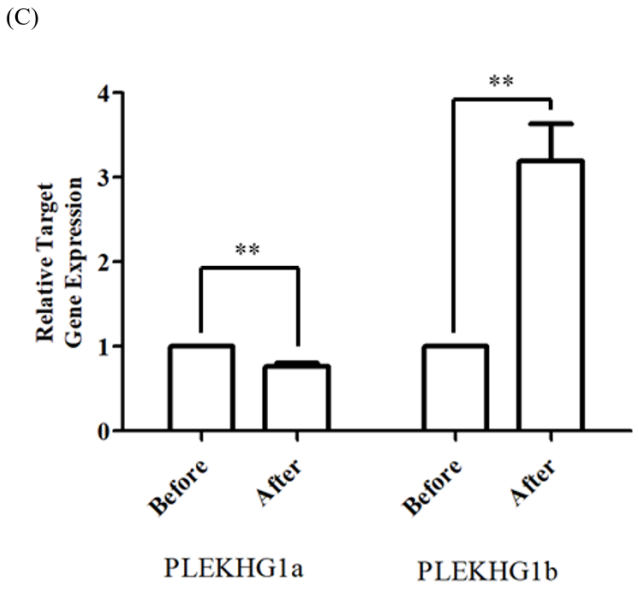


Figure 3. Expression pattern of equine *PLEKHG1* (Pleckstrin Homology And RhoGEF Domain Containing G1) alternative splicing variants. (A) Primer designs for alternative splicing variants; *PLEKHG1*a and *PLEKHG1*b. For *PLEKHG1*a transcript, the forward and reverse primers were positioned at exon 7–7 deletion and exon 8, respectively. (B) Confirmation of *PLEKHG1* alternative splicing variants. The alternative splicing variants of *PLEKHG1* gene, *PLEKHG1*a and *PLEKHG1*b, were amplified by RT-PCR. (C) Relative expression of *PLEKHG1* alternative splicing variants was analyzed by quantitative reverse-transcription polymerase chain reaction (qRT-PCR) in skeletal muscle before and after exercise. (n = 3, the ** means $p<0.05$, *** means $p<0.005$). Quantitative analysis was performed using the $2-\Delta\Delta Ct$ method. Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) was used for normalization

In case of the amino acids encoded in each transcript, the horse *PLEKHG1a* transcript encodes 1,421 amino acids whereas *PLEKHG1b* transcript encodes 1,390 amino acids. To investigate the protein structural difference between *PLEKHG1* alternative splicing variants, we predicted 3D structures of both of *PLEKHG1* transcripts with 31 amino acid deletions (Figure 4A). As a result, deleted amino acid contained two putative phosphorylation sites by protein kinase C, N-Myristoylation (Figure 4B). Many signaling pathways rapidly and reversibly convert extracellular signals into changes in gene expression. Phosphorylation of a transcription factor, often at multiple sites, is a common mechanism for responding to signaling events (KARIN and HUNTER, 1995). Many previous studies reported that protein phosphorylation is closely related to the control of protein stabilization, serving as a marker that triggers subsequent ubiquitination, in particular where ubiquitination leads to degradation (Treier et al., 1994; Fuchs et al., 1996; Magnani et al., 2000). Therefore, *PLEKHG1a* could be elaborately controlled after exercise, whereas *PLEKHG1b* could be stable after exercise due to lack of these phosphorylation sites by alternative splicing (Figure 3C). Also, *PLEKGH1a* and *PLEKHG1b* transcripts showed structural differences (Figure 4C). Alternative splicing effect on Pleckstrin homology (PH) domain (Figure 2B, dashed box). These structural differences may be explained by 31 amino acid deletions, but our prediction was based on only in silico homology. Therefore, further experimental crystal structure study should be necessary to explain this *PLEKHG1* alternative splicing effects. Generally, PH domain is known to involved in various cellular function including cell growth, cell survival through intracellular signaling (HASLAM et al., 1993; MUSACCHIO et al., 1993; INGLEY and HEMMINGS, 1994). Therefore, the different structures of the alternative *PLEKHG1* isoforms may be related to regulatory mechanism of signaling cascade.

(A)

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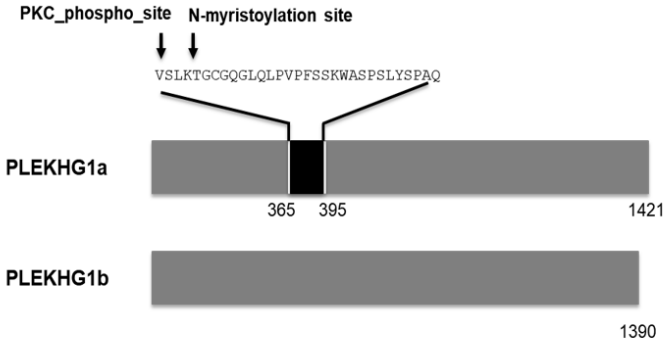
           360       370       380       390       400
PLEKHG1a TYKAHILVSLKTGCGQGLQLPVPFSSKWASPSLYSPAQCGNLMLVE
         :::::::                                 ::::::::
PLEKHG1b TYKAHIL-------------------------------CGNLMLVE
           360       370       380       390       400
```

(B)



(C)

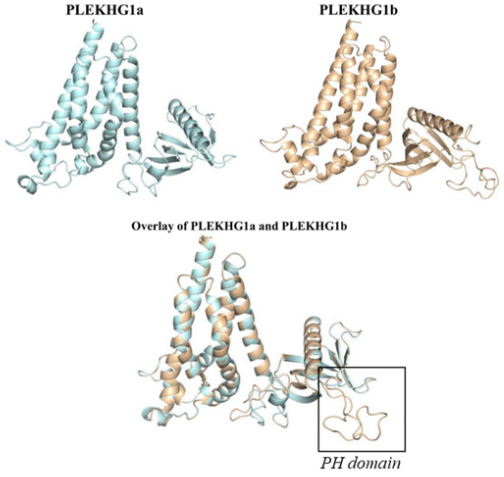


Figure 4. The comparison of amino acid sequences and protein structure between *PLEKHG1a* and *PLEKHG1b* proteins. (A) The amino sequences of *PLEKHG1a* and *PLEKHG1b* protein were aligned. And (B) the protein kinase C phosphorylation positions within the alternative splicing region were predicted (C) Structural models and overlay of both *PLEKHG1a/b* transcripts. The black rectangle indicates the structural difference between *PLEKHG1a/b* transcripts.

CONFLICT OF INTERESTS

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

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AUTHORS INFORMATION

Jeong-Woong Park: https://orcid.org/0000-0003-0885-3078

Jae-Young Choi: https://orcid.org/0000-0001-8264-1482

Kyoung Hwan Kim: https://orcid.org/0000-0003-4259-7872

Kim sujung: https://orcid.org/0000-0003-2037-0298

Jae-rung, So: https://orcid.org/0000-0001-6786-6894

Byeong-Woo Kim: https://orcid.org/0000-0003-3300-0173

Byung-Wook Cho: https://orcid.org/0000-0002-7739-1391