pISSN 2508-1357, eISSN 2508-139X
J Biomed Transl Res 2020;21(2):072-078
https://doi.org/10.12729/jbtr.2020.21.2.072
Received 23 Apr. 2020, Accepted 11 May. 2020

Research Article

Correlation between carcass grade and progesterone receptor membrane component 1 in Korean Hanwoo

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Carcass grade primarily depends on marbling of intramuscular fat, which is associated with the texture and tenderness of beef. Accordingly, various economical molecular tests for high intramuscular fat in beef have been attempted. Especially, Hanwoo (Korean Cattle) intramuscular fat has higher levels of monounsaturated fatty acids than that in the beef of other cattle. Intramuscular fats are associated with levels of lipid metabolic genes in the liver transcriptome. Therefore, hepatic triglyceride synthesis can considerably increase intramuscular fat. To investigate the relationship between hepatic lipogenesis and carcass grade, we analyzed 52 Hanwoo liver samples from domestic farms, and evaluated lipid levels and transcript levels of glucose and lipid metabolism-related genes according to carcass grade. Oil-Red-O staining revealed fatty livers in high carcass grades. Moreover, we found significantly higher levels of mRNA for lipogenesis, glycolysis, and triglyceride synthesis genes in high carcass grade livers. Importantly, progesterone receptor membrane component 1 (Pgrmc1) levels were significantly lower in high carcass grade livers. As Pgrmc1 suppression is correlated with induction of *de novo* lipogenesis (DNL) and glycolysis genes, it has a diagnostic impact for high carcass grades. These results could be used for genetic improvements in carcass grades of cattle. More importantly, as Pgrmc1 can be detected in blood peripheral nucleated cells, it also has value for rapid blood diagnosis.

Key words: cow, liver, Pgrmc1, DNL, glycolysis

Introduction

Carcass grading is the term used in carcass classification for the purpose of trading encouragement and customer satisfaction [1]. Carcass grade mainly depends on the marbling score, rather than meat color, fat color, firmness, or texture in Hanwoo. Hanwoo generally has intramuscular fat, and 1^{++} grade Hanwoo are particularly high in fat and low in moisture. Comparatively, Hanwoo possess low cholesterol levels and high levels of fatty acids including monounsaturated fatty acids (MUFA) compared to other cattle [2, 3]. Moreover, high grade Hanwoo carcasses have high levels of MUFA whereas low grade Hanwoo carcasses present high levels of saturated fatty acids (SFA) [4].

Normally, the majority of body fat consists of triglycerides (TG). Following *de novo* lipogenesis (DNL), hepatic triglycerides (TGs) are synthesized by combining three fatty acids with one glycerol molecule. During DNL, sterol regulatory element binding protein 1 (Srebp-1) is a key contributor that regulates transcriptional levels of several genes including acetyl CoA carboxylase (*Acc*), fatty acid synthase (*Fasn*), stearoyl CoA desaturase (*Scd1*), and glycerol-3-phosphate acyltransferase 1 mitochondrial (*Gpam*) [5]. In fatty acid synthesis, some of the polyunsaturated fatty acids (PUFA) are converted into MUFA by Scd1 in the endoplasmic reticulum (ER) of liver cells. Moreover, TG and MUFA synthesis is promoted by Srebp-1 [6].

Recently, whole genome sequencing of Hanwoo has been achieved [7]. Moreover, there have been several studies focusing on the expression of genes associated with marbling [8, 9]. Especially, fat-associated genes have been screened in intramuscular fat, and the level of Gpam is significantly positively correlated with carcass grade in Hanwoo [10]. While lipid metabolic genes seem to be important, another feature of high grade carcasses is the induction of glycolysis after tenderization processes such as electrical stimulation [11].

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Based on a previous report which investigated the metabolic role of Pgrmc1 under a high fat diet [12], we expected that genetic or epigenetic factors related to Pgrmc1 could influence the carcass grade of cattle. Pgrmc1 is a novel cellular membrane receptor associated with cholesterol synthesis and steroidogenesis. More importantly, Pgrmc1 interacts with the Insig-1/Scap/Srebp-1 complex and its loss triggers high levels of mature SREBP-1 protein. As high grade Hanwoo carcasses feature high levels of MUFA and lipid synthesis, we speculated that Pgrmc1 might be closely related to higher carcass grades in Hanwoo. We were able to observe the expression level of *Pgrmc1* in the livers and its association with lipids, lipid metabolism genes, and glycolytic genes.

Materials and Methods

Animals

Livers of 52 male Hanwoo were kindly provided by Institute of Health and Environment, Daejeon Metropolitan City. 1^{++} (6 cows), 1^{+} (13 cows), 1 (25 cows), and 2 (8 cows) were classified into group 1^{++}or 1^{+}, group 1, and group 2 carcass grade.

RNA isolation, reverse transcription, and qRT-PCR

Total RNA of Hanwoo liver and Hep3B cells were extracted with the TRIzol® Reagent (Thermo Fisher Scientific, MA, USA), Chloroform (C2432, Sigma-Aldrich, Gillingham, UK), Isopropanol (1.09634.1011, Merck, Darmstadt, Germany), and DEPC (E174, Amresco, West Chester, PA, USA)-treated water. cDNA was obtained by reverse transcription with 1-1.5 μg of total RNA and Reverse transcriptase kit (SG-cDNAS100, Smartgene, UK). Quantitative PCR (Real-time PCR) was performed with specific primers (Table 1) and Excel Taq Q-PCR Master Mix (SG-SYBR-500, Smartgene, Ljubljana, Slovenia). Stratagene Mx3000P (Agilent Technologies, Santa Clara, CA, USA) equipped with a 96-well optical reaction plate was used.

Oil Red O staining

For Oil-Red-O, frozen tissue were cut by 7 μm after embedded with optimal cutting temperature (OCT) compound and attached to silane coated slide. After drying 10 mins in RT, slides were fixed with formalin for 20 mins and washed with running tap water for 10 mins. Slides were proceeded to rinse step with 60% isopropanol and stained with Oil Red O working solution (3 g/L) for 15 mins. After washing with 60% isopropanol, slides were stained with hematoxylin for 30 secs and rinsed with distilled water. Region of interest was observed by light microscope after mounted in aqueous mountant.

Cell culture

Cell culture reagents were obtained from Welgene (Gyeongsan, Korea). Hep3B cells were obtained from Korean Cell Line Bank (KCLB, 88064). Hep3B cells were incubated in Dulbecco's Modified Eagle Medium (DMEM) (Welgene) containing 5% (vol/vol) foetal bovine serum, penicillin (100 U/mol) and streptomycin (100 μg/mL).

siRNA transfection

siRNA transfection was performed with lipofectamine 2000 (11668-027, Thermofisher, Waltham, MA, USA) according to the manufacturer's protocol. Negative control siRNA and PGRMC1 siRNA #1 and #2 were purchased from Bioneer (Daejoen, Korea). The sense sequences of PGRMC1 siRNA #1 and #2 were 5'-CAGUACAGUCG CUAGUCAA-3' and 5'-CAGUUCACUUUCAAGUAUCA-U-3'.

Statistical analysis

Data are reported as mean ± S.D. Differences between means were obtained by Student's *t*-test and the one-way ANOVA followed by a Dunnett post analysis was performed using Graph Pad Software (GraphPad, San Diego, CA, USA).

Results

High potential for fatty liver occurrence exists in high grade carcasses

To investigate whether fatty livers occur in high carcass grades, we performed Oil-Red-O staining to evaluate TG status. As a result, we observed fatty livers and classified them as strongly positive, slightly positive, or negative. Representative images for strongly positive, slightly positive, and negative are shown in Fig. 1. Carcasses grade $1^{++}/1^{+}$ comprised 21.1% of Oil-Red-O positive livers and carcasses grade 1/2 carcasses constituted 9.1% of Oil-

Table 1. Number and percentage of Oil-Red-O positive livers of 52 cows

Beef marbling score	Strong positive	Slight positive	Negative	Positive ratio (%)
$1^{++}/1^{+}$	1	3	15	21.1
1/2	1	2	30	9.1

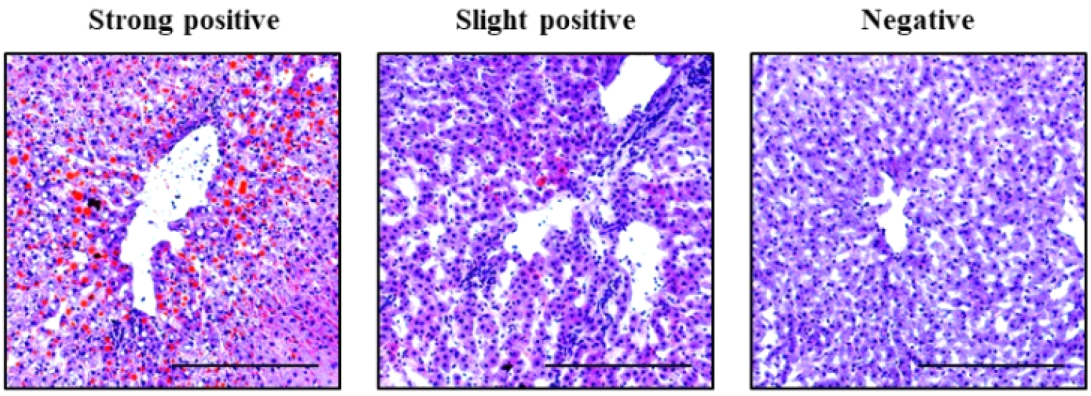

Fig. 1. Evaluation of fatty liver according to carcass grade. Representative images of Oil-Red-O staining in livers of 52 cows. Grades were classified into strong positive, slight positive, and negative (Scale bar, 200 μm).

Red-O positive livers (Table 1). As the percentage of Oil-Red-O positive livers increased with carcass grade, we speculated that fatty liver is induced in high carcass grades.

Gene levels related to *de novo* lipogenesis are upregulated in high grade carcasses

As fatty liver is induced by DNL in a series of steps, we evaluated the mRNA levels of genes associated with DNL. Unexpectedly, the mRNA levels of *Srebp-1* were not significantly different between groups (Fig. 2A). Nevertheless, as SREBP-1 protein regulates the transcription of several enzymes, we continue to monitor its mRNA levels. As a result, mRNA levels of *Acc* (4.31-fold) and *Fasn* (2.09-fold) were significantly higher (p<0.05) in carcass grade $1^{++}/1^{+}$ compared to levels in the livers

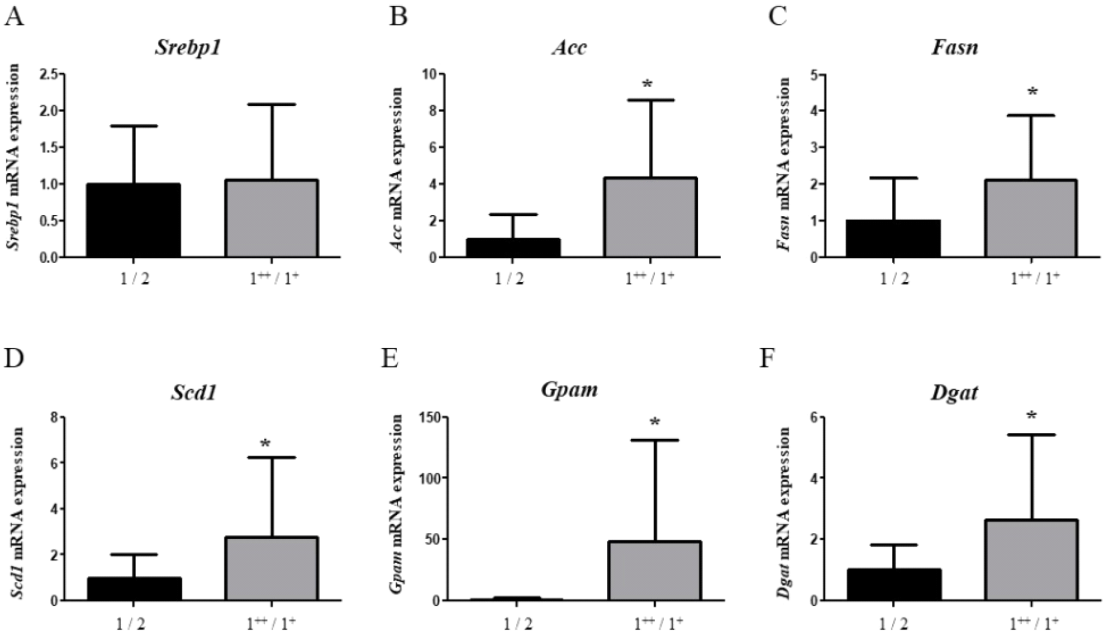

Fig. 2. mRNA levels of Srebp-1 related genes in liver. (A) mRNA levels of *Srebp-1* in liver of cow. (B–C) mRNA levels of fatty acid synthesis genes in liver of cow. (D) mRNA levels of Scd1 in liver of cow. (E–F) mRNA levels of TG synthesis genes in liver of cow. Values represent means ± S.D. $^{*}p$<0.05 vs. Carcass grade $1^{++}/1^{+}$. Data are normalized to *Rplp0* levels. Number of cows used in experiment is 50. Srebp-1, sterol regulatory element binding protein 1; ACC, acetyl-CoA carboxylase; Fasn, fatty acid synthase; Scd1, stearoul-CoA desaturase 1; Gpam, glycerol-3-phosphate acyltransferase; Dpat, diacylglycerol O-acyltransferase.

from grade 1/2 carcasses (Fig. 2B and 2C), suggesting that fatty acid synthesis was induced. Moreover, *Scd1* mRNA levels were significantly higher (p<0.05, 2.77-fold) in grade $1^{++}/1^{+}$ carcass than in grade 1 carcass (Fig. 2D), suggesting that conversion of PUFA to MUFA was triggered. Meanwhile, *Gpam* (p<0.05, 48.20-fold) and *Dgat* (Diglyceride acyltransferase, 2.654-fold) mRNA levels were significantly higher (p<0.05) in carcass grade $1^{++}/1^{+}$ than in carcass of grade 1 (Fig. 2E and 2F), suggesting that TG synthesis was promoted.

Gene levels related to glycolysis are upregulated in high grade carcasses

As glycolysis is known to be promoted in high grade carcasses, we next evaluated the mRNA levels of representative glycolytic genes. As a result, mRNA levels of *Hk1* (Hexokinase1, 2.66-fold) and *Pfk* (Phosphofructokinase, 2.26-fold) were significantly higher (p<0.05) in carcass grade $1^{++}/1^{+}$ than in carcass of grade 1 (Fig. 3A and 3B). However, there were no significant differences in the level of *Pk* (Pyruvate kinase) mRNA between groups (Fig. 3C). These results suggest that glycolysis is promoted above carcass grade 1.

Low levels of *Pgrmc1* mRNA were observed in livers from high grade carcasses

To evaluate the effect of Pgrmc1 on carcass grade, we evaluated mRNA levels of *Pgrmc1*. Data were provided for 52 Hanwoo livers. As a result, the level of *Pgrmc1* mRNA in grade grade $1^{++}/1^{+}$ carcasses was significantly lower (11%, p<0.05) than the levels in grade 1 carcass (Fig. 4A). Moreover, levels of *Pgrmc1* mRNA in carcass grade 1/2 were higher (p = 0.0169) than those in grade $1^{++}/1^{+}$ carcasses (Fig. 4B).

PGRMC1 down-regulation was correlated with glycolysis induction

As the correlation between PGRMC1 and lipogenesis *in*

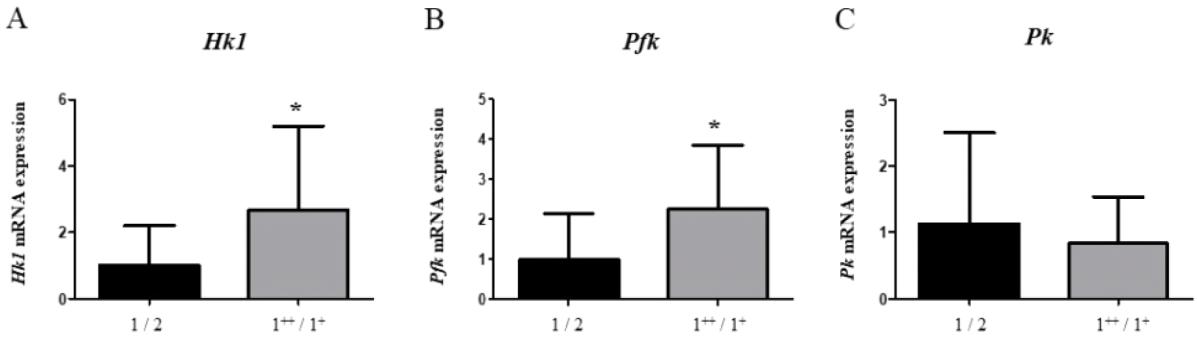

Fig. 3. mRNA levels of glycolytic genes in liver. (A–C) mRNA levels of *Hk1*, *Pfk*, and *Pk* are presented. Values represent means ± S.D. $^{*}p$<0.05 vs. Carcass grade $1^{++}/1^{+}$. Data are normalized to *Rplp0* levels. Number of cows used in experiment is 50. Hk1, hexokinase 1; Pfk, phosphofructokinase; Pk, pyruvate kinase

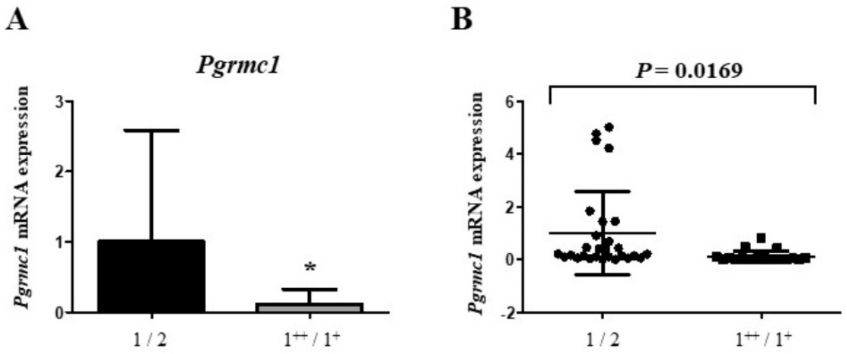

Fig. 4. mRNA level of *Pgrmc1* in liver. (A) Bar graph of Pgrmc1 mRNA levels in liver of cow. (B) Vertical scatter plot of Pgrmc1 mRNA levels in liver of cow. Values represent means ± S.D. $^{*}p$<0.05 vs. Carcass grade $1^{++}/1^{+}$. Data are normalized to *Rplp0* levels. Number of cows used in experiment is 50. Pgrmc1, progesterone receptor membrane component 1.

vitro has already been studied [12], we focused on the relationship between PGRMC1 and glycolysis in the present study. Among glycolysis genes, *Hk1* and *Pfk* levels were higher in higher grade carcasses, so their mRNA levels were analyzed in the Hep3B cell line. While *PGRMC1* level was suppressed (55%), levels of *HK1* (2.89-fold) and *PFK* (2.9-fold) were significantly higher in the *PGRMC1* siRNA group compared to the *Control* siRNA group (p<0.05, Fig. 5A–C), suggesting that glycolysis was induced after *PGRMC1* knockdown.

Discussion

The weight of Hanwoo carcasses is lower on average than that of the carcasses of US breeds of the same age at time of slaughter, suggesting less fattiness in Hanwoo carcasses than in US breeds. Intramuscular fat is affected by fattiness and is called marbling, which is a major factor in determining carcass grade [13]. When cattle take up nutrients from carbohydrate- or fat-rich diets, their livers synthesize fat using fatty acids and DNL. As a major contributor in DNL, SREBP-1 promotes visceral fat mass and MUFA levels [14]. Interestingly, Hanwoo possess high levels of MUFA due to lipogenesis in the liver.

SREBP-1 is classified into SREBP-1 and SREBP-2. While SREBP-2 plays a role in cholesterol synthesis, SREBP-1 triggers fatty acid synthesis. SREBP-1 normally exists in precursor form in the ER along with INSIG-1 and SCAP and is activated by a high carbohydrate diet. SREBP-1 then goes into the nucleus and becomes mature, called nuclear SREBP-1. Nuclear SREBP-1 can act as a transcriptional regulator for several genes, especially those related to fatty acid synthesis and TG synthesis [15]. As loss of PGRMC1 protein induces structural instability of the SREBP-1 precursor, SREBP-1 precursors are travel through the Golgi complex to the nucleus. In consequence, loss of PGRMC1 triggers lipogenesis by inducing SREBP-1 activation [12]. Therefore, the decreased *Pgrmc1* mRNA levels in carcasses grade $1^{++}/1^{+}$ suggest a decrease in PGRMC1 protein levels and an increase in SREBP-1 mature protein levels. Though *Srebp1* mRNA levels did not show significant differences, as PGRMC1 mainly influences SREBP1 protein rather than mRNA, our results are consistent with those of previous studies. Unfortunately, we could not evaluate the levels of SREBP-1 protein because of lack of host reactivity. However, mRNA levels of *Srebp-1* transcriptional genes suggest that decreased *Pgrmc1* levels caused promotion of lipogenesis and TG accumulation in liver.

In DNL, a series of steps causing lipogenesis, starts with acetyl-CoA which is then carboxylated by ACC. ACC is therefore a rate-limiting step in fatty acid synthesis. The product from acetyl-CoA is malonyl-CoA. Malonyl CoA is further saturated by several enzymes and finally, lipogenesis is terminated by FASN [16]. Therefore, it can be suggested that lipogenesis is induced in carcass grades $1^{++}/1^{+}$ because expression levels of two crucial enzymes for lipogenesis, *Acc* and *Fasn*, are increased. These two enzymes are also transcriptionally regulated by SREBP-1. Moreover, the present study is emphasized by previous studies suggesting that FASN can be used as superior genetic modification of Hanwoo [17]. After lipogenesis, SFA is desaturated into MUFA by SCD1 and is used for TG synthesis. While SCD1 is also transcriptionally regulated by SREBP-1, SCD1 overexpression is linked to metabolic disorders such as obesity and diabetes [18]. In cattle, Scd1 can also be used as genetic modification of

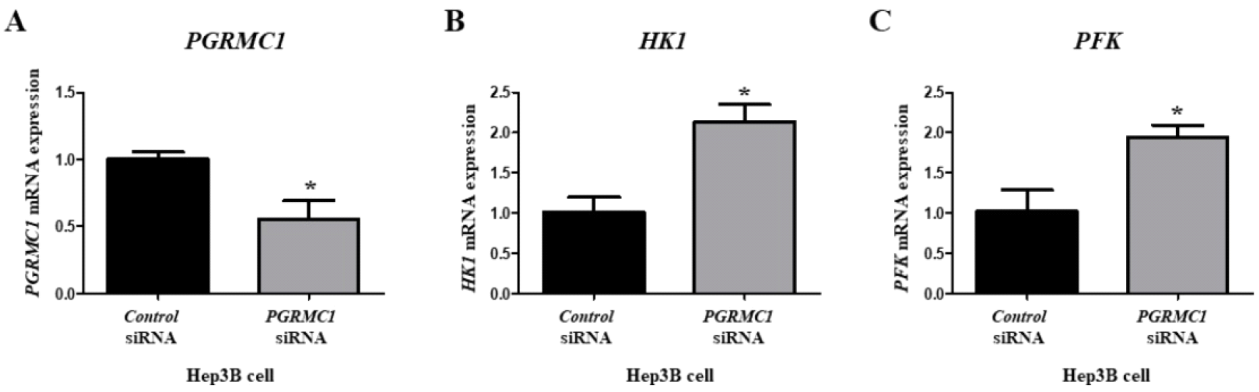

Fig. 5. *PGRMC1* knockdown increases glycolysis in Hep3B cell. (A–C) mRNA levels of *PGRMC1*, *HK1* and *PFK* are presented. Values represent means ± S.D. $^{*}p$<0.05 vs. control siRNA group. Data are normalized to *Rplp0* levels. Experiment was repeated at least 3 times. PGRMC1, progesterone receptor membrane component 1; HK1, hexokinase 1; PFK, phosphofructokinase.

Hanwoo. It is important because MUFA produced by SCD1 is a key factor in the flavor and tenderness of beef [19].

After fatty acid desaturation, MUFA combines with glycerol backbones in *de novo* TG synthesis. In TG synthesis, there are four steps including the enzymes GPAM, 1-acylglycerol-3-phosphate-O-acyltransferase (AGPAT), Phosphatidate phosphatase (PAP), and DGAT. GPAM, and DGAT are associated with the first and last steps of TG synthesis as they join fatty acyl CoA to glycerol backbones. In detail, GPAM is rate-limiting step for TG synthesis and DGAT determines flux into TG [20, 21]. More importantly, DGAT and GPAM have been suggested as candidate genes for intramuscular fat deposition in cattle [22, 23].

In DNL, acetyl CoA is synthesized from pyruvate, which is synthesized through the glycolysis pathway. In glycolysis, there are 10 steps including three irreversible steps. Three enzymes are involved in the irreversible steps: HK1, PFK, and PK. Among them, PFK is the most crucial enzyme as it controls the rate-limiting step of glycolysis [24]. By producing more pyruvate through the glycolytic pathway, it is highly likely that it is used for fatty acid synthesis in high carcass grades in the present study. Furthermore, we observed induction of glycolysis genes after *PGRMC1* knockdown *in vitro* in Hep3B cells. Through this, we confirmed that *PGRMC1* suppression leads to abundance of pyruvate sources that can be used for lipogenesis.

In conclusion, we found significant difference in *Pgrmc1* mRNA level according to carcass grade. We suggest Pgrmc1 as a valuable novel marker for producing high carcass grade cattle, which is closely related to DNL, TG synthesis, and glycolysis in the liver. The results of the present study can be used for genetic improvements in carcass grades of cattle. Since Pgrmc1 can be detected in blood peripheral nucleated cells, it also has value for rapid blood diagnosis [25]. In further studies, it will be valuable to monitor the relation between level of Pgrmc1 in blood and carcass grade in muscle. The selected cow expressing low Pgrmc1 will be used for genetic improvement.

Acknowledgments

This study was financially supported by faculty research abroad program of Chungnam National University in 2019. This work was supported by Research Scholarship of Chungnam National University.

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