Master's Thesis of Science in Agriculture

Association Study between Phospholipid Profile and SNPs Related to Fatty Acid Metabolism in Hanwoo Beef

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Abstract

Many positive physiological effects of omega-3 fatty acids have been demonstrated for over three decades. Lowering the attack rate of CVD, cancer, rheumatoid arthritis, auto-immune diseases and recurrence of myocardial infarction are representative effects of omega-3 fatty acids. Additionally, eicosanoids, the signaling molecules have contrast immune and inflammatory effects by their origin, omega-3 or omega-6. Therefore, omega-6 to omega-3 ratio is critical to maintaining immune homeostasis, normal development, and mental health. However, the modern western diets are very imbalanced in omega fatty acids ratio, and most people all over the world take much more omega-6 fatty acids than omega-3 fatty acids. Agriculture and livestock industry focused on productivity are the one of the reasons of omega fatty acid imbalance. Hanwoo industry is also focused on productivity and marbling score using maize as a main feed grain which is high in omega-6 fatty acids, and thus resulting in imbalanced omega fatty acids ratio in Hanwoo beef.

In this study, methods for beef fatty acid analysis was established and associations were studied between omega fatty acid composition in fifty-six Hanwoo beef samples and SNPs related to fatty acid metabolism. To study the associations between fatty acid composition and SNPs, instead of neutral fat, fatty acids of phospholipid was chosen since it is mainly under control of the genes related to fatty acid metabolism. Six SNPs in five genes related to fatty acid composition were selected as candidate SNPs; *FASN* (rs41919985), *SCD* (rs41255693), *FABP4* (rs41729173), *FADS1* (rs136261927 and rs42187261) and *FADS2* (rs109772589).

1. Total lipid was extracted using Folch method, and phospholipid was separated from total lipid using thin-layer chromatography. Phospholipid fraction was scraped from TLC plate and directly methylated using O'Fallon's direct methylation method. This preparation method was validated by comparing with O'Fallon's

direct methylation method and analyzing gas chromatography data of empty silica through the preparation method. The same amount of phosphatidylcholine from egg yolk was treated by above preparation method or direct methylation without pretreatment, and GC data from each method were compared. Total areas through the preparation method or direct methylation were comparable having 407 and 470, respectively.

2. Fatty acid composition in phospholipid of the samples were analyzed with GC. Genomic DNA was extracted from the fifty-six samples and genotyped for the candidate SNPs using restriction fragment length polymorphism or Sanger sequencing. In the case of rs109772589 in *FADS2*, all the samples showed the same hetero genotype, and thus it was omitted from the association analysis. On the other hand, in the case of rs41919985 in *FASN,* there were only two genotypes except AA type.

3. Associations between omega fatty acid composition and genotypes were analyzed by ANOVA. There were four SNPs showing statistically significant associations.

- GA type of rs41919985 with the highest C20:5 n-3 (p=0.027)
- CC type of rs41729173 with the lowest C22:2n-6 (p=0.049)
- AG type of rs42187261 with the lowest C20:4 n-6 (p=0.044)
- GA type of rs136261927 with the lowest C20:3n-6 (p=0.027)

The ratio of total n-3/n-6 of the individual which have GA, CC, AG and GA types in rs41919985, rs41729173, rs42187261 and rs136261927, respectively, was around 28:1. This value was the third highest grade in total n-3/n-6 ratio of the samples and much higher than the mean value of total n-3/n-6 ratio.

Consequently, these four SNPs could be applied as potential genetic markers to select Hanwoo steers in the aspect of improvement of n-3/n-6 balance in the future.

Keyword: Fatty acid, Omega-3, Omega-6, Beef, SNP, Association analysis
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List of Abbreviations

AA: Arachidonic acid
acetyl-CoA: acetyl coenzymeA
ALA: α-linolenic acid
ANCOVA: Analysis of covariance
ANOVA: Analysis of variance
BHA: Butylated hydroxyanisole
COX: Cyclooxygenase
CVD: Cardio vascular disease
DHA: Docosahexaenoic acid
ELOVL: Elongation of very-long-chain fatty acid protein
EPA: Eicosapentaenoic acid
ER: Endoplasmic reticulum
FABP4: Fatty acid binding protein 4
FADS: Fatty acid desaturase
FAME: Fatty acid methyl ester
FAO: Food and Agriculture Organization
FASN: Fatty acid synthase
FFA: Free fatty acid
GC: Gas chromatography
gDNA: Genomic DNA
HWE: Hardy-Weinberg equilibrium
LA: Linolenic acid
LOX: Lipooxygenase
MAF: Minor allele frequency
MAS: Marker-assisted selection
MUFA: Monounsaturated fatty acid
NCBI: National Center for Biotechnology Information
PCR: Polymerase chain reaction
PUFA: Polyunsaturated fatty acid
QTL: Quantitative trait loci
RFLP: Restriction fragment length polymorphsim

RT: Retention time
SCD: Stearoyl-CoA desaturase
SFA: Saturated fatty acid
SNP: Single nucleotide polymorphism
TLC: Thin-layer chromatography
Tm: Melting temperature
TR: Top round
USDA: United States Department of Agriculture
VGEF: Vascular endothelial growth factor

Ⅰ. Introduction

Modern diets have a high level of imbalance in omega fatty acids comparing to prehistoric diets because of too much intake of several grains rich in omega-6 fatty acids and modern agriculture based on grain-fed system (Simopoulos, A.P., 2002). Especially, Hanwoo industry is focused on high marbling score so that almost farmers use maize which is high in omega-6 fatty acids as a feed. Consequently, Hanwoo beef must have imbalanced n-3 to n-6 fatty acids ratio (Daley, A. C. *et al.*, 2010).

To balance n-3 to n-6 fatty acids ratio of beef, two strategies are usually used. The first strategy is feeding additives which are high in n-3 fatty acids. Using linseed as a feed additive is useful for enhancing the level of C18:3n-3 and C20:5n-3, and also fish oil increases the proportion of 20:5n-3 and 22:6n-3 in beef (Scollan, N. D. *et al.*, 2007). However, the ruminal bacteria in the ruminants hydrogenate polyunsaturated fatty acids so that fatty acids fed to the ruminants are changed into various fatty acids in the rumen and are hard to be absorbed as the original form (Weill, P. *et al.*, 2002).

The second strategy is finding genetic polymorphisms which balance n-3 fatty acids to n-6 fatty acids ratio. The amount of omega fatty acids is higher in phospholipid than neutral lipid, and phospholipid is used as building blocks of the cell membrane and mainly controlled by the genes related to fatty acid metabolism (Bourre, J. M., 2005). Therefore, many researchers have been studied on the association between

genetic polymorphisms and omega fatty acid composition. For example, Ibeagha-Awemu *et al.* reported that three single nucleotide polymorphisms (SNPs) in fatty acid desaturase 1 (*FADS1*) and 2 (*FADS2*) have significant relationships with n-3 fatty acids and n-6 fatty acids in milk fatty acids (Ibeagha-Awemu, E. M. *et al.*, 2014).

In this study, we investigated the differences in omega fatty acid composition in phospholipid of the fifty-six Hanwoo beef samples with the genotypes of six SNPs in the five genes related to fatty acid metabolism using statistical analysis. This study was also focused on the detailed method establishment for beef fatty acid analysis and SNP typing in relation to omega fatty acid-balanced meat production.

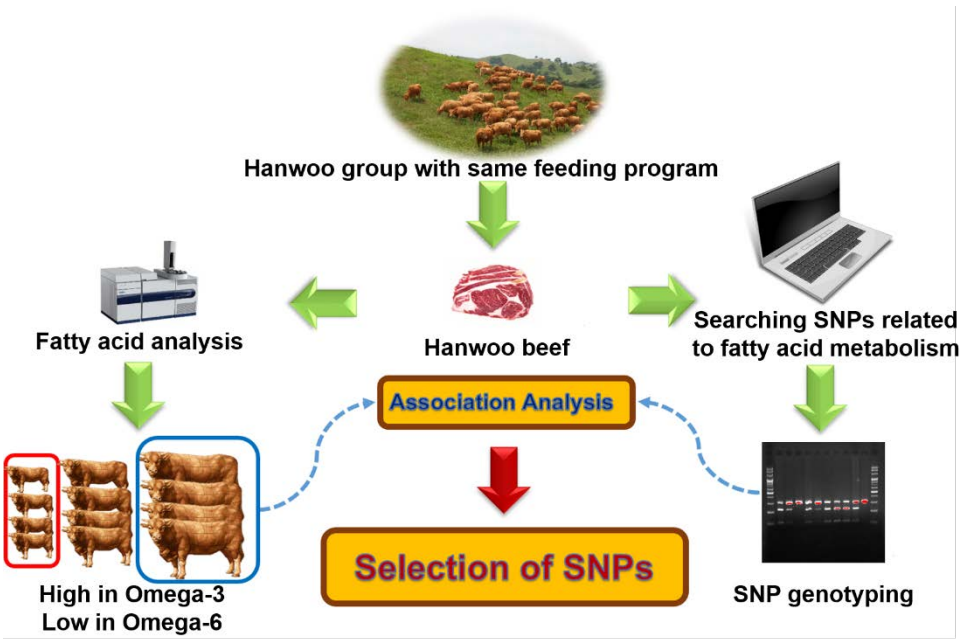


Figure 1. Scheme of the study

Ⅱ. Review of literature

1. Omega fatty acids

1) Definition of omega fatty acids

A fatty acid consists of successive carbon atoms with a carboxyl group and a methyl group at the each ends. By the nomenclature system of fatty acid, a carbon atom in a carboxyl end of a fatty acid is called Δ(delta), and another carbon in methyl end is called ω(omega). According to the existence of double bonds, fatty acids are divided into two groups, saturated fatty acids (SFAs) and unsaturated fatty acids. In detail, monounsaturated fatty acids (MUFAs) have only one double bond in the carbon chain, and polyunsaturated fatty acids (PUFAs) have two or more double bonds. Also, fatty acids are also grouped into short, long and very long in the aspect of the length of the carbon chain; however, the standards of carbon chain size may differ among researchers. (Hulbert, A. J. *et al.*, 2005).

Fatty acids are named following where the first double bond is in which carbon from methyl end or carboxyl end of the fatty acid. For example, in Figure 2, the fatty acid is made up of eighteen carbons and four double bonds, and the first double bond is at the third carbon from ω end and the another first double bond is at the sixth carbon from Δ. This fatty acid, therefore, is omega-3 fatty acid (C18:4 ω-3 or n-3) or delta-6 fatty acid (C18:4Δ-6). Usually, we call n-3 fatty

acids and omega-6 fatty acids as omega fatty acids.

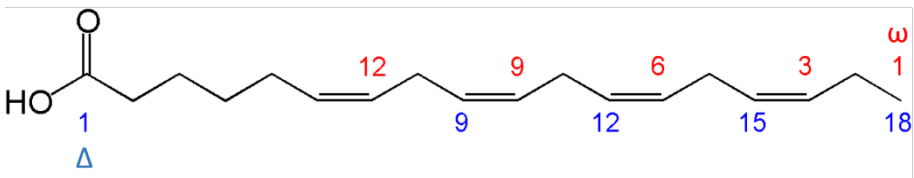


Figure 2. Fatty acid nomenclature system

2) Metabolism of omega fatty acids in mammals

There are some enzymes included in fatty acid metabolism in animals such as fatty acid desaturases which make double bonds in the carbon chain and elongases which elongate the length of the carbon chain. The desaturase and elongase system of animals can synthesize both SFAs and MUFAs using acetyl coenzymeA (acetyl-CoA). However, n-3 and n-6 cannot be synthesized *de novo* and also are not interconvertible because of the absence of $\omega-3$ and $\omega-6$ desaturase. Therefore, we must obtain n-3 and n-6 PUFAs from the diet (Hulbert, A. J. *et al.*, 2005). Especially, alpha-linolenic acid (ALA, C18:3 n-3) and linoleic acid (LA, C18:2 n-6) are the first precursors of omega PUFAs, so we call both fatty acids as essential fatty acids (EFAs). When we take the n-3 and n-6 EFAs, these are processed into longer PUFAs through fatty acid metabolism system (Guillou, H. *et al.*, 2010).

Figure 3 is a diagram of the metabolic pathway of n-3 and n-6 EFAs. FADS2 and FADS1 ($\Delta6$-desaturase and $\Delta5$-desaturase, respectively) are the acyl-CoA desaturases of membrane-bound desaturases group and the front-end desaturase which inserts a double bond between pre-existing

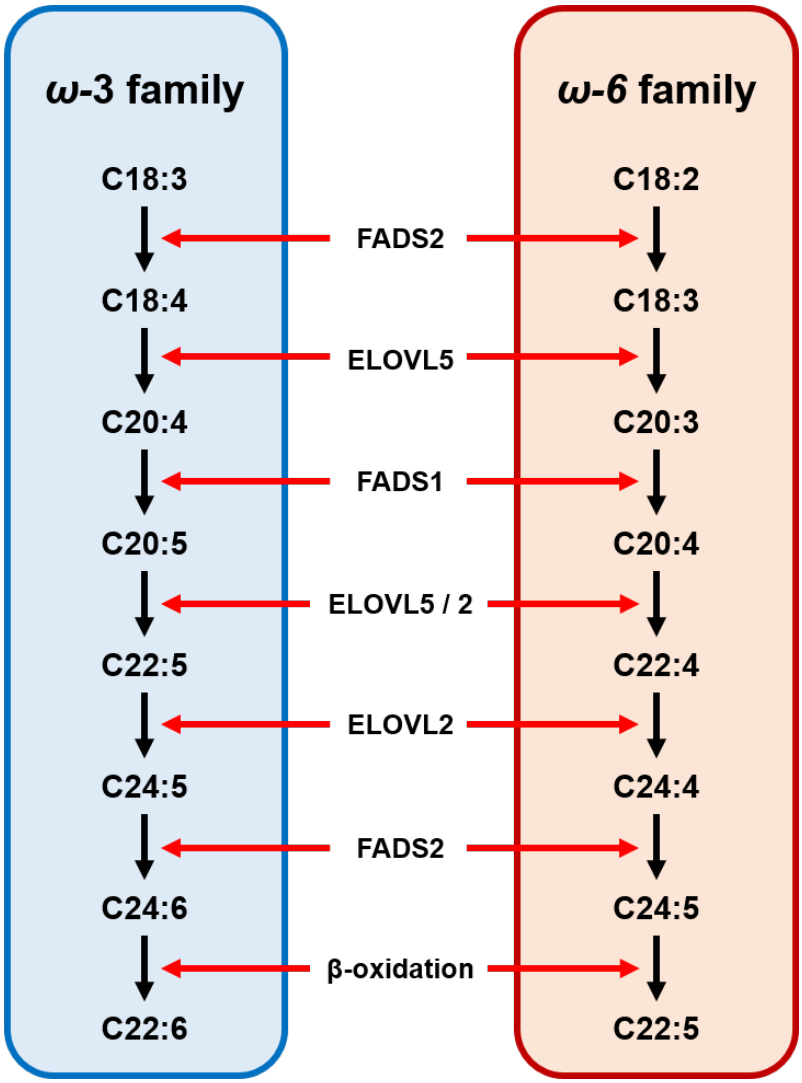


Figure 3. Omega fatty acid metabolic pathway

double bond and ⊿ carbon (front end carbon) of fatty acid. These desaturases in the endoplasmic reticulum (ER) membrane use fatty acyl-CoA and cytochrome b_5 as a substrate and an electron donor. FADS2 plays a significant role in the production of longer PUFAs because this enzyme is the initial and rate-limiting enzyme in the fatty acid metabolic pathway. FADS2 catalyzes desaturations at the ⊿6 position of C18:3 n-3 and C18:2 n-6. FADS1 also introduces a double

bond at the ⊿5 carbon of C20:4 n−3 and C20:3 n−6. Elongation of very−long−chain fatty acid proteins (ELOVLs) catalyze elongation of fatty acid carbon chains. Up to date, seven ELOVLs are identified (ELOVL1 – ELOVL7). Among these proteins, ELOVL2 and 5 make PUFAs longer selectively. β−Oxidation also is involved in fatty acid metabolism, and make C24:6 n−3 and C24:5 n−6 shorter (Nakamura, M. T. and Nara, T., 2004; Guillou, H. *et al.*, 2010). The most important thing in the metabolic pathway of omega fatty acids is that fatty acids share and compete for the same enzyme at the each metabolic step (Garcia, P. T., 2011).

Besides, fatty acid metabolism may occur quite differently in phospholipid and neutral lipid. Phospholipid is the primary building block of the cell membrane. On the other hand, neutral lipid is mainly deposited in adipose tissue. Saturated fatty acids are more deposited than unsaturated fatty acids (Garcia, P. T., 2011). In other words, unsaturated fatty acids are mostly used to phospholipid and saturated fatty acids are involved in neutral lipid. Also, Bourre, in his review paper (Bourre, J. M., 2005), argued that fatty acids of phospholipid are under control of the genes related to fatty acid metabolism, but fatty acids of neutral lipid are mainly influenced by the diet. Thus, the fatty acid compositions of phospholipid and neutral lipid are very different each other (Buchanan, J. W. *et al.*, 2015). In the article, while the percentage of PUFA in phospholipid was 35%, the percentage of PUFA in neutral lipid was only around 2.3%. Therefore, fatty acid analysis in beef should be conducted separately in phospholipid and neutral lipid, and it is only reasonable to study the association between fatty acid composition of phospholipid but not neutral lipid in Hanwoo beef and SNP types in the genes related to fatty acid metabolism.

3) Physiological effects of omega fatty acids

After the metabolic pathway, PUFAs from C18:3 n−3 and C18:2 n−6 to C22:6 n−3 and C22:5 n−6 become primary components of the cell membrane as building blocks of phospholipid. These components of phospholipid bilayer have the significant influence on the physical properties of cells. For example, the length and the number of double bonds of fatty acids in the cell membrane are leading factors of the membrane fluidity. Additionally, the type of fatty acids affects physiological functions such as cell signaling, cell division, inflammation and gene expression (Hulbert, A. J. *et al.*, 2005; Guillou, H. *et al.*, 2010).

Many positive physiological effects of n−3 fatty acids have been demonstrated for over three decades. Firstly, the health benefits of n−3 fatty acids were discovered from Inuits who took mainly seafood and had the low attack rate of heart disease, asthma and type 1 diabetes mellitus. Since then, many beneficial health effects of n−3 fatty acids have been observed. Lowering the attack rate of cardiovascular disease (CVD), cancer, rheumatoid arthritis, auto−immune diseases and recurrence of myocardial infarction are representative effects of n−3 fatty acids (Calder, P. C., 2006; Simopoulos, A. P., 2008). Additionally, ALA, eicosapentaenoic acid (EPA, C20:5 n−3) and docosahexaenoic acid (DHA, C22:6 n−3) effect on brain structure and function, and cognitive functions. The lack of these fatty acids causes the structural changes in the cell membrane of brain, neuron, myelin and nerve terminal. As a result, the membrane fluidity is changed, and it leads to neurosensory and behavioral disorders (Bourre, J. M., 2005).

In contrast with n−3 fatty acids, very high level of n−6 fatty acids has adverse physiological effects which make the physiological condition pro−thrombotic and pro−aggregative increasing vasospasm, vasoconstriction and blood viscosity. It decreases bleeding time, and patients with diabetes and atherosclerotic diseases have shorter bleeding time than healthy people (Simopoulos, A.P., 2002).

4) Eicosanoids

20−carbon PUFAs like arachidonic acid (AA, C20:4 n−6), dihomo−γ−linolenic acid (DGLA, C20:3 n−6) and EPA are the precursor of the eicosanoid family. A word, 'eicosanoid,' is originated from the Greek *eicosa* meaning twenty. Eicosanoids are signaling molecules made by enzymatic oxidation of 20 carbon PUFAs, and have multiple subfamilies (prostaglandins, thromboxanes, leukotrienes, *etc.*). In eicosanoids synthesis, firstly, phospholipase A_2 disassembles cell membrane phospholipid into a phosphate group and fatty acids. After that, 20−carbon fatty acids are oxidized by few oxygenases such as cyclooxygenase (COX) and lipoxygenase (LOX). Consequently, newly synthesized eicosanoids induce local cellular responses by binding to eicosanoid receptor with autocrine and paracrine (Figure 4). Eicosanoids derived from n−6 fatty acids normally have pro−inflammatory effects such as stimulating white blood cells, aggregating platelets, vasoconstriction, *etc.*, but other eicosanoids derived from n−3 have anti−inflammatory effects (Funk, C. D., 2001; Calder, P. C., 2013).

Both groups of eicosanoids have contrast immune and

inflammatory effects and complementarity so that n−6 to n−3 ratio is critical to maintaining immune homeostasis, normal development, and mental health. However, a very high n−6 to n−3 fatty acids ratio is found in the modern diets (Simopoulos, A. P., 2011).

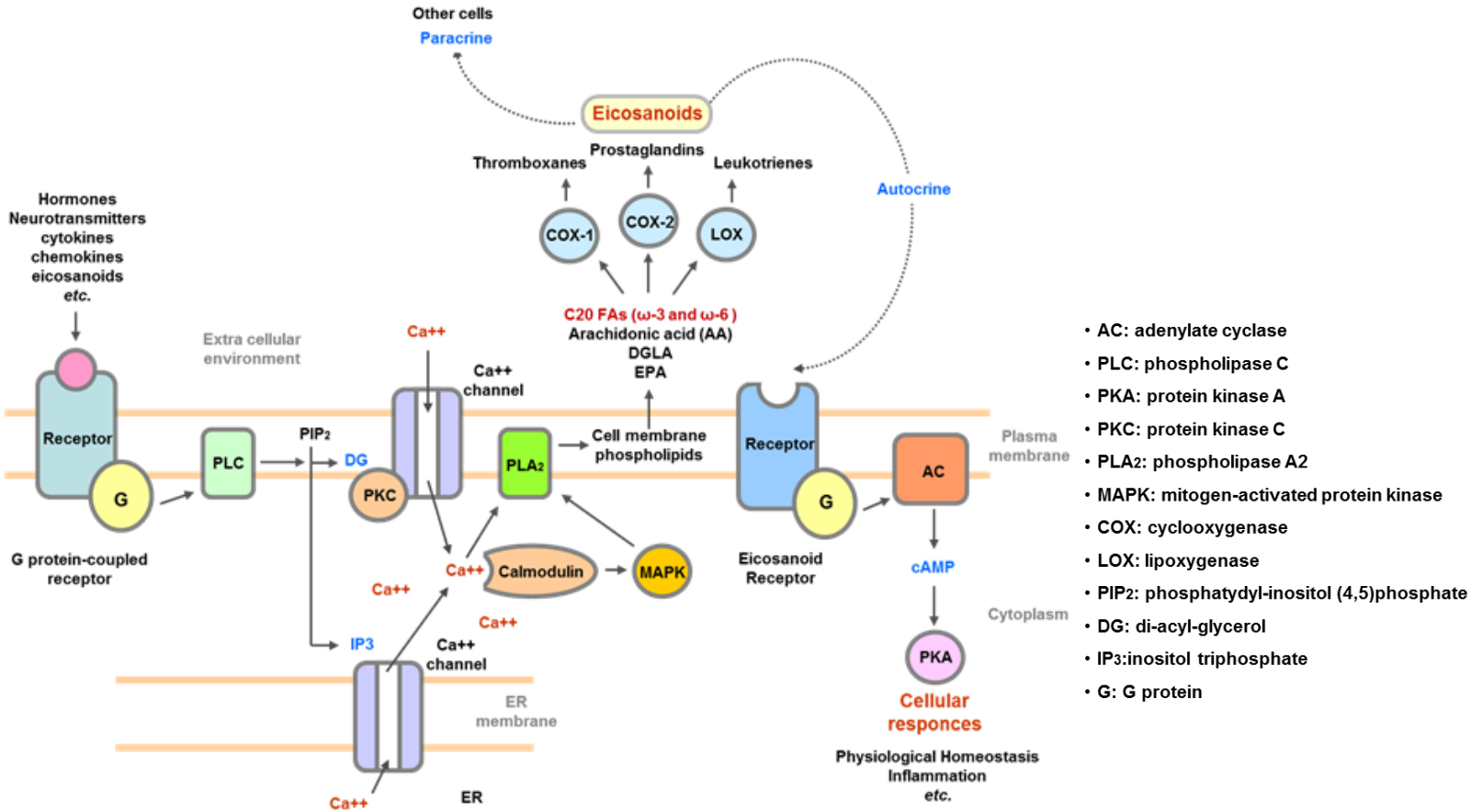


Figure 4. Eicosanoid synthesis
(from Prof. Sang-kee Kang, Seoul National University, Korea)

2. Imbalance of omega fatty acids

1) Imbalance in human

Simopoulos argued that n−6 to n−3 ratio of the modern western diet which is 15 to 16.7 is very imbalanced, in her review article (Simopoulos, A.P., 2002). Due to the intake of the current food imbalance in omega fatty acids, many people suffer from diseases like CVD, autoimmune disease, *etc*. Also, she emphasized three reasons of omega fatty acids imbalance in the modern diet; large−scale production of soybean and corn oils high in n−6 fatty acids, three major grains high in n−6 fatty acids (maize, rice, and wheat) occupying more than 90% of world's cereal production (FAOSTAT, 2013) and modern livestock industry based on productivity using grain feed.

(1) Current state of the modern diet regarding omega fatty acids

Since twenty years ago, the health beneficial effects of fatty acids, especially omega fatty acids have been reported. Kris−Etherton *et al.* surveyed the intake of PUFAs by sex, age and year, and the ratio of n−6 to n−3 fatty acids in the US food supply (Kris−Etherton, P. M. *et al.*, 2000). According to their paper, from 1989 to 1991, male and female aged twenty to forty−nine took 16 and 11 g/d of LA, and 2 and 1g/d of ALA, respectively. In the US food supply, total ratio of n−6 to n−3 was 10.6:1 at 1994. Similarly, Sugano and Hirahara surveyed fatty acids intake and PUFA proportion in the serum of

Japanese people (Sugano, M. and Hirahara, F., 2000). The intake ratio of n−6 to n−3 had stayed at around 4:1 from 1971 to 1995. Total n−6 to n−3 fatty acids proportions of Japanese people's serum were around 9, 6, 5, 4.5:1 and 6:1 in 20's, 30's, 40's, 50's and all ages, respectively.

Harika *et al.* reported a review paper about fatty acids intake of 40 countries (Harika, R. K. *et al.*, 2013). In this article, fifteen countries' intake ratios of LA and ALA were from 3.9 to 28.7:1. Hungary was the country having the highest raio, and Finland was the country having the lowest ratio. Additionally, Harika *et al.* highlighted the point that three countries, Mexico, Hungary and South Korea, did not meet the recommended ALA range (0.5 ~ 2% of energy intake).

Lastly, Simopoulos asserted that our ancestors' diet living with hunt and gather lifestyle was very well−balanced from every aspect comparing with the modern people's diet (Simopoulos, A.P., 2011). She suggested current intake ratios of n−6 to n−3 in various populations. In her paper, urban India had the highest ratio from 38 to 50:1, and the United Kingdom and the United States were 15:1 and 16.74:1, respectively. Japan had the lowest ratio, 4:1 except to Greece before 1960's (1 ~ 2:1) and Paleolithic people (0.79:1). Overall, the modern western diets are very imbalanced in omega fatty acids ratio. Far East Asian countries including Korea have relatively lower n−6 to n−3 ratio, but their ratios are not ideal too except Japan which has 4:1 ratio.

(2) The importance of omega fatty acids balance

As mentioned above, 20-carbon omega fatty acids of membraneous phospholipid may be metabolized into eicosanoids through oxidation. The eicosanoids from n-6 fatty acids are pro-inflammatory, and the other eicosanoids from n-3 fatty acids are anti-inflammatory. Therefore, well-balanced ratio of omega fatty acids is crucial for immune homeostasis and normal development. Here is some evidence for this theory.

Kang and his colleagues (2004) have studied on the balance of omega fatty acids. They cloned cDNA of *fat-1* gene encoding n-3 desaturase from *Caenorhabditis elegans*. After that, they supplemented the cDNA and n-6 fatty acids to cardiomyocytes of normal rat and human breast cancer cells. As a result, the cells converted n-6 fatty acids into n-3 fatty acids, and the ratio of n-6 to n-3 in the culture was almost 1:1 (Ge, Y. L. *et al.*, 2002; Kang, J. X. *et al.*, 2004). As another case, Cheng *et al.* (2015) made transgenic beef cattle using *fat-1* gene. The ratio of n-6 to n-3 of the transgenic calves was around 0.95:1 in contrast with that the ratio of n-6 to n-3 of the negative controls was 5.33:1 (Cheng, G. *et al.*, 2015).

PUFAs in phospholipid are controlled by the genes related to fatty acid metabolism, but these fatty acids, especially omega fatty acids, are obtained from the diet. Thus, it is important which kinds of and how much fatty acids we take. Ambring *et al.* reported that the Mediterranean diet balanced in omega fatty acids ratio reduces n-6 to n-3 ratios in serum phospholipid, platelets and leukocytes and vascular endothelial growth factor (VGEF)[①] in healthy people (Ambring, A. *et al.*, 2006). Maillard

[①] VGEF : the index of bronchial asthma, diabetes mellitus and CVDs

et al. presented the positive association between n−6 to n−3 ratio of adipose tissue and the risk of breast cancer with breast carcinoma patients in Tours, France (Maillard, V. *et al.*, 2002). The omega fatty acids ratio of adipose tissue can be used for the marker of past dietary intake of fatty acids. Above this, many papers have been reporting the benefits of the well-balanced dietary ratio of n−6 / n−3.

(3) The recommended ratio of omega fatty acids

As the importance of the ratio of omega fatty acids has become a significant interest, internationally authoritative institutes and scholars have suggested the ideal ratios of omega fatty acids.

In Sugano and Hirahara's paper, the recommended dietary n−6 to n−3 ratios in the world from late in 1980's to early in 1990's are well-organized (Sugano, M. and Hirahara, F., 2000). The USA and Canada's governments suggested 4 to 10:1 as an ideal ratio, and Japanese government and North Atlantic Treaty Organization recommended the ratio of 4:1. Food and Agricultural Organization (FAO)'s ratio was 5 to 10:1. However, Sugano and Hirahara pointed out that these ratios were not based on a firm theoretical basis. Kris-Etherton *et al.* stated 2.3:1 as the recommended rate in their paper (Kris-Etherton, P. M. *et al.*, 2000). They argued that it was difficult to achieve the recommended ratio with the dietary life of the day, and increasing n−3 fatty acids and decreasing n−6 fatty acids in the diet are the only way. Simopoulos is one of the well-known scholars in this field. She has argued that the ratio of n−6 / n−3 in the diet should be decreased at least

2:1 until recent exemplifying the case of Paleolithic people, transgenic animals, *etc.* (Simopoulos, A.P., 2011)

However, Trautwein had a different opinion. In her view, total ratio of n−6 / n−3 is not that much important because each n−3 and n−6 fatty acids have different physiological effects, and it is more important to establish the recommended allowance of each fatty acids (Trautwein, E. A., 2001). Actually, after late in 2000’s, international organizations have announced the recommend allowance of each fatty acids in priority rather than total n−6 / n−3 ratio (FAO and WHO, 1994; FAO, 2010; EFSA, 2010; FAO, 2013). Nevertheless, it is an evident fact that we should eat more n−3 fatty acids.

2) Imbalance in animals

To meet the need of omega fatty acid ratio in human, food materials, especially animal foods such as beef, pork, chicken, fish, *etc*. were also focused on the omega fatty acids ratio. Livestock industry use some grains high in n−6 fatty acids as main feeds. In consequent, n−6 fatty acids in animals and even fishes have been increased. It can be easily noticed from comparing between domestic animals and wild animals (Simopoulos, A.P., 2002).

Simopoulos showed the differences between the fatty acid composition of domestic animals and wild animals. In the case of eggs from pastured Greek chickens, the n−6 / n−3 ratio was 1.3:1. On the other hand, the n−6 / n−3 ratio of the standard US Department of Agriculture (USDA) egg was 19.9:1. Similarly, the n−6 to n−3 ratio was also different in the wild salmon and the cultured salmon. The wild one had the

n−3 / n−6 ratio of around 11:1 and the cultured one had the ratio of around 6:1 (Weber, P. C., 1989). The fatty acids composition of salmon from USDA data (Table 1) has a bigger difference between farmed and wild than Weber's data.

Table 1. n-3 to n-6 ratio of salmon

	Farmed (g/100g)	Wild (g/100g)
Total fat	13.4	63
Total n3	0.25	0.20
Total n−6	0.10	0.02
n−3/n−6	2.6	11.7

(The data from USDA National Nutrient Database for Standard Reference, Release 28. Version Current: September 2015)

Likewise, milk and cheese from grazing dairy cows have the lower n−6 / n−3 ratio than 1:1, and milk and cheese from dairy cows raised by USDA have the higher ratio than 1:1. Even EPA and DHA were not detected from USDA milk and cheese (Simopoulos, A.P., 1998). Daley *et al.* released a review article comparing fatty acids profiles between grass−fed and grain−fed beef (Daley, C. A. *et al.*, 2010). They put the results of seven research teams together. According to these results, the highest n−6 / n−3 ratio and the lowest ratio in grass−fed beef were 3.72:1 and 1.44:1, respectively. On the other hand, the maximum n−6 / n−3 ratio and the minimum ratio in grain−fed beef were 13.6:1 and 3:1, respectively.

(1) Studies on enhancing omega-3 fatty acids in cattle using high omega-3 feed additives

In the history of the USA domestic cattle industry, most of the cattle had been raised on the meadow until 1940's. Since 1950's, to improve productivity and marbling, the farmers have used grain feedstuffs. Recently, many farmers come back to the traditional way following well-being trend despite lower productivity (Daley, C. A. *et al.*, 2010).

However, the other farmers stick to grain feed system for efficiency, and many researchers have studied on increasing n-3 fatty acids in cattle without raising on grass. Raes *et al.* reviewed about enhancing n-3 fatty acids in cattle using feed additives (Raes, K. *et al.*, 2004). They are focused on fish oil and fish meal, and linseed. It is because fish oil and fish meal have abundant EPA and DHA, and linseed is very high in ALA and also have very low n-6 to n-3 fatty acids ratio of around 0.3:1. In the data of feeding fish oil or fish meal, n-3 fatty acids, ALA, EPA, and DHA, were increased, and n-6 fatty acids, LA and AA were decreased in longissimus muscles of cattle. Also, similar patterns of data came from linseed feeding trials, but the effect of increasing EPA and DHA was little bit weaker than fish stuff (Mandell, I. B. et al., 1997; Choi, N. J. *et al.*, 2000; Scollan, N. D. *et al.*, 2007, Raes, K. *et al.*, 2003).

Raes and her researchers (2004) emphasized that the diet has an effect on the fatty acid composition of animals. However, they also pointed out that the relationship between the dietary fatty acids and the fatty acid composition in beef is less evident than in pork because of bio-hydrogenation of ruminal microbes.

(2) The limitations of feed additives in cattle

As mentioned above, bio-hydrogenation is a major limitation of feed additives to enhancing n-3 fatty acids in cattle. When cattle eat esterified lipid, lipolysis and hydrogenation are occurred by ruminal microbes. After lipolysis, free fatty acids (FFAs) are released from lipid, and ruminal microbes hydrogenate unsaturated fatty acids among FFAs. Finally, the unsaturated fatty acids are changed into more saturated fatty acids (Jenkins, T. C., 1993). Due to bacterial bio-hydrogenation in the rumen, cattle have much more SFAs and MUFAs than PUFAs in comparison with monogastric animals although feed additives such as fish oil and linseed high in n-3 PUFAs are fed (Weill, P. *et al.*, 2002). Particularly, dietary ALA is hydrogenated on the level of around 80% (Doreau, M. and Ferlay, A., 1994).

Aside from bio-hydrogenation, there are some other limitations of feed additives to be used to improve n-3 fatty acids ratio in beef. Palatability is one of them. For example, perilla contains around 64% ALA of total fatty acids, however, unfortunately, it has the bad palatability. The last one is the price of feed additives. Fish oil and fish meal, and linseed are relatively expensive. Besides, in the case of linseed, the additional cost is needed to process linseed. The seed coat of linseed cannot be digested by animal's digestive system so that the seed should be crushed, extruded or expanded. Additionally, linseed has linamarin, an anti-nutritional compound, and it makes the usage of linseed as a feed additive hard (Raes, K. *et al.*, 2004).

3. SNP typing for genes related to fatty acid metabolism in cattle

1) Application of SNPs to improve n-3/n-6 balance in cattle

Genetic approaches are one of the options to overcome the previously mentioned limitations of feed additives if it is working. Furthermore, it may create a synergy effect with feed additives.

Cheng's paper mentioned in the previous chapter, is a good example of genetic approaches. He and his research staffs made transgenic cattle which have the well-balanced omega fatty acids ratio (Cheng, G. *et al.*, 2015). However, there are some serious problems. First of all, the success rate is very low. They used ninety-four synchronized recipients and obtained twenty transgenic calves by caesarean section. After 111 days from the birth, only three calves survived. The second problem is that it costs too much. Experts in various fields are needed to make transgenic animals. The last one is the ethical issue. Transgenic animals are related to not only animal welfare but also GMO issue. The animal lovers and people against GMO will not consume transgenic beef.

Genotyping is a practical alternative for making a transgenic animal. Traditional breeding has made tremendous advancement in the livestock industry. However, it takes too much time, and individual selection is hard in some traits such as fatty acid composition and quality grade which are not exposed. In the breeding of present day, the marker-based selection is utilized actively. The marker-based selection

consists of three steps. The first step is finding some genes or quantitative trait loci (QTL) related to a trait interested. One phenotype is expressed by the network of many genes and QTL. Thus, excavating underlying genes or QTL associated with the trait of interest is not easy. The second step is identifying DNA variants or polymorphisms in the gene or QTL. Lastly, association study between identified genetic variations and the trait of interest should be conducted. If there is a significant difference among each group by the variations, it can be used the variations as a genetic marker for selection. This kind of selection is called as marker-assisted selection (MAS). Up to now, more than 1123 QTL in over 101 traits have been identified in cattle. The majority of variations in these QTL are SNPs. Consequently, we can use SNPs as selection markers. Some SNPs are already used as selection markers. The position of SNPs brings out differences in gene expression. For example, SNPs in exon make differences in amino acid sequence, and SNPs in promoter region may affect the level of gene expression (Ibeagha-Awemu, E. M. *et al.*, 2008).

Figure 5 shows the schematic effects of SNPs according to the position. SNPs are divided into two groups, synonymous and nonsynonymous. Synonymous SNP does not affect amino acid sequence. Nonsynonymous SNP is divided to missense and nonsense. Missense SNP changes only one amino acid. However, nonsense SNP makes stop codon early so that the amino acid sequence affected from nonsense SNP is shorter than the original protein.

2) Studies on SNPs related to beef quality

There are many studies on SNPs for enhancing beef quality such as marbling score, fatty acids composition, *etc. FABP4* (Fatty acid-binding protein 4), *FASN* (Fatty acid synthase) and *SCD* (Stearoyl-CoA desaturase) are the representative genes related to meat quality. The SNP, rs41729173, in *FABP4* is a missense SNP and significantly associated with marbling scores and subcutaneous fat depth (Michael, J. J., *et al.*, 2006). Additionally, rs41729173 has effects on fatty acid composition, especially LA, AA and EPA (Dujkova, R., *et al.,* 2015). In the case of *FASN,* rs41919985 changes amino acid sequence from Threonine to Alanine. It results in variation of fatty acid composition in adipose fat and milk and the content of C20:3 n-6 (Li, C., *et al.*, 2012). rs41919985 is also significantly associated with marbling score (Oh, D. Y., *et al.*, 2012). Taniguchi *et al.* reported that rs41255693 in *SCD* affecting amino acid sequence (Valine to Alanine) have a significant effect on MUFA content and melting point in intramuscular fat (Taniguchi, M., *et al.*, 2004). In Li' s paper, rs41255693 also affects other fatty acids composition of beef as well as MUFA content and melting point (Li, C*., et al.*, 2012).

Ibeagha-Awemu and her research team found three SNPs (rs136261927, rs42187261, rs109772589) in *FADS* gene cluster have the significant association with three milk PUFAs, C20:3 n-6, C20:4 n-6 and C20:5 n-3. rs136261927 and rs42187261in *FADS1* are linked to C20:3 n-6 and C20:4 n-6, and C 20:5 n-3, respectively. rs109772589 in *FADS2* is also associated with C20:3 n-6 and C20:4 n-6 (Ibeagha-Awemu, E. M. *et al.*, 2014).

In this study, these six SNPs in five genes related to fatty acids metabolism in cattle were studied in Korean native cattle, Hanwoo in relation to omega fatty acid composition.

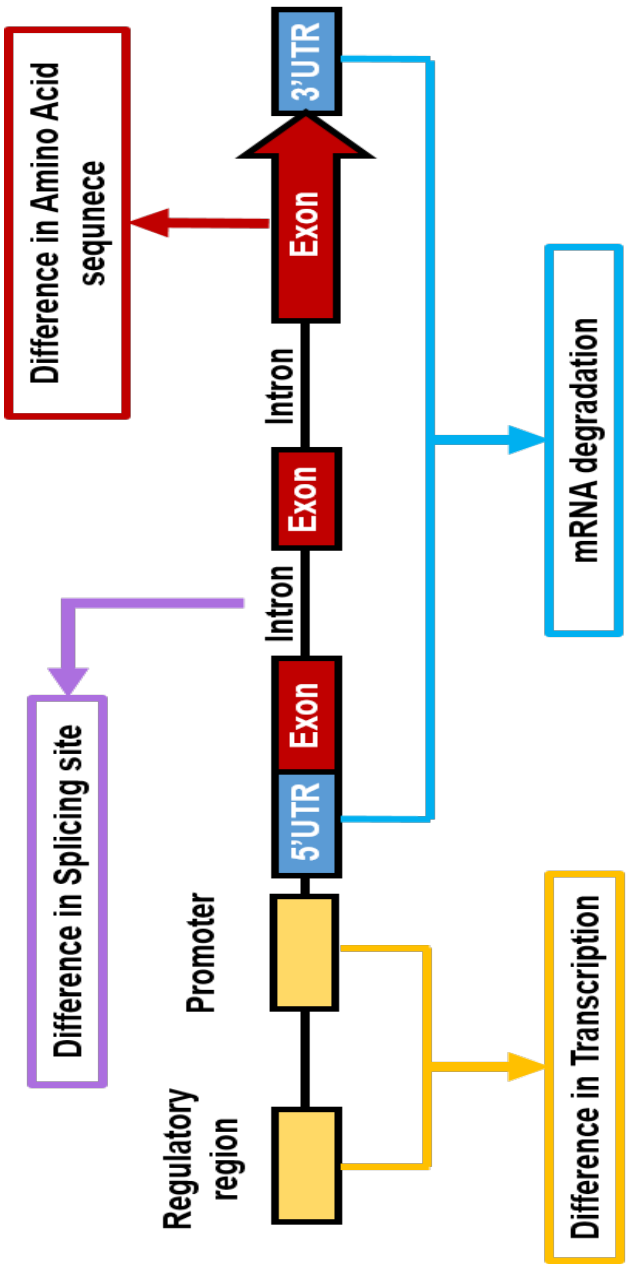


Figure 5. Different effects of SNPs according to positions

Ⅲ. Materials and Methods

1. Beef sample

1) Carcass grade of the samples

The beef samples of fifty-six Hanwoo steers were collected from October to November of 2015 at Pyeongchang province, Korea Federation of Livestock Cooperatives. The collected beef samples were from seven producers with three feeding programs. Table 2 shows detailed information about the producers and the feeding programs of the samples. Information on carcass grade and quality grade are also collected (Table 3). Carcass grade and quality grade determine the price of beef. Especially, quality grade and yield index are the primary factors for the price.

2) Sampling part for fatty acid analysis

Top round (TR) was used for fatty acid analysis because TR has the lower intramuscular fat and the higher proportion of phospholipid which has more PUFAs than neutral lipid (Buchanan, J. W. *et al.*, 2015). To analyze the association between fatty acid composition of beef and the genotypes of SNPs in the genes related to fatty acid metabolism, phospholipid fraction is better because the neutral fat composition is greatly affected by diet.

Table 2. Producers and feeding programs of the samples

Producers								Feeding programs		
n	AJH	AYW	HYR	KHD	LCS	LJW	YJG	P	NH	WS
56	8	5	3	16	2	13	9	43	11	2

(The names of producers and feeding programs were written in abbreviation.)

Table 3. Carcass grade and quality grade of the samples

Mean ± SD (n=56)					Quality grade (n=56)			
Age (day)	BFT (mm)	LMA (cm^2)	DW (kg)	Yield index	1++	1+	1+	2
925±26	14±4	95±9	458±46	63.92±3.07	11 (19.6%)	29 (51.8%)	15 (26.8%)	1 (1.8%)

(SD, standard deviation; BFT, backfat thickness; LMA, longissimus muscle area; DW, dressed weight)

2. SNP typing

The six SNPs were chosen in the five genes related to fatty acid metabolism from the references to observe the differences in omega fatty acids ratio by the genotypes; rs41919985 in *FASN*, rs41255693 in *SCD*, rs41729173 in *FABP4,* rs42187261 and rs136261927 in *FADS1* and rs109772589 in *FADS2*.

After genomic DNA (gDNA) extraction, SNP typing can be conducted using sequencing techniques such as Sanger sequencing or restriction fragment length polymorphism (RFLP). RFLP consists of three steps, Polymerase Chain Reaction (PCR), restriction enzyme digestion and gel electrophoresis. RFLP and Sanger sequencing (Macrogen, Korea) were used for five SNPs (rs41919985, rs41255693, rs41729173, rs136261927, and rs109772589) and for one SNP (rs42187261), respectively due to that there is no suitable restriction enzyme for the SNP.

1) Genomic DNA extraction

Ghatak's method was used for extracting gDNA from beef with minor modifications (Ghatak, S. *et al.*, 2013). 25mg of a beef sample was chopped and placed in a 1.5ml microtube with 300㎕ of tissue lysis buffer(10mM Tris-HCl, 10mM EDTA, 100mM NaCl, 2% SDS, pH 7.5.). 5㎕ of 20mg/ml proteinase K (Biosesang, Korea) and 20mg/ml RNase A (Thermo Fisher, USA) were added to the tube, and the tube was incubated at 56℃ for around 3 hours until the beef sample was melted

entirely with occasional gentle inverting. After the incubation, 300㎕ of 7M ammonium acetate (Biosesang, Korea) was added to the tube, and the tube was inverted several times smoothly. The tube was centrifuged at 13,000rpm and 20℃ for 15 minutes, and then, 600㎕ of the supernatant was transferred into a new 1.5ml microtube. 600㎕ of 2-propanl (Merck, Germany) was added into the tube, and the tube was chilled at -20℃ for 1 hour. The tube was centrifuged at 13,000rpm and 4℃ for 10 minutes. The supernatant was discarded, and 250㎕ of 70% ethanol (Merck, Germany) was added and followed by centrifugation at 13,000 rpm and 4℃ for 5 minutes. After the centrifugation, ethanol was removed, and the pellet was dried entirely at room temperature. At last, 50㎕ of TE buffer was added to dissolve genomic DNA, and the tube was frozen at -20℃ for storage.

2) RFLP

RFLP analysis was conducted on the five SNPs. Table 4 shows the information of six SNPs from National Center for Biotechnology Information (NCBI) and the effects of the SNPs on omega fatty acids ratio from the references.

(1) Primer design

Primer design is the first step of PCR. Primers were designed using NCBI primer BLAST. Table 5 is the information of the primers. The primer pairs were synthesized (Bioneer, Korea) and dissolved in TE buffer at 10pmol/㎕.

(2) Temperature gradient PCR

Temperature gradient PCR was used to optimize melting temperature (Tm) of the primer pairs. The range of temperature was followed the suggested temperature from NCBI primer BALST. Hot start PCR premix with dye (Biofact, Korea) was used for PCR and followed the manual of the manufacturer's. Figure 6 is a diagram of PCR. PCR was carried out for fifty-six samples with five primer pairs using proper Tms from the results of temperature gradient PCR as figure 6.

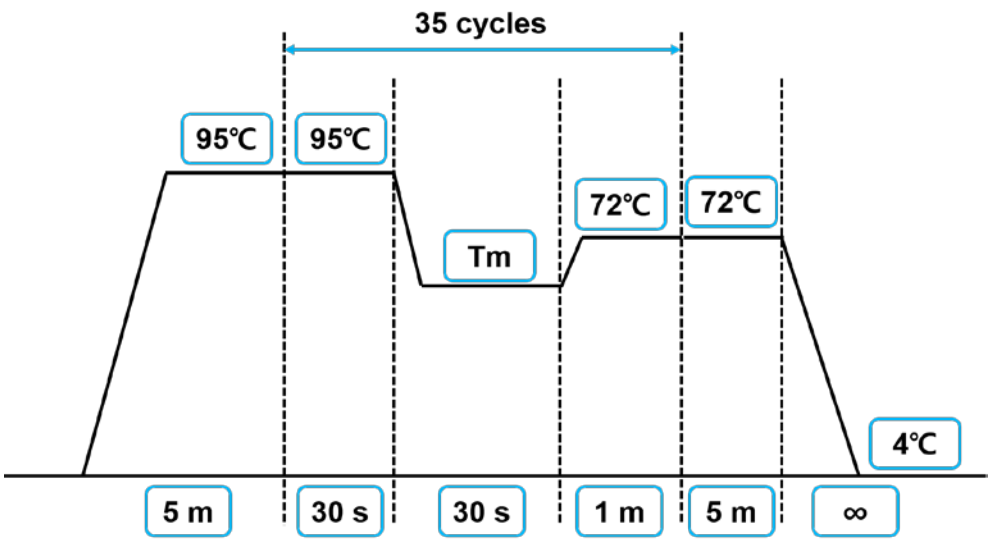


Figure 6. The diagram of PCR
(m, minute; s, second; Tm, melting temperature)

Table 4. The information of the SNPs

rs No. (gene, NCBI ID.)	Position (Chr No.)	Allele	Function	The effects on omega fatty acids ratio
rs41919985 (*FASN*, 281152)	51,402,032 (19, Exon 36)	A / G	missense (Ala - Thr)	AA - the lower concentration of beef C20:3 n-6 ($p < 0.05$) (Li et al., Animal Genetics, 2011).
rs41255693 (*SCD,* 280924)	21,263,681 (26, Exon 3)	C / T	missense (Ala - Val)	CC - the lower concentration of beef 20:2n-6 ($p < 0.05$) (Li et al., Animal Genetics, 2011).
rs41729173 (*FABP4,* 281759)	46,832,359 (14, 3' UTR)	C / G	3' UTR variant	GG - the higher concentration of beef C18:2 n-6; CC - the higher concentration of beef C20:4 n-6, C20:5 n-3 and C22:6 n-3 (Dujkoba et al., ACTA VET. BRNO., 2015).
rs42187261 (*FADS1*, 533107)	40,942,263 (29, Exon 7)	A / G	synonymous (Tyr-Tyr)	AA - the highest increase in milk C20:5 n-3 ($p<0.05$) (Ibeagha-Awemu et al., BMC Genetics., 2014).
rs136261927 (*FADS1*, 533107)	40,949,430 (29, Intron)	A / G	intron variant	GG - the highest increase in milk C20:3 n-6 ($p<0.05$) (Ibeagha-Awemu et al., BMC Genetics., 2014).
rs109772589 (*FADS2*, 521822)	41,082,035 (29, 3' UTR)	A / G	3' UTR variant	GG - the highest increase in milk C20:3 n-6 and C20:4 n-6 ($p<0.05$) (Ibeagha-Awemu et al., BMC Genetics., 2014).

(From NCBI; rs No., reference SNP ID; Chr No., chromosome number; aa. amino acid; Ala, alanine; Thr, threonine; Val, valine; Tyr, tyrosine; 3'UTR, 3' untranslated region)

Table 5. The information of the primers

rs No. (gene)	Forward primer(bp)	Reverse primer(bp)	Tm(℃)	AL(bp)
rs41919985 (*FASN*)	5’ -CTTCACAGAGCT GACGGACT-3’ (20)	5’ -CTTAGCCCTCG TCTGCAGG-3’ (19)	65	311
rs41255693 (*SCD*)	5’ -ACCTGGTGTCCTGTT GTTGTGCTTC-3’ (25)	5’ -GATGACCCACTCT TCTATTTACGC-3’ (24)	54.5	568
rs41729173 (*FABP4*)	5'-ATATAGTCCATAGG GTGGCAAAGA-3' (24)	5'-AACCTCTCTTTGAA TTCTCCATTCT-3' (25)	61	452
rs42187261 (*FADS1*)	5'-CTGGCACCTGT GAAGGAAAT-3' (21)	5'-CTGTGTAACCC ACACAAACC-3' (21)	60	523
rs136261927 (*FADS1*)	5'-AGCGGGAGAAAT GGAAGGTG-3' (20)	5'-TCATGAAGGCCA ACCCTGTC-3' (20)	61	311
rs109772589 (*FADS2*)	5'-ATGGGACAAAGA GGGGTTGG-3' (20)	5'-GCGCTGAACAAG GAAGTGAGA-3' (21)	61	380

(rs No., reference SNP ID; Tm, melting temperature; AL, amplicon length)

(3) Restriction enzyme digestion

After PCR, restriction enzyme digestion was conducted to type SNP. In this study, four restriction enzymes were used (New England Biolabs, U.K.; Enzynomics, Korea), and proper enzymes for each SNP were searched by using NEB cutter V2.0 program (New England Biolabs, http://nc2.neb.com/NEBcutter 2). Restriction enzyme digestion was carried out following the manufacturer's condition. Restriction enzymes have a specific recognition sequence, and an SNP should be in the sequence to conduct RFLP. For example, if the SNP changes a single nucleotide in the recognition sequence, the restriction enzyme will not cut PCR products. PCR products may or may be not digested depending on SNP type. Figure 7 shows the position of each SNPs and the recognition sequences of each restriction enzymes in the sequences of PCR products. Consequently, band patterns of each samples was checked using agarose gel electrophoresis. In Table 6, there is the information of restriction enzyme and restriction fragments by the SNPs.

(4) Gel electrophoresis

Genomic DNA and the products of restriction enzyme digestion were observed on an agarose gel. SEKEM LE agarose (Lonza, Switzerland), 0.5×TBE buffer (Biosesang, Korea) and Eco staining solution (Biofact, Korea) were used for making agarose gel. Genomic DNAs were checked on 1% agarose gel with 0.5×TBE buffer. 1kbp size marker (T&I, Korea) was usded and 5㎕ of gDNA was loaded with 1㎕ of 6×loading dye (Biofact, Korea). Electrophoresis was conducted at 100V for

40 minutes.

5㎕ of restriction enzyme digestion products and 100bp size marker (Biofact, Korea) were loaded on 2% agarose gel with 0.5×TBE buffer, and the products are separated for 25 minutes at 100V. After electrophoresis, we observed the agarose gel under UV (260nm) and analyzed the images using Chemi-Doc (Bio-Rad, USA). In the case of restriction enzyme products, each sample had different patterns of restriction fragments by the SNP types, and each sample was genotyped comparing the bands and size marker in the pictures.

Figure 7. The position of SNPs and the recognition sequence of restriction enzyme in PCR product (5' and 3' primer sequences are underlined)

(c) rs41729173 (*FABP4*), MspA1I

5'AACCTCTCTTTGAATTCTCCATTCTTA
AGTAATTTCAAAGTGAGAGTTTAACTC
TCTGCTTTTGTAAAGTAAATTATTATGA
TCAGAAACTTAATTTACCTCTTCTGGCT
TCTCTGTATCTTGGTGTTATGCATATAG
TGTGTGTGTGTGTGTGTCTGTGTGTGT
CTGTGTGTGCTAAGTCGCTTCAGTTGT
GTCTGACTCTTTGTGATCCTATGGACT
ATAGGCCACCAGGCTCCTCTGTCCATG
GGATTCTCCAGGCAAGGATACTGGAG
TGAGTTGCCATGCCCTCCTCCAGGGG
ATCTTCCTGACCCAGGGATCAAACCTG
CATCTCTTATGTCTCCCACATTGG
(CAG ↓↑ CGG)
GGTTTTTCATCGCTAACGCCACCTATG
CATATGGTGCATGTGTGCATGCTAAGC
TGCTTCAGTTGTTTCGAACTCTTTGCCA
CCCTATGGACTATAT3'

(d) rs136261927 (*FADS1*), Aci I

5' TCATGAAGGCCAACCCTG
TCTTCTTCCTGCTGTACCTG
CTGCACATCCTGCTGCTGGA
CGTCGCTGCCTGGCTCACTC
TCTGGCTCTTTGGGACATCC
TTGGTGCCCTTCCTCCTGTG
TTCCGTGCTGCTCAGTATAG
TTCAGGTGAGAGCCCTGCCT
CACCCAGCATCCATAGGGT
AAGTTCTTTGTACCCCTTAG
GAGGCCACTGAGGACACA
(G ↓ CG ↑ G)
GAGAGTTGGTACAGGAAGA
GAACGCCTGTTCCATTGTCT
GTGGCTTCTCTCTGCTCATC
TTCACCACACATGCACCTTC
CATTTCTCCCGCT3'

(e) rs109772589 (*FADS2*), Fau I

5'ATGGGACAAAGAGGGGTTGGGGC
CGAAGGCCAGACAGCTGAGGGGAG
AGGAAAGTGAGAGGGTGGGGAACC
TGGCGGCCACTGGGAGTATCCAGA
GCTGCTGCATCTGGAAATTAACCCA
CTAACCCGTAGATAGCTCCAGGGG
AGG
(GCGGG)
GCAG ↓ GA ↑ GGACCAGCTCTGAC
TGGTGATGGACCTGAGGGTTGCCAT
GGCAACAAGGGATCAGACTGCCTG
AAGCCCTCCCACTGAGAACTGGCC
GCCCAAAGAGCCACTGTCCCTCCCC
TGAGGCAGACTGGATCCATAGGGA
ACTGACCTTAATTTTTATCATGTTGC
TTCCCCACCTCTACACTTTTGGAAAT
AAATGATTTTATTCTCACTTCCTTGT
TCAGCGC3'

Figure 7. The position of SNPs and the recognition sequence of restriction enzyme in PCR product
(5' and 3' primer sequences are underlined, continued)

Table 6. The information of the restriction enzymes and the restriction fragments

rs No. (gene)	RE(℃)	Restriction fragments(bp)		
rs41919985 (*FASN*)	Msc I (37)	AA (196/44)	AG (311/196/114)	GG (311)
rs41255693 (*SCD*)	Aci I (37)	CC (348/163/58)	CT (406/348/163/58)	TT (406/163)
rs41729173 (*FABP4*)	MspA1I (37)	GG (352/100)	GC (452/352/100)	CC (452)
rs136261927 (*FADS1*)	Aci I (37)	GG (216/95)	GA (311/216/95)	AA (311)
rs109772589 (*FADS2*)	Fau I (55)	GG (224/156)	GA (380/224/156)	AA (380)

(rs No., reference SNP ID; RE, restriction enzyme; bp, base pair)

3. Fatty acid analysis

Fatty acid analysis consists of two steps, lipid extraction and gas chromatography (GC) analysis. The preparation step for GC has three steps. First, total lipid is extracted from beef samples. Second, total lipid is separated into polar lipid (especially phospholipid) and neutral lipid (mainly triglyceride) by thin-layer chromatography. At last, separated lipid is methylated. GC analysis is the most popular and efficient tool for fatty acid analysis. Figure 8 is a diagram representing whole steps for fatty acid analysis.

1) Preparation methods for fatty acid analysis

(1) Total lipid extraction

Folch method (Folch, J. *et a*l., 1957) was used for extracting total lipid. This process was established six decades ago, but still it is a standard method for lipid extraction. Folch method was used with minor modifications following other reports to meet our circumstances (Toschi, T. G., *et al.*, 2003; Tanamati, A., *et al.*, 2005). Folch method used for total lipid extraction method is as follow.

The frozen meat sample at −80℃ was crushed using a hammer. During this step, the sample was re-frozen by soaking in liquid nitrogen and repeated several times until the samples were broken entirely. After that, 1g of the sample was put into a glass tube with 20ml of Folch solvent, a mixture of chloroform/methanol (2:1. v/v) and 150㎕ of 10% butylated hydroxyanisole (BHA). The sample, then, was incubated at

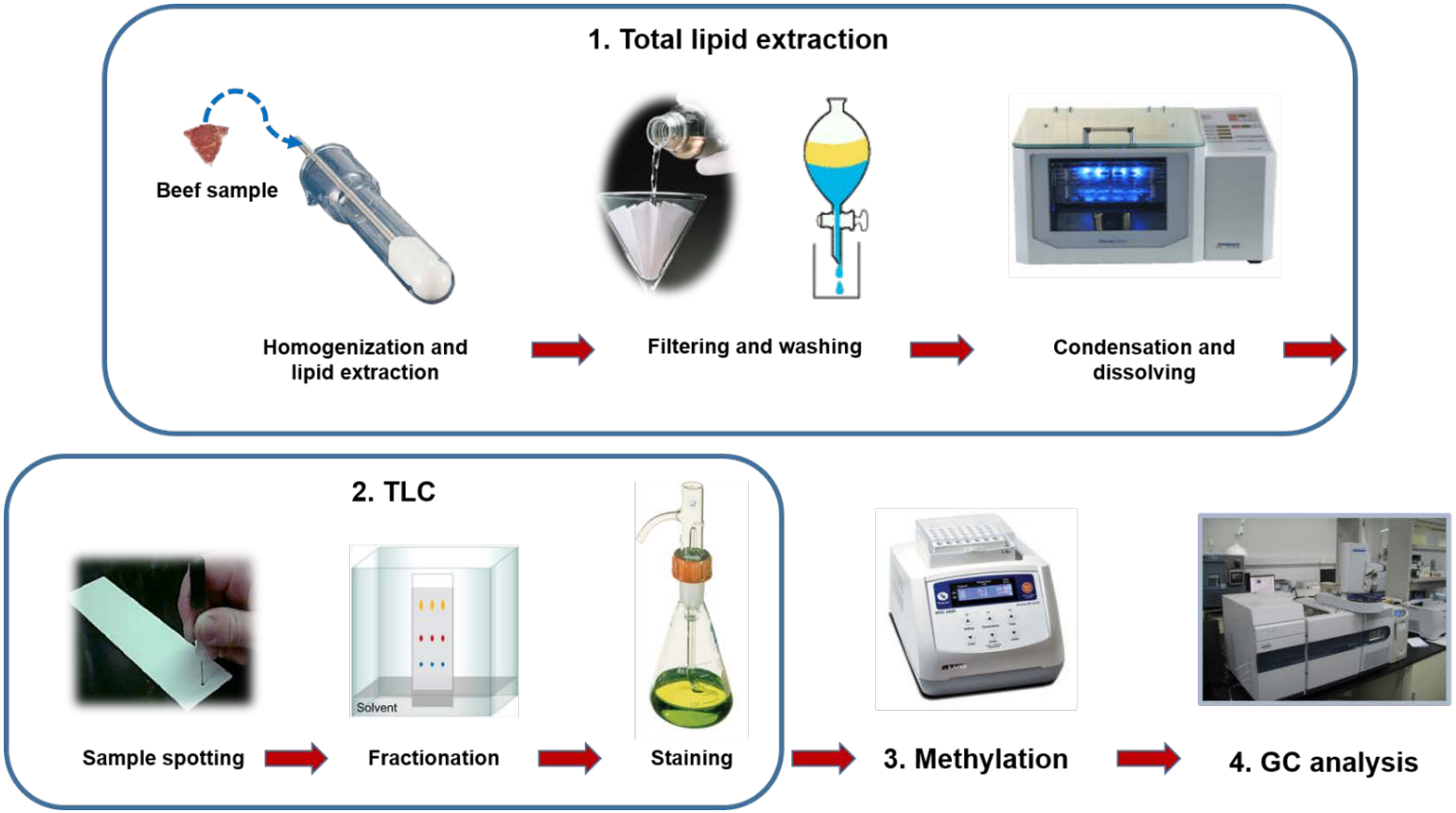


Figure 8. The diagram of fatty acid analysis

room temperature for 30 minutes with rotating at 50rpm, and filtered through filter paper (Whatman, USA) into a new glass tube with 4ml of 0.9% NaCl solution. The solution was incubated again in the same way. After the second incubation, the solution was centrifuged at 3,000 rpm for 10 minutes. The upper phase of the solution was removed by aspiration. This washing step was repeated several times. After washing step, anhydrous sodium sulfate was added one or two times with micro-spatula and stirred enough until there were no clumps. The solution was filtered again in the same way, and the filtered solution was condensed under nitrogen gas (99.99%) at 40℃ and 20 psi for one hour. The condensate was dissolved in Folch solvent, and stored at -20℃.

(2) Thin-layer chromatography

Thin-layer chromatography (TLC) is the most efficient technique for separation of complex lipid mixture. PUFAs of interest are mainly in phospholipid and under control of genes related to fatty acid metabolism. Therefore, TLC should be done for an analysis of fatty acid composition in phospholipid. Fatty acid analysis of phospholipid will tell us the relationship between fatty acid composition and genotypes accurately without masking from the effect of neutral lipid which in turn reflects the diet lipid composition.

The first step of TLC is preparing a TLC plate (Merck, Germany). A new plate was put in a glass tank containing chloroform/methanol 1:1, v/v, and the tank was covered with a glass lid until the plate was wetted entirely. After that, the plate was taken out and dried under the fume hood. The second step

is applying the sample on the spot of 2.5cm from the bottom, and then the plate was put in a glass tank containing diethyl ether/methanol /acetic acid 90:1:1, v/v. The tank was covered until the solvent reached under 10cm from the top of the plate, the plate was dried under the fume hood, and primuline (Sigma-Aldrich, Germany) solution was sprayed with a glass atomizer. Primuline solution was made up of 100mg of primuline in 200ml of acetone/water mixture (4:1, v/v). The plate was dried in an oven at 55℃ for two minutes. Under UV light (340nm), separated lipids were observed. Phospholipid stays on the spot, and neutral lipid is moved with the solvent line. The last step is scraping off the lipid fraction from the plate and eluting the lipid. Phospholipid was recovered by 5ml of chloroform/methanol/water (5/5/1, v/v) and 5ml of water mixture. After brief vortexing and centrifugation, polar lipids are in the lower chloroform phase (www.cyberlipid.org). The chloroform phase was transferred to a new glass tube, dried entirely under 20psi nitrogen gas at 40℃, and dissolved into 2ml of Folch solvent.

Phosphatidylcholine from egg yolk (>60%) and mono / di / triglyceride mix (Sigma Aldrich, Germany) were used to validate the position of polar lipid and neutral lipid on silica plate. Phosphatidylcholine is a main component of cell membrane and a type of phospholipid. Two kinds of standard lipid were dissolved in Folch solvent at 1% of concentration.

(3) Methylation

For gas chromatography, fatty acid should be methylated. Methylation makes acidic ends from lipid and makes fatty acids more hydrophobic. GC columns for fatty acid analysis are

usually hydrophobic. Methylated fatty acids are called as fatty acid methyl esters (FAMEs). FAMEs are stuck to the inside wall of GC column due to hydrophobic effect, and then, come away from the wall following gas flow by their weight.

Direct methylation method (O'Fallon, J. V. et al., 2007) was used for methylation. This process is very simple and can be applied to various sample types easily. First, 1g of a sample was put into a glass tube with 5.3ml of methanol and 700㎕ of 10N KOH, incubated in a water bath at 55℃ for an hour and a half, and briefly vortexed every 20 minutes. After incubation, the tube was chilled, and 580㎕ of 24N H_2SO_4 was added. The tube was incubated and chilled as before. Lastly, 3ml of hexane was added to the tube, and the tube was vortexed for 5 minutes and centrifuged at 3,000 rpm and 20℃ for 5 minutes. After that, upper phase (hexane) was transferred to a new GC-vial (Agilent, USA).

2) Validation and optimization of the methods

Lipid extraction efficiency was not good using above method. Thus, method was optimized for the lipid extraction and TLC separation of phospholipid and neutral lipid. During optimization, top round beef produced in Korea not included in the fifty-six samples was used as samples. Phosphatidylcholine from egg yolk and mono / di / triglyceride mix was also used in validation of TLC step.

(1) Validation and optimization of lipid extraction

Lipid recovery efficiency of Folch method was compared with direct methylation method. In this procedure, two GC data which were obtained from directly methylated lipids from 1g of beef without extraction step, and lipids extracted and methylated from same 1g of beef were compared. Comparing two data, some steps of Folch method were modified (Figure 9). The washing step of total lipids was carried out once, and the step of anhydrous sodium sulfate was omitted. After lipid extraction, lipids were dissolved in 5.3ml methanol. The sample grinding also modified: homogenization by a homogenizer (IKA, Germany) or crushing with liquid nitrogen. After extraction, all lipids were directly methylated using O'Fallon's method.

After this optimization, other factors were tested. Effects of increasing extraction time (30min / 1h / 2h / 4h) and the amount of sample (1g / 2g / 3g), dissolving 1ml of hexane after methylation and excluding washing step in Folch method were tested.

(2) Optimization of TLC and methylation

To increase the phospholipids concentration after TLC separation and methylation step, a few steps were optimizaed. First, the amount of the sample applied on TLC plate was increased from 10㎕ to 20㎕, and decreased the volume of Folch solvent in dissolving step from 2ml to 500㎕, total 8-fold increase of lipid concentration. Besides, lipid elution step from silica powder was omitted. Second, direct methylation method was scaled down to 1/10 except hexane. 500㎕ of hexane was

used for dissolving fatty acid methyl esters in a 2ml glass tube. We call this downscaled direct methylation as small scale direct methylation. Third, the amount of sample applied on TLC plate was increased to 80㎕ and extracted total lipid was dissolved in 250㎕ of chloroform (Figure 10 for details).

After this optimization of the process, recovery rate of TLC step was tested. Two GC from directly methylated 80㎕ of 1% phosphatidylcholine in a state of the solution and after TLC running and scraping were compared. Also, directly methylated empty TLC silica was analyzed in GC-FID to confirm background noise of silica.

3) The established preparation method

For GC analysis of Hanwoo beef phospholipids, the established preparation method was used. Total seven steps in the overall preparation method were modified. In homogenization step of Folch method, homogenizer was used for grinding 2g of beef sample instead of crushing 1g of beef sample with liquid nitrogen. Washing step was conducted only one time, and anhydrous sodium sulfate was omitted. After condensation, extracted total lipid was dissolved with 250㎕ of chloroform, and 80㎕ of lipid sample was spotted to TLC plate. Phospholipid fraction from the TLC plate was scraped and directly methylated without elution step using small scale direct methylation. Figure 11 is a simplification of the established preparation method in this research.

4) GC-FID

Gas chromatography-flame ionization detector (GC-FID, Agilent 7890B, USA) was used for fatty acid analysis. The column was SP-2560 (ID 0.25mm * length 100m; Sigma-Aldrich, Germany), and FAME 37 (Sigma-Aldrich, Germany) was used as a reference fatty acid for peak identification and quantification. The running condition of GC-FID was followed the FAME 37 manual (oven temperature: 140℃ for 5 min; Ramp: 240℃ at 4℃/min and hold for 28 min; injector and detector temperature: 260℃; split ratio: 1:30; injection volume: 1$\mu\ell$).

After GC-FID, retention time (RT) and area value of each fatty acid peak, and peak pattern of the sample were obtained. Each peak was identified comparing with FAME 37 peak pattern. These data were processed for association analysis between fatty acid composition and the genotype.

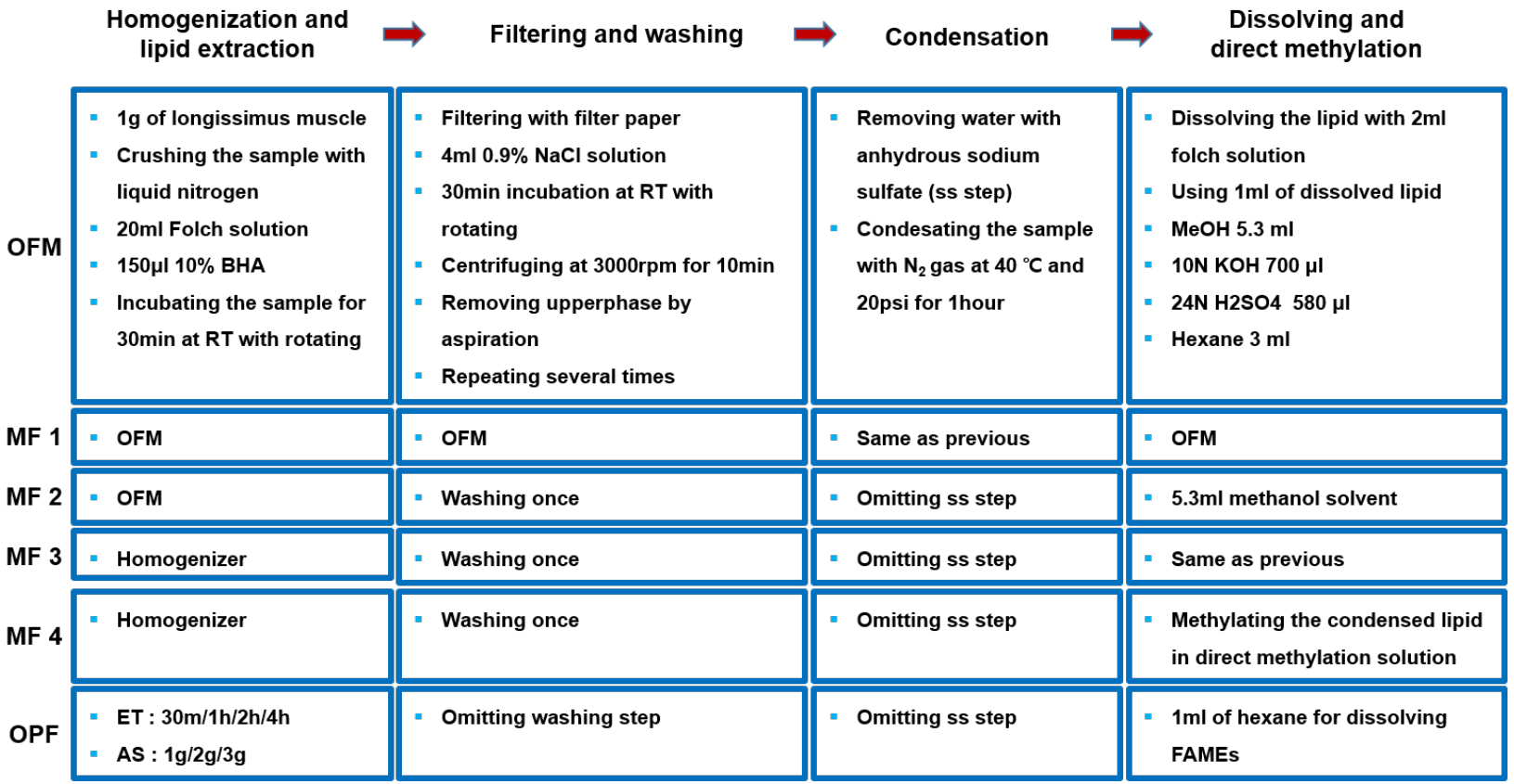


Figure 9. The simplification of modified Folch method

(OFM, original Folch method; MF, Modified Folch method; OPF, optimization of Folch method; ET, extraction time; AS, amount of sample; m, minuet; h, hour)

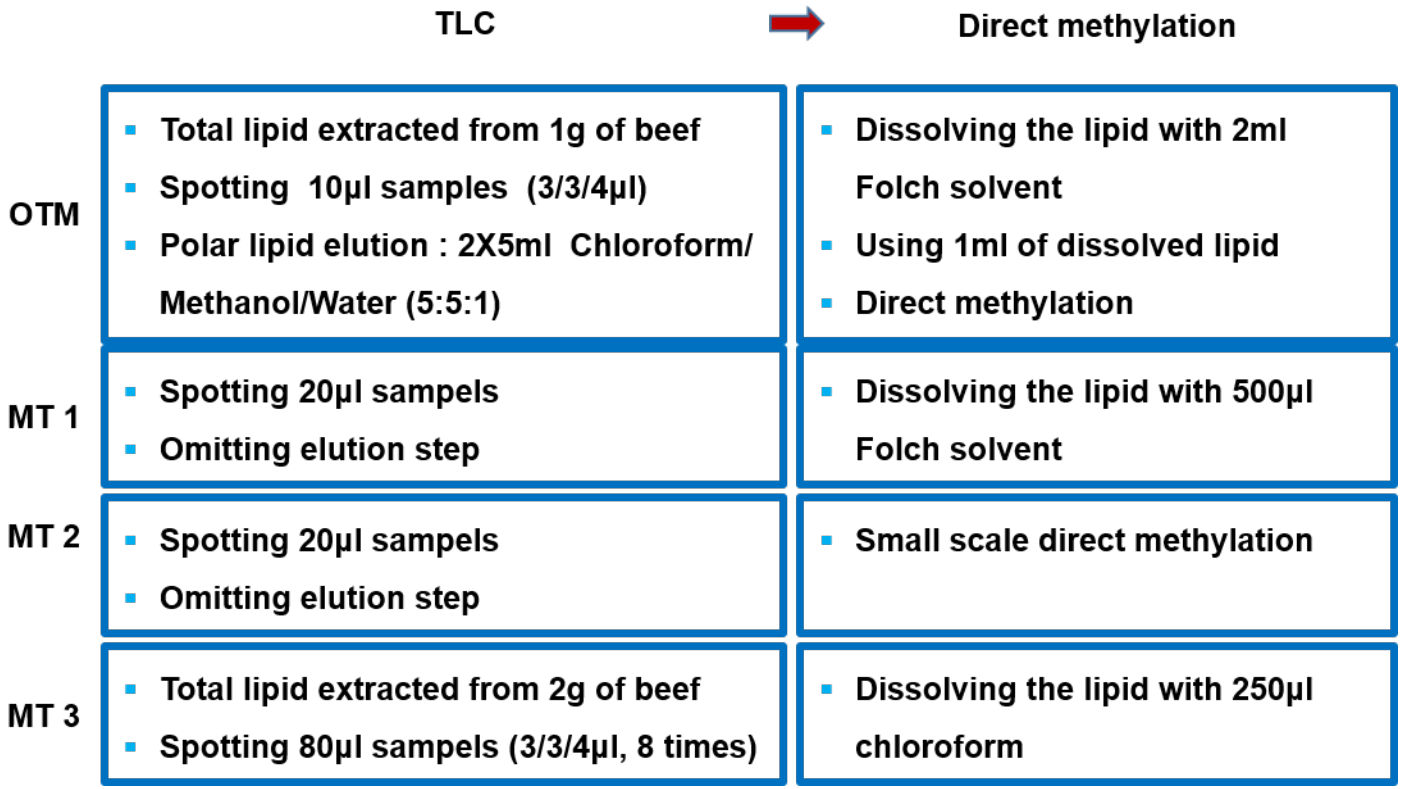


Figure 10. The simplification of modified TLC
(OTM, original TLC method; MT, modified TLC method)

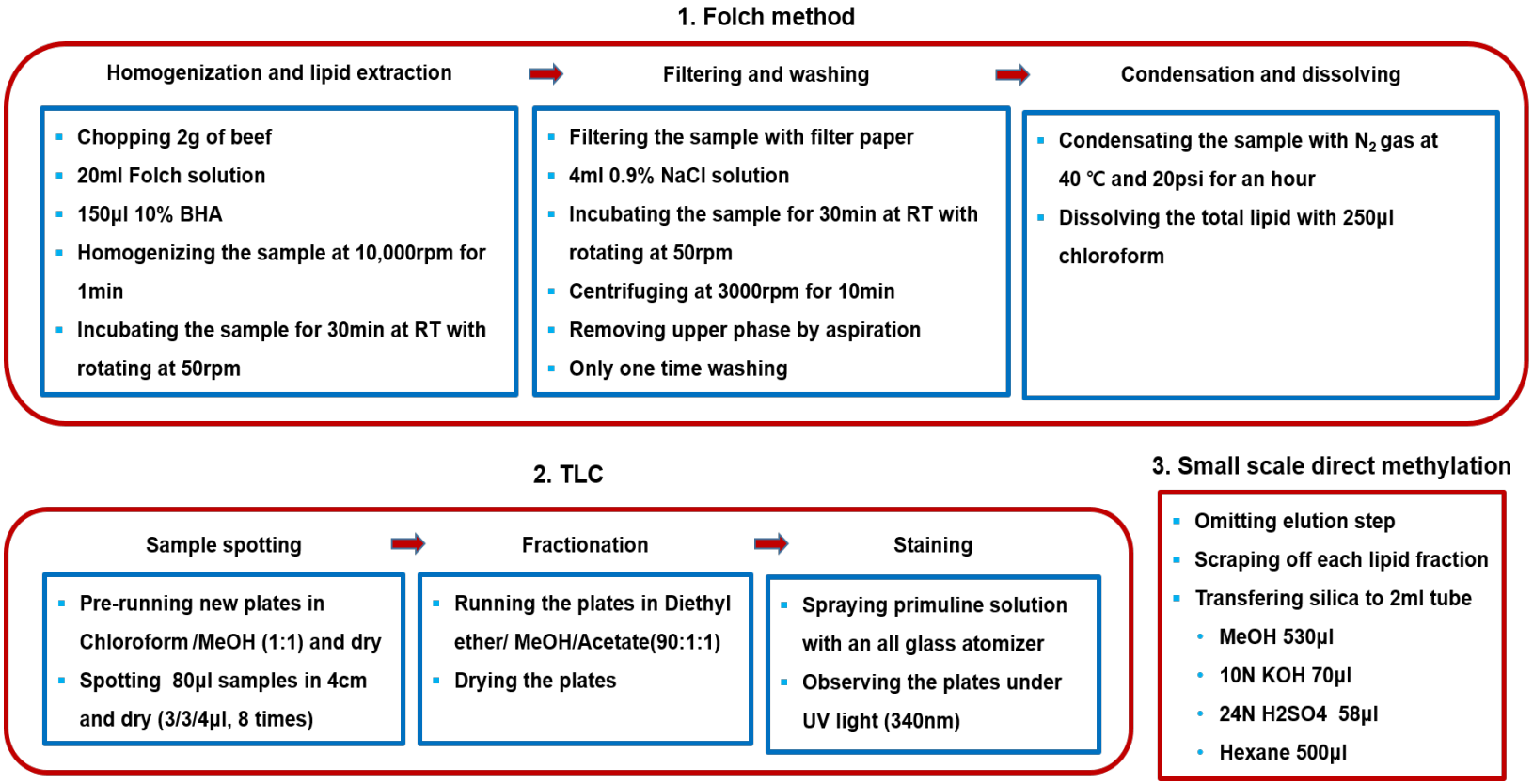


Figure 11. The established preparation method

4. Statistical analysis

To confirm the association between omega fatty acid composition and the genotypes of the fifty-six samples, the statistical program, SPSS v. 22 (IBM, USA) was used for statistical analysis, one-way analysis of variance (ANOVA) (Mannen, H., 2011) and analysis of covariance (ANCOVA).

1) ANOVA

ANOVA is a useful tool to analyze mean differences of a dependent variable among or between groups in an independent variable. One-way ANOVA can be applied to analyze when the dependent variable is continuous, and the independent variable is categorical and has two groups or more. In other words, the association can be analyzed between three genotypes of each SNPs and ratios of each omega fatty acids in fifty-six beef samples using ANOVA. If p-value of ANOVA is less than 0.05, there is a significant difference among each group.

2) ANCOVA

ANCOVA is similar with ANOVA at the point of analyzing mean differences of a continuous dependent variable among or between groups in a categorical independent variable, but more independent variables can be added and other continuous variables out of interest as known as covariates can be controlled. In this study, the age of each sample was set up as

the covariate, and the differences in omega fatty acids ratios by the SNPs, the producers and the feeding programs were observed. Along with ANOVA, a significant probability of ANCOVA is 0.05.

Ⅳ. Results and Discussion

1. Carcass grade of Hanwoo steers used in this study

Table 7 shows a carcass grade-comparison of Hanwoo steers in this study (56 steers produced from Pyeongchang area in Korea) and the average values of steer beef grade in Korea from October to November of 2015 (www.ekapepia.com). Apperance rate of 1++ and 1+ quality grade of Hanwoo steers from Pyeongchang area is over 71%, and it is much higher than that of nationwide (57.6%). Besides, there is no grade 3 in Pyeongchang. In the case of yield grade, nationwide data is better than that from Pyeongchang.

Table 7. Carcass grade comparison between steers in this study and average values in Korea during same period

Carcass grade		Korea (n=61,003)	Pyeongchang (n=56)
Quality grade	1++	10,376 (17.0%)	11 (19.6%)
	1+	24,764 (40.6%)	29 (51.8%)
	1+	17,052 (28.0%)	15 (26.8%)
	2	8,254 (13.5%)	1 (1.8%)
	3	511 (0.8%)	-
Yield grade	A	12,766 (21.0%)	9 (16.1%)
	B	28,944 (47.4%)	24 (42.9%)
	C	19,247 (31.6%)	23 (41.1%)
Off grade		46 (0.08%)	-

2. SNP typing of Hanwoo beef

1) gDNA extraction from the beef samples

Genomic DNA was extracted from top round part of the fifty-six Hanwoo beefs. After extraction, eight random samples of gDNA were tested for the quantity and quality using Chemi-Doc (Bio-Rad, U.S.) and Nanodrop (Thermo Fisher, USA). Figure 12 and Table 8 show the conditions of the representative gDNA tested.

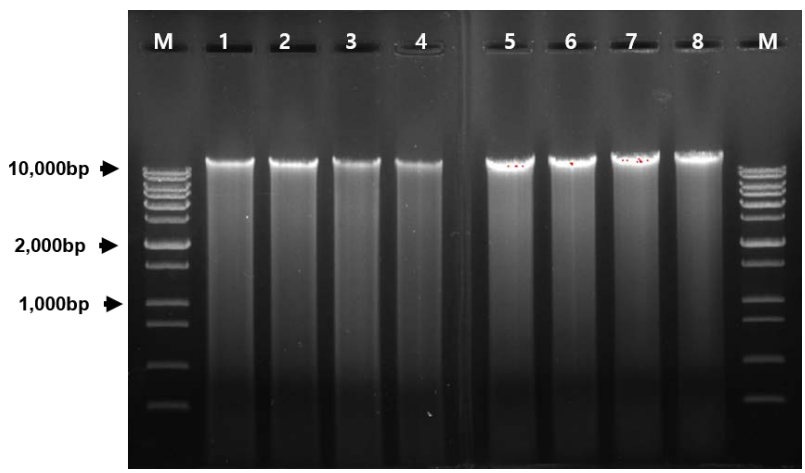


Figure 12. The electrophoresis pattern of the representative gDNA samples

Table 8. Concentration and purity of the representative gDNA samples

	1	2	3	4	5	6	7	8
NA (ng /㎕)	1303	444	592	543	439	457	286	677
260/280	1.8	1.79	1.88	1.85	1.87	1.88	1.88	1.85
260/230	1.64	1.3	2.14	1.94	2.02	2.05	1.99	2.03

(NA, nucleic acid concentration)

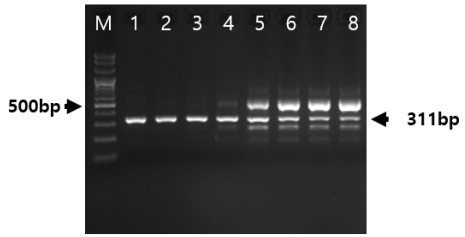
2) Optimization of PCR condition using gradient PCR

Figure 13 shows agarose gel electrophresis pattern of temperature gradient PCR products of six primer sets. Temperature gradient PCR for primers of rs41919985 (*FASN*) (a) was conducted at range from 55℃ to 65℃. Under 61.3℃ of annealing temperature, several nontarget bands were appeared besides the target band (311bp). Thus, 65℃ was used as Tm for rs41919985 (*FASN*). Temperature gradient PCR for primers of rs41255693 (*SCD*) (b) was carried out at range from 54℃ to 62℃. At 54℃, one thick band was showed. It seemed that Tm variation greatly affects the PCR. Consequently, 54.5℃ was set for optimal Tm for rs41255693 (*SCD*). For rs41729173 (*FABP4*), two run conditions were tested for Temperature gradient PCR. The ranges of two PCRs were from 56℃ to 59℃ and from 59℃ to 62℃, respectively. In the first gradient PCR (c), several bands below the target band were produced at all Tms. In the second gradient PCR (d), 61.4℃ and 60.9℃ showed best results so that 61℃ was set as optimal Tm for rs41729173 (*FABP4*). At last, temperature gradient PCRs for rs136261927 (*FADS1*) (e) and rs109772589 (*FADS2*) (f) were conducted at the same range from 57.5℃ to 61℃. At all Tms, both PCRs produced good quality products without contamination bands. 61℃ was set as optimal Tm for rs136261927 (*FADS1*) and rs109772589 (*FADS2*). Using these optimal Tm conditions, the fifty-six beef samples were analyzed for SNP typing.

3) The SNP typing of the fifty-six beef samples

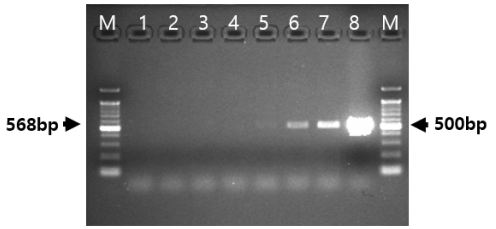
As mentioned above, RFLP was conducted for five SNPs (rs41919985, rs41255693, rs41729173, rs136261927 and rs109772589), and Sanger sequencing was used for rs42187261. Figure 14 shows the representative RFLP patterns for 5 SNP types. Table 9 shows the frequencies and benefited verification of the genotypes as a marker in the fifty-six samples. In the case of rs109772589 (*FADS2*) genotype, all fifty-six samples had the same 'GA' hetero. Thus, rs109772589 was excluded from association analysis. Minor allele frequency (MAF), Heterozygosity and P-value of Hardy-Weinberg equilibrium (HWE) are the indexes for the utility of genotypes as a marker (Oh, D. Y., et al., 2013). If minor allele frequency and heterozygosity of one SNP were very low, the SNP is already fixed at one of the genotypes, and it means the SNP is not useful as a selection marker. P-value of Hardy-Weinberg equilibrium means re-occurrence of the same genotype frequency in next generation. The genotype frequency of one SNP will appear in next generation if HWE is higher than 0.05.

(a) rs41919985 (*FASN*)



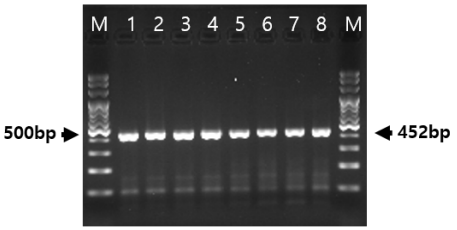
No	1	2	3	4	5	6	7	8
Tm (℃)	65	64.3	63.1	61.3	59	57.3	56	55

(b) rs41255693 (*SCD*)



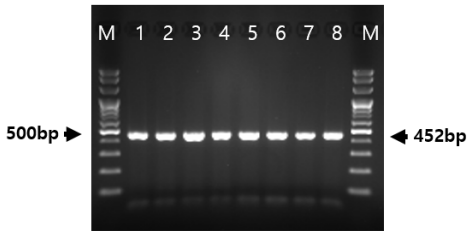
No	1	2	3	4	5	6	7	8
Tm (℃)	62	61.4	60.4	58.9	57.1	55.5	54.6	54

(c) rs41729173 (*FABP4*) #1



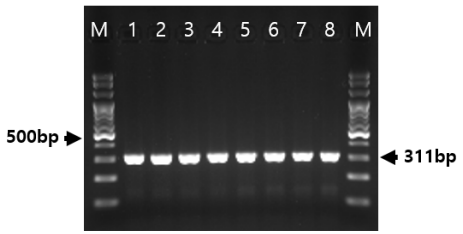
No	1	2	3	4	5	6	7	8
Tm (℃)	59	58.8	58.4	57.9	57.2	56.7	56.3	56

(d) rs41729173 (*FABP4*) #2



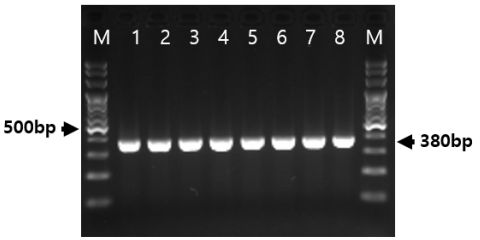
No	1	2	3	4	5	6	7	8
Tm (℃)	62	61.8	61.4	60.9	60.2	59.7	59.3	59

(e) rs136261927 (*FADS1*)



No	1	2	3	4	5	6	7	8
Tm (℃)	61	60.8	60.4	59.7	58.9	58.3	57.8	57.5

(f) rs109772589 (*FADS2*)



No	1	2	3	4	5	6	7	8
Tm (℃)	61	60.8	60.4	59.7	58.9	58.3	57.8	57.5

Figure 13. Optimization of Tms for the five pairs of primers
(Tm, melting temperature; M, size marker; bp, base pair)

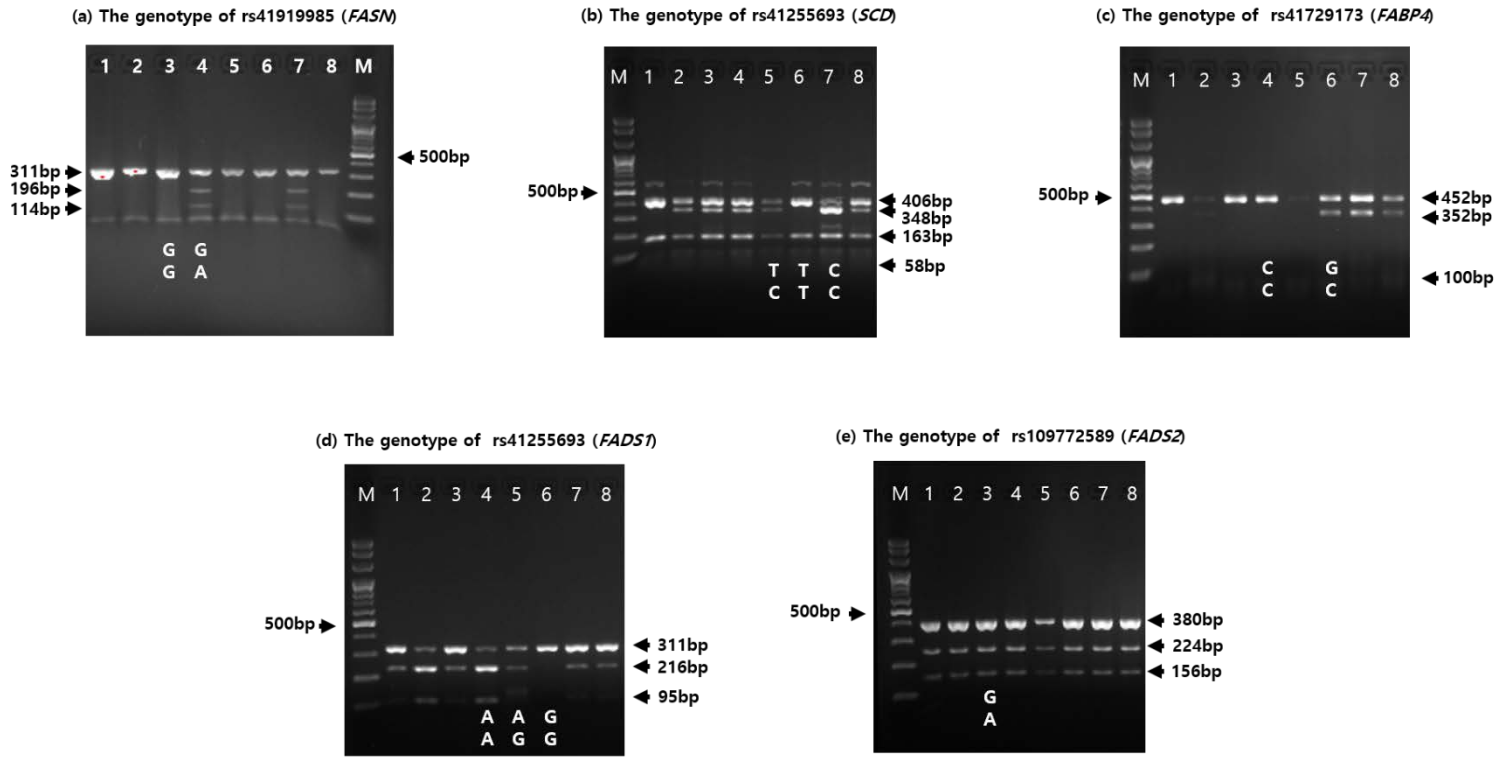


Figure 14. The representative data of RFLP
(bp, base pair; M, size marker)

Table 9. The frequencies and statistical values of the genotypes in the fifty-six samples

rs No.	Gene	Allele	Count	Genotype	Count	Frequency	MAF	H	HWE
		G	95	GG	39	0.70			
rs41919985	*FASN*	A	17	GA	17	0.30	0.15	0.26	0.18
				AA	0	0.00			
		T	58	TT	13	0.23			
rs41255693	*SCD*	C	54	TC	32	0.57	0.48	0.50	0.28
				CC	11	0.20			
		C	85	CC	33	0.59			
rs41729173	*FABP4*	G	27	CG	19	0.34	0.24	0.37	0.59
				GG	4	0.07			
		G	57	GG	17	0.30			
rs136261927		A	55	GA	23	0.41	0.49	0.50	0.18
	FADS1			AA	16	0.29			
		A	79	AA	29	0.52			
rs42187261		G	33	AG	21	0.38	0.29	0.42	0.46
				GG	6	0.11			

(rs No., reference SNP ID; MAF, minor allele frequency; H, heterozygosity; HWE, P-value of Hardy-Weinberg equilibrium)

3. Establishment of the preparation methods

1) Extraction of total lipid from the beef samples

In modified Folch method (MF) 1, total area of GC peaks was 377 but, direct methylation method gave 18936 area value which is fifty-fold higher one. Additionally, physiologically important but very low concentration fatty acids such as C20:5 n-3 and C22:6 n-3 were not detected in MF 1. In MF 2, lipid extraction efficiency was greatly improved having seventeen-fold increase of total area compared to MF 1. In the case of MF 3, total area was decreased in half compared to MF 2 despite using homogenizer. In MF 4, condensed lipids were directly methylated in direct methylation solution which solubilized and methylated very efficiently. As a result, the total area in MF 4 was over 10,000, 33% improved compared to MF 2. Although it was much lower than that of DM, all the n-3 and n-6 fatty acids were detected, and fatty acids composition in % was similar with DM.

Next, total lipid extraction conditions were varied to optimize the extraction efficiency. MF 4 was basal method, and sample amount (1g / 2g / 3g), extraction time (0.5h / 1h / 2h), washing (once / no washing) and solvent amount (3ml / 1ml) were varied (Table 11). 2g of beef sample gave 1.8× total area compared to MF 4 in which 1g sample, 0.5h extraction time, washing once and 3ml hexane were used.

In the extraction time, the longer extraction time reduced the total area. Omitting washing step reduced the total area in half. When solvent amount reduced to one third, lipid concentration improved 1.9-fold, but not the expected level of

Table 10. GC results prepared with modified Folch methods

	MF 1		MF 2		MF 3		MF 4		DM	
Fatty acids	Area	%	Area	%	Area	%	Area	%	Area	%
C14:0	11.93	3.16	166.40	2.48	91.49	2.66	325.45	3.02	594.50	3.14
C14:1	3.31	0.88	52.92	0.79	22.87	0.66	92.82	0.86	1.15	0.01
C15:0	1.33	0.35	19.52	0.29	11.44	0.33	38.66	0.36	70.90	0.37
C15:1	–	–	–	–	–	–	1.38	0.01	5.01	0.03
C16:0	106.83	28.30	1668.80	24.91	895.59	26.03	2933.36	27.26	5090.35	26.88
C16:1	14.21	3.76	272.40	4.07	105.07	3.05	395.80	3.68	823.98	4.35
C17:0	3.76	1.00	61.34	0.92	32.88	0.96	115.63	1.07	174.26	0.92
C17:1	–	–	52.52	0.78	–	–	86.40	0.80	153.82	0.81
C18:0	51.31	13.59	870.97	13.00	492.38	14.31	1490.79	13.85	2043.59	10.79
C18:1 n-9	163.83	43.40	3375.30	50.39	1526.53	44.37	4687.91	43.56	9036.40	47.72
C18:2 n-6	12.85	3.41	3.95	0.06	155.70	4.53	325.24	3.02	531.12	2.80
C20:0	–	–	5.06	0.08	2.76	0.08	9.04	0.08	8.38	0.04
C18:3 n-6	–	–	–	–	1.16	0.03	4.73	0.04	6.22	0.03

(MF, modified Folch method; DM, direct methylation method)

Table 10. GC results prepared with modified Folch methods (continued)

	MF 1		MF 2		MF 3		MF 4		DM	
Fatty acids	Area	%	Area	%	Area	%	Area	%	Area	%
C20:1 n−9	−	−	19.87	0.30	6.41	0.19	22.84	0.21	31.12	0.16
C18:3 n−3	1.01	0.27	15.59	0.23	9.53	0.28	26.59	0.25	55.40	0.29
C21:0	3.06	0.81	63.55	0.95	28.53	0.83	103.69	0.96	166.67	0.88
C20:2 n−6	−	−	−	−	1.25	0.04	3.67	0.03	4.07	0.02
C20:3 n−6	1.16	0.31	16.15	0.24	16.94	0.49	28.92	0.27	39.90	0.21
C22:1 n−9	−	−	−	−	−	−	0.75	0.01	5.81	0.03
C20:3 n−3	−	−	−	−	−	−	1.89	0.02	2.46	0.01
C20:4 n−6	2.90	0.77	34.14	0.51	37.74	1.10	59.13	0.55	83.25	0.44
C22:2 n−6	−	−	−	−	−	−	0.45	0.00	2.59	0.01
C24:0	−	−	−	−	−	−	0.44	0.00	−	−
C20:5 n−3	−	−	−	−	2.36	0.07	2.96	0.03	3.26	0.02
C22:6 n−3	−	−	−	−	−	−	2.65	0.02	2.20	0.01
Total	377.50	100.00	6698.47	100.00	3440.63	100.00	10761.17	100.00	18936.39	100.00

(MF, modified Folch method; DM, direct methylation method)

3-fold. Considering these results of optimization, the sample amount and the extraction time were set to 2g and 30 minutes, respectively.

Table 11. Optimization of total lipid extraction method

Sample	Total area	Extraction time	Total area	Other variations	Total area
1g	10762	0.5h	10762	H 3ml	10762
2g	18178	1h	8522	H 1ml	19041
3g	13517	2h	7603	No washing	5575
		4h	4631		

(H, hexane for dissolving FAMEs; 1g sample / 0.5h extraction / washing once/ 3ml hexane of modified Folch method 4 were varied in sample, extraction time, washing and solvent)

2) TLC separation of phospholipid and methylation

Figure 15 shows the positions of polar lipid including phospholipids and neutral lipid separated by TLC. Two polar lipids, phosphatidylcholine and the polar lipid from meat sample did not move from the spotting point. But, neutral fat, mono / di / triglyceride mix and the sample moved with the solvent front.

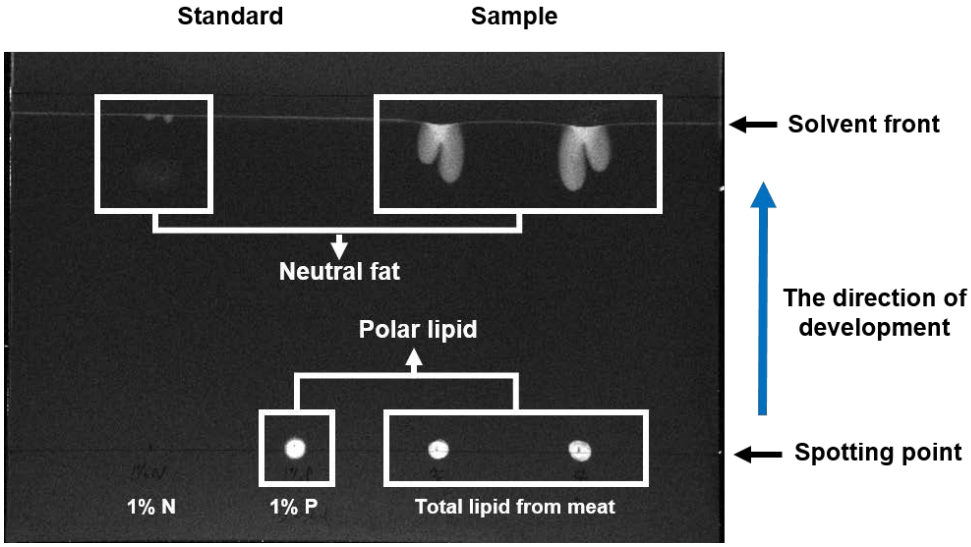


Figure 15. TLC separation of polar lipid and neutral fat
(N, mono / di / triglyceride; P, phosphatidylcholine from egg yolk)

TLC conditions were optimized through three trials. Table 12 is results of TLC optimization. In the first trial (MT 1), the total area of phospholipid fraction was very low, and only major fatty acids were detected such as C 18:1 n−9, C 18:2 n−6, C 20:4 n−6, *etc*. In the second trial (MT 2), phospholipid fraction was methylated using small scale direct methylation. The total area of the second trial was 57.47, six−fold higher than MT 1 which was perfectly matched with the concentration effect from 6−fold less solvent in MT 2 (3ml vs. 500㎕). To increase the total area, third trial (MT 3) condition was modified to 2×

sample amount (2g), 1/2 solvent (250㎕), and 4× lipid spotting on TLC plate (80㎕) compared to MT 2. Theoretically, in MT 3, it should have 16-fold higher total area over MT 2 and actual value (924 / 57) was over 16-fold. Moreover, all the fatty acids including n-3 and n-6 PUFAs were detected in MT 3. Thus, MT 3 method was used for TLC analysis of fifty-six samples.

The effects of TLC on fatty acid recovery was tested (Table 13). To study this, 80㎕ of 1% phosphatidylcholine was run on a TLC plate and phosphatidylcholine spot was scraped and fatty acid was analyzed. Control was 80㎕ of 1% phosphatidylcholine directly processed without TLC step for GC analysis. TLC step had 87% recovery rate of fatty acid. Blank silica powder showed minor noise background peaks having ~1% of total area.

Table 12. The results of modified TLC

	MT 1		MT 2		MT 3	
Fatty acids	Area	%	Area	%	Area	%
C14:0	–	–	0.59	1.02	1.07	0.12
C14:1	–	–	–	–	–	–
C15:0	–	–	2.17	3.77	1.58	0.17
C15:1	–	–	–	–	4.50	0.49
C16:0	1.29	14.91	6.93	12.05	144.27	15.60
C16:1	–	–	–	–	9.36	1.01
C17:0	–	–	1.01	1.75	3.04	0.33
C17:1	–	–	–	–	4.36	0.47
C18:0	1.43	16.51	9.35	16.27	117.12	12.66
C18:1 n-9 c	2.00	23.12	12.47	21.70	213.09	23.04
C18:2 n-6 c	2.42	27.95	15.62	27.18	228.50	24.71
C20:0	–	–	–	–	0.78	0.08
C18:3 n-6	–	–	–	–	2.41	0.26
C20:1 n-9	–	–	–	–	2.36	0.26
C18:3 n-3	–	–	0.61	1.07	4.06	0.44
C21:0	–	–	–	–	1.90	0.21
C20:2 n-6	–	–	–	–	2.43	0.26
C20:3 n-6	0.39	4.48	2.48	4.32	40.98	4.43
C22:1 n-9	–	–	–	–	–	–
C20:3 n-3	–	–	–	–	1.35	0.15
C20:4 n-6	1.13	13.03	6.25	10.88	137.35	14.85
C22:2 n-6	–	–	–	–	1.24	0.13
C24:0	–	–	–	–	–	–
C20:5 n-3	–	–	–	–	2.34	0.25
C22:6 n-3	–	–	–	–	0.72	0.08
Total	8.66	100.00	57.47	100.00	924.80	100.00

(MT, modified TLC)

Table 13. The effects of TLC on fatty acid recovery

	TLC		No TLC		Silica
Fatty acids	Area	%	Area	%	Area
C14:0	0.895	0.220	1.076	0.229	–
C14:1	–	–	–	–	–
C15:0	0.310	0.076	0.394	0.084	0.713
C15:1	–	–	–	–	–
C16:0	123.048	30.215	140.986	29.959	1.624
C16:1	4.962	1.218	6.403	1.361	–
C17:0	0.776	0.191	0.945	0.201	–
C17:1	–	–	–	–	–
C18:0	61.692	15.149	71.513	15.196	1.478
C18:1 n-9	110.685	27.179	131.077	27.853	0.538
C18:2 n-6	67.203	16.502	77.047	16.372	–
C20:0	0.339	0.083	0.407	0.087	–
C18:3 n-6	0.525	0.129	0.601	0.128	–
C20:1 n-9	0.745	0.183	0.744	0.158	–
C18:3 n-3	1.269	0.312	1.332	0.283	–
C21:0	–	–	–	–	–
C20:2 n-6	1.003	0.246	1.117	0.237	–
C20:3 n-6	1.177	0.289	1.737	0.369	–
C22:1 n-9	–	–	–	–	–
C20:3 n-3	–	–	–	–	–
C20:4 n-6	25.151	6.176	27.031	5.744	–
C22:2 n-6	–	–	–	–	–
C24:0	–	–	–	–	–
C20:5 n-3	–	–	–	–	–
C22:6 n-3	7.464	1.833	8.188	1.740	–
Total	407.245	100.000	470.599	100.000	4.353

(TLC, 80㎕ of 1% phosphatidylcholine after TLC; No TLC, 80㎕ of 1% phosphatidylcholine without TLC)

4. Association analysis between omega fatty acids ratio and SNP types

Total lipids of the fifty-six samples were extracted, separated and methylated using the established preparation method, and analyzed with GC-FID. Table 14 shows means, standard deviation, maximum and minimum for individual fatty acid in phospholipid of the fifty-six beef samples. After that, the mean differences between the ratios of omega fatty acids were observed by the SNP types using ANOVA and ANCOVA. During ANCOVA, samples raised by the producer, LCS and the feeding program, WS were discarded because the number of the discarded samples were only two out of fifty-six, and it might have made statistical errors. On the other hand, during ANOVA, the whole fifty-six samples were used since the producers and the feeding programs were not included in ANOVA as independent variables.

1) Association analysis by the producers or the feeding programs and SNP types

ANCOVA was conducted twice, and the first trial was focused on the SNP types and the producers. At each SNP, the fifty-four samples were divided into six groups by the producers and subdivided into three or two groups by the SNP types. In the first trial, CG and CC type of rs41729173 in *FABP4* and AG type of rs42187261 in *FADS1* were significantly associated with lowest the amount of C20:2 n-6

(p=0.012) and C20:4 n−6 (p=0.023), respectively. The second trial was conducted using the feeding programs and the SNP types as independent variables. At each SNP, the fifty−four samples were divided into three groups by the feeding program and subdivided into three or two groups by the SNP types. As a result of the second trial, CC type of rs41255693 in *SCD* was significantly associated with the highest amount of C18:3 n−3 in comparison with other types (p=0.004). Additionally, CC type and CG type of rs41729173 in *FABP4* were significantly associated with the lowest amount of C18:3 n−6 (p=0.04) and C20:2 n−6 (p=0.001), respectively.

However, all these results were biased due to the very few number of samples. In other words, the fifty−four samples were divided and subdivided by the producers or the feeding programs and the SNP types. Consequently, some groups had just only one sample, and it made the biased results. Thus, the results of ANCOVA are not showing in this study.

Table 14. Fatty acid composition of the fifty-six beef samples

Fatty acids (%)	Mean	SD	Max	Min
C14:0	0.260	0.386	2.968	0.105
C14:1	0.009	0.027	0.149	0.000
C15:0	0.162	0.059	0.369	0.081
C15:1	0.795	0.323	1.750	0.182
C16:0	13.310	2.361	24.219	10.053
C16:1	1.381	0.690	5.565	0.606
C17:0	0.307	0.152	1.322	0.149
C17:1	0.134	0.075	0.314	0.000
C18:0	14.410	1.842	20.927	8.983
C18:1 n-9	18.422	5.827	42.102	0.679
C18:2 n-6	29.123	4.555	36.029	8.183
C20:0	0.136	0.136	0.959	0.000
C18:3 n-6	0.255	0.118	0.620	0.122
C20:1 n-9	0.200	0.073	0.423	0.049
C18:3 n-3	0.401	0.100	0.703	0.165
C21:0	0.201	0.114	0.912	0.087
C20:2 n-6	0.273	0.123	0.779	0.121
C20:3 n-6	5.096	1.118	6.932	0.143
C22:1 n-9	0.014	0.022	0.094	0.000
C20:3 n-3	0.099	0.029	0.160	0.000
C20:4 n-6	14.437	2.750	19.737	3.693
C22:2 n-6	0.108	0.033	0.175	0.000
C24:0	0.006	0.015	0.071	0.000
C20:5 n-3	0.331	0.227	1.582	0.066
C22:6 n-3	0.129	0.234	1.802	0.000
SFA	28.792	2.262	40.139	25.851
MUFA	20.955	6.252	48.359	4.961
PUFA	50.253	6.783	57.951	14.055
Total n-3/n-6	0.020	0.007	0.053	0.010

(Mean, mean percentage value of fatty acid composition; SD, standard deviation; Max and Min, maximum and minimum percentage value of fatty acid composition)

2) Association analysis by SNP types

After ANOVA, the results of ANOVA were interpreted in three aspects. First, the data were selected if the p-values of the data were less than 0.05. Second, the data was compared with the effects of SNPs in the references. Third, data which did not follow a certain tendency was sorted out.

(1) Association-significance between omega fatty acids ratio and SNP types

Table 15 shows the selected data of which p-values are less than 0.05. GA type of rs41919985 in *FASN* was significantly associated with increasing the amount of C20:5 n-3 (p=0.027). However, AA type of rs41919985 was not observed in the fifty-six samples. Therefore, further study should be conducted with more samples to confirm the effects of rs41919985. CC type of rs41729173 in *FABP4* was significantly associated with the lowest amount of C22:2 n-6 (p=0.049), and AG type of rs42187261 in *FADS1* was significantly associated the lowest amount of C20:4 n-6 (p=0.042). GA type of rs136261927 in *FADS1* was significantly associated with the lowest amount of C20:3 n-6 (p=0.044) and also the lowest ratio of C20:3 n-6 to C20:4 n-6 (0.027). These results of the four SNPs did not follow a certain tendency.

Nevertheless, four SNPs may need further study using more samples to conclude the results. Especially, rs42187261 and rs136261927 in *FADS1* were significantly associated with

the amount of C20:4 n−6, and the amount of C20:3 n−6 and the ratio of C20:3 n−6 to C20:4 n−6, respectively. FADS1 is the enzyme which changes C20:3 n−6 to C20:4 n−6. Therefore, it is reasonable that the groups by the SNP types have different mean values of C20:3 n−6 and C20:4 n−6. If the groups by the SNPS types of rs42187261 and rs136261927 in *FADS1* have different mean values of C20:3 n−6 and C20:4 n−6 in the further study with more samples, the SNPs can be used as genetic markers to select individuals which have lower major n−6 fatty acid, C20:4 n−6.

Table 15. The association between genotypes and fatty acid composition with p-value less than 0.05

SNP	FA	G	n	Mean	SD	SE	p
rs41919985 (*FASN*)	C20:5 n−3	GA	17	0.430	0.337	0.082	0.027
		GG	39	0.285	0.141	0.023	
rs41729173 (*FABP4*)	C22:2 n−6	GG	4	0.105	0.029	0.015	0.049
		CG	19	0.123	0.025	0.006	
		CC	33	0.100	0.035	0.006	
rs42187261 (*FADS1*)	C20:4 n−6	AA	29	14.859	2.272	0.422	0.042
		AG	21	13.282	3.163	0.690	
		GG	6	15.929	2.075	0.847	
rs136261927 (*FADS1*)	C20:3 n−6	AA	16	5.434	0.526	0.131	0.044
		GA	23	4.639	1.518	0.316	
		GG	17	5.336	0.612	0.148	
	C20:3 n−6 /C20:4 n−6	AA	16	0.395	0.070	0.017	0.027
		GA	23	0.323	0.100	0.021	
		GG	17	0.381	0.082	0.020	

(FA, fatty acid; G, genotypes; n, the number of samples; Mean, mean percentage value of fatty acid composition; SD, standard deviation; SE, standard error; p, p-value of ANOVA)

(2) Implications for the association between omega fatty acids ratio and SNP types

Table 16 shows the data of the SNPs mentioned in the references. AA genotype of rs41919985 in *FASN* was significantly associated with lower concentrations of C20:3n−6 in comparison with GG genotype, and CC genotype of rs41255693 in *SCD* was significantly associated with lower concentrations C20:2n−6 in comparison with TT genotype ($P<0.05$) (Li et al., Animal Genetics, 2011).

GG type of rs41729173 in *FABP4* was significantly associated with the highest amount of C18:2 n−6 ($p=0.031$) and total n−6 fatty acids ($p=0.03$) of Aberdeen Angus beef. CC type of rs41729173 in *FABP4* was significantly associated with the highest amount of C20:4 n−6 ($p=0.044$), C20:5 n−3 ($p=0.044$) and total n−3 fatty acids ($p=0.044$) of Blonde d'Aquitaine beef (Dujkoba et al., ACTA VET. BRNO., 2015).

AA type of rs42187261 in *FADS1* was significantly linked to the highest increase in milk C20:5 n−3 ($p=0.004$). GG type of rs136261927 in *FADS1* was significantly linked to the highest increase in milk C20:3 n−6 ($p=0.0003$) (Ibeagha−Awemu et al., BMC Genetics, 2014).

In this study, all the data of the SNPs mentioned in the references had higher p−values than 0.05 except rs136261927 in *FADS1* (0.044). Besides, almost data had different tendency with the references. It may be due to few samples or physiological and genetic character of Hanwoo. Additionally, Dujkoba’s analysis was also conducted with very few samples (seventeen samples in Aberdeen Angus beef and sixteen samples in Blonde d'Aquitaine beef), and GG type of

rs41729173 in *FABP4* was only one in Blonde d'Aquitaine beef. Thus this association results are hardly conclusive.

Table 17 shows the data showing some tendency though p-values were higher than 0.05 and association was not mentioned in the references. This study and the references were conducted with different breed of cow and lipid (phospholipid vs. total lipid). Therefore, different results can be possible. These associations should be confirmed by further analysis with more samples

Table 16. The association between genotypes and fatty acid composition in comparison with the references

SNP	Reference	FA	G	n	Mean	SD	SE	p
rs419199859 (*FASN*)	Li, 2011.	C20:3 n-6	GA	17	4.921	1.25	0.30	0.49
			GG	39	5.146	1.06	0.17	
rs41255693 (*SCD*)	Li, 2011.	C20:2 n-6	TT	13	0.286	0.16	0.04	0.42
			TC	32	0.281	0.12	0.02	
			CC	11	0.228	0.04	0.01	
rs41729173 (*FABP4*)	Dujkoba, 2015.	C18:2 n-6	GG	4	30.243	5.06	2.53	0.65
			CG	19	29.549	3.21	0.74	
			CC	33	28.554	5.16	0.90	
		C20:4 n-6	GG	4	13.814	1.40	0.70	0.56
			CG	19	14.925	2.19	0.50	
			CC	33	14.139	3.12	0.54	
		C20:5 n-3	GG	4	0.210	0.07	0.03	0.27
			CG	19	0.388	0.32	0.07	
			CC	33	0.310	0.16	0.03	
		total n3/n6	GG	4	0.015	0.00	0.00	0.18
			CG	19	0.022	0.01	0.00	
			CC	33	0.019	0.01	0.00	
rs42187261 (*FADS1*)	Ibeagha-Awemu, 2014.	C20:5 n-3	AA	29	0.377	0.28	0.05	0.19
			AG	21	0.259	0.14	0.03	
			GG	6	0.345	0.14	0.06	
rs136261927 (*FADS1*)	Ibeagha-Awemu, 2014.	C20:3 n-6	AA	16	5.434	0.53	0.13	0.04
			GA	23	4.639	1.52	0.32	
			GG	17	5.336	0.61	0.15	

(FA, fatty acid; G, genotypes; n, the number of samples; Mean, mean percentage value of fatty acid composition; SD, standard deviation; SE, standard error; p, p-value of ANOVA)

Table 17. The association between genotypes and fatty acid composition showing some tendency

SNP	FA	G	n	Mean	SD	SE	p
rs41255693 (*SCD*)	C18:3 n-6	TT	13	0.285	0.14	0.04	0.40
		TC	32	0.254	0.13	0.02	
		CC	11	0.219	0.03	0.01	
	C18:3 n-3	TT	13	0.370	0.08	0.02	0.37
		TC	32	0.402	0.10	0.02	
		CC	11	0.427	0.11	0.03	
	C20:3 n-3	TT	13	0.097	0.04	0.01	0.95
		TC	32	0.100	0.03	0.01	
		CC	11	0.100	0.01	0.00	
	total n3/n6	TT	13	0.022	0.01	0.00	0.50
		TC	32	0.019	0.01	0.00	
		CC	11	0.018	0.00	0.00	
rs41729173 (*FABP4*)	C20:3 n-6	GG	4	5.381	1.04	0.52	0.43
		CG	19	5.295	0.49	0.11	
		CC	33	4.916	1.35	0.24	
rs42187261 (*FADS1*)	C18:3 n-6	AA	29	0.230	0.09	0.02	0.20
		AG	21	0.268	0.14	0.03	
		GG	6	0.318	0.13	0.05	
	C22:2 n-6	AA	29	0.111	0.03	0.00	0.74
		AG	21	0.106	0.04	0.01	
		GG	6	0.101	0.05	0.02	
	C20:3 n-6 /C20:4 n-6	AA	29	0.353	0.07	0.01	0.31
		AG	21	0.383	0.12	0.03	
		GG	6	0.325	0.03	0.01	
rs136261927 (*FADS1*)	C18:3 n-6	AA	16	0.240	0.10	0.03	0.50
		GA	23	0.243	0.10	0.02	
		GG	17	0.282	0.15	0.04	
	C20:5 n-3	AA	16	0.366	0.35	0.09	0.70
		GA	23	0.327	0.16	0.03	
		GG	17	0.298	0.17	0.04	
	Total n3/n6	AA	16	0.019	0.01	0.00	0.96
		GA	23	0.019	0.01	0.00	
		GG	17	0.020	0.01	0.00	

(FA, fatty acid; G, genotypes; n, the number of samples; Mean, mean percentage value of fatty acid composition; SD, standard deviation; SE, standard error; p, p-value of ANOVA)

Ⅴ. Conclusion

1. SNPs associated with fatty acid metabolism in Hanwoo steers in this study

Six SNPs in five genes related to fatty acid metabolism were typed using RFLP and Sanger sequencing. Three genotypes were detected in four SNPs in *SCD, FABP4* and *FADS1* (rs41255693, rs41729173, rs136261927 and rs42187261). However, in the case of rs41919985 in *FASN* and rs109772589 in *FADS2,* three genotypes were not detected.

FASN is a multifunctional enzyme complex that regulates *de novo* biosynthesis of long chain fatty acid, and rs41919985 is significantly associated with marbling score and MUFAs content in beef (Zhang, S. *et al.*, 2008; Oh, D. Y., *et al.*, 2012; Bhuiyan, M. S. A., *et al.*, 2009). In Oh and Bhuiyan's paper, frequencies of rs41919985 minor allele, 'A' were very low in Korean native cattle, Hanwoo as 0.12 and 0.16, respectively. We assumed that is because of breeding focused on marbling score.

In this study, rs109772589 in *FADS2* showed only one, GA genotype. GA type is a hetero type, and it is very unlikable that the genotypes of all fifty-six samples are hetero. The enzyme, FADS2 is the initial and rate-limiting enzyme of omega fatty acid metabolic pathway. Therefore, rs109772589 has potential as a genetic marker to produce high n-3 beef, and genotypes of rs109772589 should be observed with more samples.

2. Establishment of specialized sample treatment method for the analysis of omega fatty acid balance in beef

Folch method, TLC and direct methylation was used for extracting total lipid from beef sample (Folch, J. *et a*l., 1957), separating total lipid (www.cyberlipid.org) into phospholipid and neutral lipid, and methylating phospholipid (O'Fallon, J. V. *et al.*, 2007), respectively. Each method is very popular and relatively simple. However, these methods are individual procedure, and there were trials and errors to combine these methods as one procedure. Thus, the preparation method was validated and optimized for fatty acid analysis.

First, direct methylation of 1g beef was conducted with or without Folch method. The total area of 1g beef after Folch method was lower than the total area of directly methylated 1g beef, but the former was enough to measure fatty acid composition of phospholipid and reasonable considering that whole 1g of beef was not melted in Folch solvent.

Second, Folch method was optimized to extract much total lipid from the beef sample adding variety to the amount of the sample, the extraction time, the volume of hexane and the washing step. As a result, 2g of the beef sample and extraction for 30 minutes were most efficient. In the case of the washing step, one time was most effective. The total areas were decreased significantly when the washing step was conducted for several times or was not carried out. 3ml of Hexane for dissolving FAMEs from the beef sample after direct methylation was adequate. The volume of hexane was

decreased at 1ml to concentrate FAMEs, and 3ml was chosen as the volume of hexane because the total area of the sample using 1ml hexane was not meet three times of the total area of the sample using 3ml of hexane.

Third, optimization of TLC step was conducted to increase the recovery rate. Removing the elution step of TLC was the first step for increasing the recovery rate. Also, the amount of the sample loaded on the TLC plate was increased from 10㎕ to 80㎕, and the solvent for dissolving total lipid was changed from 2ml of Folch solvent to 250㎕ of chloroform. Direct methylation was scaled down to 1/10 except hexane. Hexane for dissolving FAMEs from phospholipid of the beef sample was decreased from 3ml to 500㎕.

Fourth, after optimization of TLC step, modified TLC step was validated using phosphatidylcholine from egg yolk and empty silica. The total areas and the rates of detected fatty acids were almost same between directly methylated phosphatidylcholine and phosphatidylcholine after modified TLC. Besides, empty silica through modified TLC did not affect the result of GC-FID.

Finally, three methods were combined as one preparation method for fatty acid analysis, and fatty acid compositions of phospholipid extracted from the fifty-six beef samples were analyzed by GC-FID. However, the more samples were analyzed with this combined method, the more unusual peak pattern of GC-FID became. The plastic 2ml microtubes used in small scale direct methylation are weak in strong acid. Therefore, we assumed that some materials melted from the plastic tube stuck to the GC column and it made the peak pattern unusual.

3. Future-applications of the SNPs as genetic markers

We searched candidate SNPs which may effect on omega fatty acid composition in beef. Fatty acid synthase (FASN) plays a major role in producing *de novo* long chain fatty acids (Roy, R. *et al.*, 2005). rs41919985 in this gene is significantly associated with marbling score and fatty acid composition in beef (Zhang, S. *et al.*, 2008; Oh, D. Y., *et al.*, 2012; Bhuiyan, M. S. A., *et al.*, 2009; Li, C., *et al.*, 2012).

Stearoyl-CoA desaturase (SCD) is the enzyme catalyzing $\Delta 9$ desaturation of SFAs to MUFAs, and many researchers have studied in association with improved unsaturated fatty acids in beef (Taniguchi, M., *et al.*, 2004; Mannen, H., 2011). rs41255693 in *SCD* have been reported to be associated with unsaturated fatty acid composition in beef of many breeds (Oh, D. Y., *et al.*, 2013; Dujkova, R., *et al.*, 2015).

Fatty acid binding protein 4 (FABP4) is an intracellular lipid binding protein and have a high affinity with long chain fatty acids and hydrophobic ligands. This enzyme is involved in fatty acid metabolism by uptake and transferring fatty acids (Michael, J. J., *et al.*, 2006). Dujkova *et al* reported that rs41729173 in *FABP4* was significantly associated with fatty acid composition in beef (Dujkova, R., *et al.*, 2015).

Fatty acid desaturase (FADS) 1 and 2 are the enzymes involved in fatty acid metabolism. FADS2 plays a significant role in the production of longer PUFAs as the initial and rate-limiting enzyme. FADS2 catalyzes desaturations at the $\Delta 6$ position of C18:3 n-3 and C18:2 n-6. FADS1 also introduces a double bond at the $\Delta 5$ carbon of C20:4 n-3 and C20:3 n-6

(Nakamura, M. T. and Nara, T., 2004; Guillou, H. *et al.*, 2010). rs136261927 and rs42187261in *FADS1* are linked to C20:3 n−6 and C20:4 n−6, and C 20:5 n−3, respectively. rs109772589 in *FADS2* is also associated with C20:3 n−6 and C20:4 n−6 (Ibeagha−Awemu, E. M. *et al.*, 2014).

Based on the above references, six SNPs in five genes related to fatty acid metabolism were chosen, and associations were analyzed between the genotypes and omega fatty acid composition with fifty−six Hanwoo beef samples. Except rs109772589 in *FADS2* which did not have variations in the genotype, associations between five SNPs and omega fatty acid compositions were analyzed by ANOVA and ANCOVA. The results of ANCOVA was biased by the few number of samples. Also, many of the results of ANOVA did not meet p−value or follow a certain tendency, or were different with the references.

However, there were some significant results. GA type of rs41919985 in FASN was significantly associated with the highest amount of C20:5 n−3 (p=0.027). CC type of rs41729173 in FABP4 was significantly associated with the lowest amount of C22:2n−6 (p=0.049). AG type of rs42187261 in FADS1 was significantly linked to the lowest concentration of C20:4 n−6 (p=0.044), and GA type of rs136261927 in FADS1 was significantly linked to the lowest amount of C20:3n−6 (p=0.027). In the fifty−six samples, there was only one individual which have GA, CC, AG and GA types in rs41919985, rs41729173, rs42187261 and rs136261927, respectively. The ratio of total n−3/n−6 of this individual was around 1:28. This value was the third highest grade in total n−3/n−6 ratio of the fifty−six samples and much higher than the mean value of total n−3/n−6 ratio (mean = around 1:51; SD =

0.00736).

In conclusion, these four SNPs could be applied as potential genetic markers to select Hanwoo steers in the aspect of improvement of n−3/n−6 balance in the future.

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Ⅶ. Abstract in Korean

오메가 3 지방산은 심혈관계 질환, 암, 류머티스성 관절염, 자가면역질환 등의 발병률과 심근경색의 재발률을 억제하는 효과가 알려져 있으며, 이러한 생리학적 효능은 지난 30년 동안 꾸준히 밝혀져 왔다. 또한, 오메가 지방산의 비율은 면역 항상성과 신체의 정상적인 발달 및 정신 건강에 매우 큰 영향을 미치는 것으로 밝혀졌다. 이는, 생리 신호전달 물질이면서 20개의 탄소로 이뤄진 오메가 지방산으로부터 생성되는 Eicosanoid와 연관이 있다. Eicosanoid는 오메가 3 지방산으로부터 생성된 것과 오메가 6 지방산으로부터 생성된 것이 서로 상반된 면역, 염증 작용을 조율하기 때문이다. 그러나, 현대의 서구 식단은 오메가 지방산의 불균형이 매우 심각하고, 전세계의 많은 사람들이 오메가 3 지방산보다 훨씬 많은 양의 오메가 6 지방산을 섭취하고 있는 실정이다. 이러한 현상의 이유로 생산성에 초점을 맞춘 농축산업을 들 수 있다. 특히, 한우산업은 쇠고기 근 내 지방을 높이기 위해 오메가 6 지방산이 매우 높은 옥수수를 농후사료로 사용하고 있다. 따라서, 한우 쇠고기 내 오메가 지방산의 비율은 매우 불균형적이라고 할 수 있다.

본 연구에서는, 쇠고기, 특히 인지질 내 지방산 분석을 위한 전처리 방법을 확립하였고, 56두의 한우 쇠고기 지방산과 지방산 대사와 관련된 유전 자 내의 단일 염기 다형성 (SNP) 간의 연관성을 분석하였다. 지방산 조성과 유전자형의 연관성을 연구하기 위해, 지방산 분석은 지방산 대사 관련 유전자의 조절을 받는 인지질을 대상으로 이루어졌고 사료성분에 주로 영향을 받는 중성지방은 연구에서 배제하였다. 문헌조사를 통하여, 5개의 지방산 대사 관련 유전자 내에서 총 6개의 SNP를 후보로 선정하였다; *FASN* (rs41919985), *SCD* (rs41255693), *FABP4* (rs41729173), *FADS1* (rs136261927, rs42187261), *FADS2* (rs109772589).

1. 쇠고기 내 전체 지질을 추출하기 위하여 Folch 법을 사용하였고, 추출한 전체 지질에서 인지질을 분리하기 위해 thin-layer chromatography (TLC) 를 사용하였다. 분리된 인지질을 TLC plate에서 긁어 내어 O'Fallon의 direct methylation method를

사용하여 메틸화하였다. 본 연구에서 확립한 전처리 법의 효율은 같은 양의 난황 유래 phosphatidylcholine를 각각 O'Fallon의 direct methylation method와 해당 전 처리법으로 지방산을 처리한 후 얻은 gas chromatography (GC) 데이터를 비교 분석하여 검증하였다. 두 데이터의 총 면적 값이 각각 407과 470으로 유사하였다. 더하여, 지방산을 처리하지 않은 TLC plate의 silica는 GC 데이터에 거의 영향을 미치지 않았다.

2. 본 연구에서 확립한 전처리 법으로 56두의 쇠고기 인지질 내 지방산 조성을 분석하고 후보 SNP에 대한 유전자형을 제한효소 단편 다형화 현상 (RFLP) 과 Sanger 법을 이용하여 분석하였다. *FADS2* 유전자 내 rs109772589의 경우, 모든 샘플이 같은 이형이어서 연관분석에서 제외 하였고, *FASN* 유전자 내 rs41919985의 경우 AA형이 발견되지 않았다.

3. 분산분석을 이용하여 오메가 지방산 조성과 유전자형 간의 연관분석을 수행한 결과, 4개의 SNP와 특정 지방산 조성이 유의적인 연관성이 있음을 알 수 있었다.

- rs41919985의 GA type과 가장 높은 C20:5 n−3 함량 (p=0.027)
- rs41729173의 CC type과 가장 낮은 C22:2n−6 함량 (p=0.049)
- rs42187261의 AG type과 가장 낮은 C20:4 n−6 함량 (p=0.044)
- rs136261927의 GA type과 가장 낮은 C20:3 n−6 함량 (p=0.027)

실험에 사용된 56두의 샘플 중에서 단 하나의 개체만이 rs41919985, rs41729173, rs42187261, rs136261927 에서 각각 GA, CC, AG, GA 유전자형을 가졌는데, 이의 오메가6/오메가3 비율은 약 28:1로 56두 중 세 번째로 높은 수치였고 평균치보다도 훨씬 높았다.

따라서, 본 연구에서 유의성을 가진 4개의 SNP는 추후 한우의 오메가 지방산 대사와 연관된 유전자 마커로서 잠재력을 가지고 있다고 판단된다.