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Research Article
Association Study and Expression Analysis of Stearoyl Co-A Desaturase as a Candidate Gene for Fatty Acid Composition in Indonesian Crossbred Chickens

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Abstract

Background and Objective: The stearoyl-CoA desaturase (SCD) gene encodes an enzyme involved in fatty acid (FA) biosynthesis. The aim of the present study was to investigate the association and expression of SCD as a candidate gene for FA composition in Indonesian crossbred chickens. **Materials and Methods:** Sixty-two Indonesian crossbred, unsexed chickens (F2 Kampung×broilers) were used. FA composition was measured at 12 weeks of age from thigh meat of crossbred chicken. **Results:** A single nucleotide polymorphism in coding region c.17492542 C>G of SCD was associated with FA composition, including both unsaturated [linoleic (C18:2n6c) and eicosadienoic (C20:2) acids] and saturated [lauric acid (C12:0)] forms. SCD mRNA expression analysis in liver revealed 6 chickens with extremely high and low FA compositions, of them, high FA birds (n = 3) had higher unsaturated and lower saturated FAs, while the low FA group (n = 3) had lower unsaturated and higher saturated FA levels. SCD expression was higher ($p<0.05$) in tissues collected from high FA chickens than low FA chickens. **Conclusion:** These results will improve the understanding of SCD function in FA composition and will shed light on SCD as a potential candidate in the selection of chickens with higher levels of unsaturated and lower levels of saturated FA.

Key words: Polymorphism, liver, mRNA, fatty acid composition, Indonesian crossbred chickens

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Fatty acid (FA) composition plays an important role in meat quality in terms of nutritional value and flavor[1]. In particular, polyunsaturated FAs (PUFAs), including C18:2, C20:4 and C22:6, positively correlate with meat flavor and are associated with human health[2]. However, the genetics behind the FA composition of chicken meat is poorly understood. A deeper knowledge of the genetic mechanisms behind FA production is important for generating more effective biomarker-assisted breeding and selection of chickens with higher PUFA levels, leading to economic benefits in the chicken production industry and for human health.

Using quantitative real time PCR analysis of chicken liver tissues, our group recently identified higher stearoyl-CoA desaturase (SCD) gene expression in chickens with higher PUFA levels versus those with lower levels[3]. SCD is a microsomal enzyme that catalyzes synthesis of monounsaturated FAs from saturated fatty acyl-CoAs[1]. The preferred substrates for SCD are palmitoyl and stearoyl-CoA, which are converted to palmitoleoyl and oleoyl-CoA, respectively[4]. This function could directly affect the FA composition in muscle. *SCD* has been mapped to GGA6 in chickens, which is located within the genomic region of a quantitative trait loci (QTL) for intramuscular and abdominal fat, hence, it is considered a positional and functional candidate gene for FA metabolism[5]. While investigation of SCD has previously done in cattle and pigs, study of SCD in chickens is lacking. In cattle and pigs, SCD has been linked to FA composition profiles[5-9]. A previous study reported the presence of two single nucleotide polymorphisms (SNPs) in *SCD* (g.3728 A>G and g.12903 G>A) but only one (g.3728 A>G) was found to be associated with myristoleic acid (C14:1), palmitic acid (C16:0) and palmitoleic acid (C16:1) production in Cobb 500 chickens (n = 95)[5]. The present study aimed to identify the relationships between the SCD SNP c.17492542 C>G and expression of SCD with FA composition in Indonesian crossbred chickens.

MATERIALS AND METHODS

Birds: Sixty-two Indonesian crossbred chickens (F2 Kampung × broilers) were reared under identical feeding conditions. At 12 weeks of age, birds were sacrificed and had approximately 1.6 kg of slaughter weight per chicken. Carcass and meat quality data were collected according to guidelines of the Indonesian performance test. Tissues from breast muscle were used for genomic DNA isolation and FA composition analysis.

FA composition analysis: Total lipids in each sample were extracted from breast muscle using chloroform-methanol (2:1) according to a previously reported procedure[10]. FA methyl esters were prepared from the extracted lipids with BF3-methanol (Sigma-Aldrich, St. Louis, MO, USA) and separated on a HP-6890N gas chromatograph (Hewlett-Packard, Palo Alto, CA, USA) as described previously[11]. A split inlet (split ratio, 50:1) was used to inject samples into a 30 m × 0.25 mm × 0.25 μm Omegawax 320 capillary column (Supelco, Bellefonte, PA, USA). The oven temperature was kept at 150°C for 3 min before being raised in 2.5°C min^{-1} intervals to 180 for 5 min, followed by an increase to 220 for 25°C in 2.5°C min^{-1} intervals. The inlet temperature was 210°C. Air was the carrier gas at a constant flow of 0.7 mL min^{-1}.

DNA isolation, SNP identification and genotyping: Using RNA deep sequencing analysis, several SNPs in chicken SCD by comparing the unsaturated FA contents of chicken breast muscle was previously described[3]. Among them, c.17492542 G>C was selected for investigation in the current association study. For this purpose, genomic DNA was isolated from the breast muscle and liver tissue of chickens for genotyping according to a standard phenol-chloroform method[12]. *In silico* analysis of genomic sequences by comparing several sequences from a publicly available database (NCBI) revealed possible targets for polymerase chain reaction (PCR) amplification. A working solution with a final concentration of 50 ng μL^{-1} of DNA was prepared and stored at 4°C for further analysis. PCR was performed in a 20 μL volume containing 2 μL of genomic DNA, 1× PCR buffer (with 1.5 mM $MgCl_2$), 0.25 mM dNTP, 5 pM of each primer and 0.1 U of Taq DNA polymerase (GeneCraft). Genotyping was performed using PCR-restriction fragment length polymorphism (RFLP). PCR products were analyzed using 1.5% agarose gels and digested by the restriction enzyme *Hinf1* for SCD (New England Biolabs, Ipswich, UK). Digested PCR-RFLP products were resolved on 2% agarose gels. The PCR-RFLP pattern, GenBank accession number and primer sequences used are listed in Table 1.

Selection of animals for mRNA expression analysis: Liver tissues from 6 chickens with divergent FA compositions were collected for mRNA analysis. These chickens were divided equally into two groups (n = 3 each) based on their unsaturated (C18:2n6c and C20:2) and saturated (C12:0) FA levels. The high FA composition birds (HFA) had comparatively higher unsaturated and lower saturated FA levels, while low FA composition birds (LFA) had higher saturated and lower

Table 1: GenBank accession numbers and primer sequences

Gene name	Accession number	Primer sequence	Application	Size (bp)	Tm (°C)	Enzyme	SNP	Digested fragment length (bp)
SCD	NC_006093.4	F: 5′-GAT GAG CTG CCC CCA GAA C-3′ R: 5′-AAG CTG CTG TCC CGC TAT G-3	Genotyping	407	61	*Hin*fI	g. 17492542 C>G	GG : 373 and 34 CC : 407 GC : 407, 373 and 34
SCD	XR_001467161.1	F: 5′-CTG GAA CAG AGA AAC CTT TG-3′ R: 5′-CTG TGA TAG GTT CCA AAA GC-3	qPCR	220	55			
GAPDH	NM_204305.1	F: 5′-TGA TGG TCC ACA TGG CAT CC-3′ R: 5′-GGG GAG ACA GAA GGG AAC AGA-3	qPCR	153	55			

SCD: Stearoyl-CoA desaturase, GAPDH: Glyceraldehyde-3-phosphate dehydrogenase, Tm: Temperature, SNP: Small nucleotide polymorphism

unsaturated FA levels. Significant differences between the two groups were determined using the proc GLM test in SAS.

mRNA expression by quantitative real time (qRT)-PCR: Since FAs are metabolized and catabolized in the liver of chickens, liver tissues were selected for our mRNA expression study. Total RNA was extracted from the liver tissues of HFA and LFA (n = 6 total) chickens using Tri-Reagent according to the manufacturer's instructions (Sigma-Aldrich). Total RNA was treated using an on-column RNase-free DNase set (Promega, Wisconsin, USA) and quantified by NanoDrop spectrophotometry (ND8000, Thermo-Scientific, Massachusetts, USA). RNA quality was assessed using an Agilent 2100 Bioanalyzer (data not shown) and RNA Nano 6000 Labchip kit (Agilent Technologies, California, USA). RNA integrity was examined by 2% agarose gel electrophoresis. In all cases, cDNA were synthesized by RT-PCR using 2 µg of total RNA, SuperScript II reverse transcriptase (Invitrogen, California, USA) and oligo(dT)12 primers (Invitrogen). SCD-specific primers for qRT-PCR were designed using Primer3 software[13] (Table 1). In each run, the 96-well microtiter plate contained each cDNA sample and a no-template control. qRT-PCR was conducted with the following program: 40 cycles at 95°C for 3 min and 95°C for 15 sec/60°C for 45 sec EC on a Step One Plus qPCR system (Applied Biosystem, California, USA). Each PCR reaction contained, 10 µL of iTaqTM SYBR® Green Supermix with Rox PCR core reagents (Bio-Rad, California, USA), 2 µL of cDNA (50 ng μL^{-1}) and an optimized amount of primers mixed with double-distilled H_2O to a final reaction volume of 20 µL/well. All samples were analyzed twice (technical replication) and the geometric mean of the Ct values was used for mRNA expression profiling. Glyceraldehyde-3-phosphate dehydrogenase was used for normalization of the target gene. ΔCt values were calculated as the difference between the target and reference genes: ($\Delta Ct = Ct_{target}$-$Ct_{housekeeping\ gene}$)[14]. The final results were reported as fold-changes calculated from ΔΔCt values.

Statistical analysis: The association between c.17492542 G>C of SCD and FA composition was analyzed using the General Linear Model procedure (SAS Institute Inc., Cary, USA). The model was fitted with genotype (3 levels) and sex (2 levels) as fixed effects according to the following equation:

$$Y_{ij} = \mu + genotype_i + sex_j + e_{ij} \quad (1)$$

where Y_{ij} is the FA composition, µ is the overall mean of FA compositions, $genotype_i$ is the fixed effect of the i-th genotype (j = 1, 2 and 3), sex_j is the fixed effect of j-th sex (male/female)

and e_{ij} is the residual error. Least square mean values for loci genotypes were compared by t-test and p-values were adjusted by Tukey-Kramer correction[15,16].

Differences in SCD expression were analyzed with a paired t-test, a $p<0.05$ indicated statistically significant differences.

RESULTS

Phenotypic profile: The phenotypic profile (Table 2) shows the descriptive statistics for FA composition in Indonesian crossbred chickens (F2 Kampung×broilers). Twenty-one FA compositions, including total saturated FAs, PUFAs and monounsaturated FAs, were detected in each sample. Eight saturated FAs were found, namely lauric acid (C12:0, 0.04%), myristic acid (C14:0, 0.54%), pentadecanoic acid (C15:0, 0.09%), palmitic acid (C16:0, 19.65%), heptadecanoic acid (C17:0, 0.17%), stearic acid (C18:0, 5.26%), arachidic acid (C20:0, 0.16%) and behenic acid (C22:0, 0.06%). Monounsaturated FAs included C14:1, C16:1, C18:1n9t and C18:1n9c, while PUFAs included C18:2n6c, C18:3n6, C20:2, C20:4n6 and C22:6n3. The level of total saturated FAs was also lower than that of monounsaturated FAs and PUFAs (Table 2). The descriptive statistics and General Linear Modle for FA concentrations for HFA and LFA groups are described in Table 1. The coefficient of variation ranged from 0.68% to 10.8%, indicating high homogeneity within each group. The concentration of all FAs measured were significantly different ($p<0.01$) between the HFA and LFA groups (Table 1).

Gene polymorphism: A synonymous SNP g.17492542 C>G in SCD was confirmed by PCR-RFLP and 3 genotypes were found in our population: CC (407 bp), GC (407, 373 and 34 bp) and GG (373 and 34 bp) (Fig. 1). The CC genotype was more rare compared to GG and CG genotype in F2 Kampung×broilers population. The genotypic frequency of g.17492542 C>G polymorphism in SCD was detected by Hardy-Weinberg Equilibrium ($p<0.05$). The number of animals per genotype and allele frequency of the SNP is presented in Table 3.

SNP association with FA composition: Association analysis of SCD (g.17492542 C>G) with FA composition revealed a significant ($p<0.05$) association with unsaturated (C18:2n6c and C20:2) and saturated (C12:0) FAs (Table 4). Chickens with the homozygous CC genotype were associated with lower levels of unsaturated FAs (C18:2n6c and C20:2) and higher saturated FAs (C12:0) (Table 4). However, this association should be validated in a larger population.

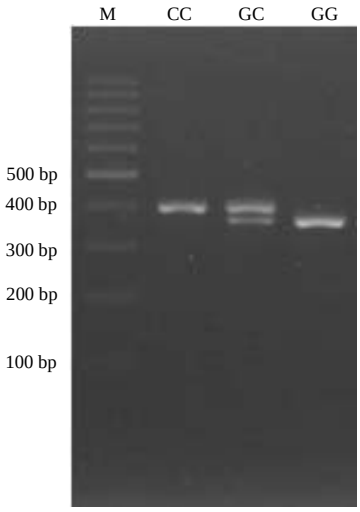

Fig. 1: PCR-RFLP genotyping results for SCD

Table 2: Fatty acid composition in Indonesian crossbred chickens (F2 Kampung×broilers)

Trait	Mean (%)	SD
Fat content	4.61	1.56
Lauric acid, (C12:0)	0.04	0.01
Myristic acid, (C14:0)	0.54	0.05
Myristoleic acid, (C14:1)	0.08	0.03
Pentadecanoic acid, (C15:0)	0.09	0.04
Palmitic acid, (C16:0)	19.65	1.62
Palmitoleic acid, (C16:1)	2.46	0.75
Heptadecanoic acid, (C17:0)	0.17	0.03
Stearic acid, (C18:0)	5.26	0.73
Elaidic acid, (C18:1n9t)	0.15	0.03
Oleic acid, (C18:1n9c)	31.31	1.99
Linoleic acid, (C18:2n6c)	20.64	1.78
Arachidic acid, (C20:0)	0.16	0.04
γ-Linoleic acid, (C18:3n6)	0.10	0.02
Cis 11, 14-Eicosedienoic Acid, (C20:2)	0.16	0.03
Behenic acid, (C22:0)	0.06	0.03
Arachidonic acid, (C20:4n6)	0.70	0.27
Docosahexaaonic (C22:6n3)	0.07	0.03
Fatty acid total	81.68	4.29
Saturated fatty acid	20.72	1.64
Monounsaturated fatty acid	33.39	2.52
Polyunsaturated fatty acid	21.66	1.85

SD: Standard deviation

SCD mRNA expression in divergent FA chickens: qRT-PCR analysis showed the abundance of SCD transcripts in birds with divergent FA composition in muscles. The results showed that SCD mRNA was differentially regulated between birds with high and low FA compositions ($p<0.05$) in the liver. Higher transcript abundance was detected in the liver of chickens with high fatty acid composition (HFA) compared to chickens with low fatty acid composition (LFA) (Fig. 2).

Table 3: Number of birds per genotype and allele frequency of each small nucleotide polymorphism

		Genotype frequency (%)			Allele frequency (%)	
Sample	N	CC (n = 2)	GC (n = 31)	GG (n = 29)	C	
Indonesian crossbred chicken						
(F2 Kampung×Broiler)	62	0.03	0.50	0.47	0.28	0.72

Table 4: Genotype and association analysis of the stearoyl-CoA desaturase (SCD) gene

	Genotype (%)		
Trait	CC (n = 2)	CG (n = 29)	GG (n = 31)
Fat content	6.48±3.24[a]	4.30±1.44[b]	4.80±1.52[ab]
Lauric acid, (C12:0)	0.07±0.04[a]	0.04±0.01[b]	0.04±0.01[b]
Myristic acid, (C14:0)	0.54±0.06	0.53±0.06	0.55±0.04
Myristoleic acid, (C14:1)	0.08±0.00	0.08±0.02	0.08±0.04
Pentadecanoic acid, (C15:0)	0.08±0.01	0.09±0.01	0.10±0.06
Palmitic acid, (C16:0)	19.69±1.71	19.24±1.79	20.09±1.34
Palmitoleic acid, (C16:1)	2.49±0.13	2.48±0.66	2.43±0.87
Heptadecanoic acid, (C17:0)	0.16±0.01	0.17±0.02	0.17±0.02
Stearic acid, (C18:0)	4.66±0.33	5.14±0.67	5.43±0.79
Elaidic acid, (C18:1n9t)	0.13±0.02	0.15±0.03	0.15±0.03
Oleic acid, (C18:1n9c)	29.57±1.58	31.17±1.98	31.59±2.00
Linoleic acid, (C18:2n6c)	18.44±1.90[b]	20.81±1.69[a]	20.62±1.82[a]
Arachidic acid, (C20:0)	0.13±0.03	0.17±0.04	0.16±0.03
γ-Linoleic acid, (C18:3n6)	0.09±0.00	0.09±0.02	0.10±0.02
Cis 11, 14-Eicosedienoic Acid, (C20:2)	0.13±0.03[b]	0.17±0.02[a]	0.16±0.03[ab]
Behenic acid, (C22:0)	0.04±0.01	0.06±0.03	0.05±0.02
Arachidonic acid, (C20:4n6)	0.52±0.16	0.73±0.27	0.68±0.27
Docosahexaaonic (C22:6n3)	0.04±0.01	0.07±0.03	0.06±0.02
Fatty acid total	76.84±1.73[b]	81.23±4.72[ab]	82.49±3.91[a]
Saturated fatty acid	20.70±1.78	20.31±1.82	21.17±1.35
Monounsaturated fatty acid	32.26±1.73	33.87±2.31	34.25±2.79
Polyunsaturated fatty acid	19.22±2.09[b]	21.87±1.68[a]	21.61±1.96[a]

Mean±SD are units of percentage fatty acid composition, [ab]Mean value with different superscript letters in the same row differ significantly at p<0.05

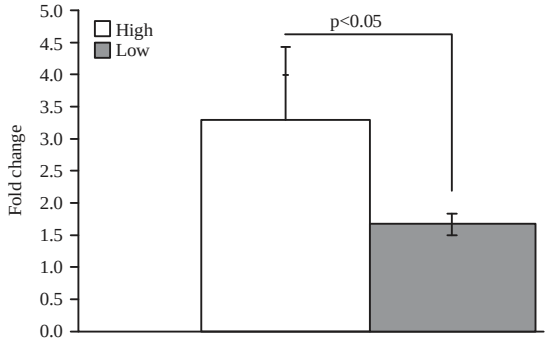

Fig. 2: mRNA expression of SCD in the liver HFA and LFA chickens

DISCUSSION

SCD plays important roles in FA production in the muscle of chickens. Significant differential expression of SCD was found in a previous study[5] and its physiological roles and chromosomal position, which incorporates several QTL related to FA production in chickens, were of particular interest. Furthermore, exonic SNP g.17492542 C>G in SCD was found to be significantly associated with unsaturated (C18:2n6c and C20:2) and saturated (C12:0) FA composition. In a previous study of broiler chickens, g.3728 A>G of *SCD* was significantly associated with monounsaturated FAs (C14:1 and C16:1) and saturated FAs (C16:0) but not with unsaturated Fas[5].

SCD is a microsomal enzyme that catalyzes the synthesis of monounsaturated FAs from saturated fatty acyl-CoAs. The preferred substrates for SCD are palmitoyl and stearoyl-CoA, which are converted to palmitoleoyl and oleoyl-CoA, respectively[4]. Hence, its function likely directly affects the FA composition in muscle. In paritcular, palmitoleic acid (C16:1) is considered to have a positive effect by reducing bad cholesterols[17], fat deposition in blood vessels and subsequent blood clot formation[18].

The present study showed the SCD polymorphism g.17492542 C>G is associated with C18:2 and C20:2 composition in Indonesian crossbred chickens (F2 Kampung×broilers). These results are in agreement with a previous study which showed that SCD is significantly higher (p<0.05) in Lantang pigs that have higher PUFA levels (C18:2

and C20:2)[1]. Moreover, homozygous GG genotype birds had increased C18:2 and C20:2 levels and decreased C12:0. High intake of saturated FAs can result in elevated plasma cholesterol, which leads to cardiovascular disease. Saturated FAs, such as C12:0, C14:0 and C16:0, have deleterious influences on cardiovascular health[19]. In contrast, linoleic acid has been shown to positively correlate with meat flavor and human health[1,2,17]. Moreover, C18:2, C20:4 and C22:6 PUFAs have also been shown to have a positive correlation with the flavor of meat[2], these PUFAs are high in Lantang pig muscles and considered flavor precursors in meat, indicating these pigs have better pork flavor intensity[20]. Additionally, the content of these PUFAs in Lantang pig meat is beneficial to human health due to their many functions in the human body[21].

The present study showed that SCD mRNA expression upregulated in liver tissues from chickens with high unsaturated FA levels (C18:2 and C20:2) in muscles (Fig. 2). This result may indicate SCD's functions with regard to FA metabolism in the liver. The expression pattern of SCD was also consistent with a previous study which used FA transcriptome profiling[1]. These results suggest that SCD expression is higher in chickens with higher C18:2, C18:3, arachidonic acid (C20:1, C20:3 and C20:4) and C20:5 levels, as in pigs.

Gebauer *et al.*[22] reported that appropriate dietary intake of eicosapentaenoic acid (C20:5n-3) and docosahexaenoic acid (C22:6n-3) reduced the risk of cardiovascular diseases. Moreover, PUFAs are required for the normal sperm, retina and brain lipid development and composition, as well as for optimal maturation of visual and cortical function in preterm infants[21]. In the present study, over expression of SCD led to an increased proportion of PUFAs, including C18:2 and C20:2 and reduced C12:0 in chicken muscles. These results suggest that SCD has other ways of affecting FA composition besides acting as a desaturase. Alterations in this desaturation ratio have been linked to cardiovascular disease, obesity, diabetes and cancer[23-27] and have also been correlated with longevity[28]. Recent evidence indicates that SCD also plays an important role in defining plasma and tissue lipid profiles[24].

In chickens, SCD is assigned to chromosome GGA6q17 (QTL database). In pigs, several studies have reported that SCD maps to SSC14q27[5,29,30], which incorporates the QTLs for FA muscle content[31,32]. Hence, SCD is an attractive positional candidate gene[9]. In fact, findings to date have suggested there is genetic variation in SCD affecting FA composition of muscles and adipose tissue. Heightened expression and activity of SCD1 has been shown to correspond with low rates of FA oxidation[28]. Moreover, Dobrzyn *et al.*[33] reported that SCD1-deficient mice have increased energy expenditure and upregulated expression of several genes encoding enzymes necessary for FA β-oxidation. Although the mechanism is not known, FA metabolism could affect cellular FA uptake[34]. Therefore, an increased proportion of PUFAs may be due to changes in FA metabolism or uptake related to up regulation of SCD.

CONCLUSION

SCD may control other FA desaturases or elongases required for PUFA synthesis. It is also possible that Indonesian crossbred chickens have good flavor intensity and human health benefits because of its higher C18:2 content. Therefore, the association and expression of SCD in chickens could be an important indicator of better quality meat. However, these results require validation with large sample sizes and in other commercial breeds.

SIGNIFICANCE STATEMENT

The present study describes for the first time an association between expression of SCD polymorphisms and FA composition in Indonesian crossbred chickens, implicating SCD as an important candidate for selection of birds with high meat and nutritional quality. The results of the present study serve to improve the current understanding of SCD function and shed light on its potential as a marker of high unsaturated and low saturated FA composition in chicken meat.

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