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Association and Expression of Cluster of Differentiation 4 (CD4) Gene in IPB-D2 Chicken Related to Immunocompetence Index

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ABSTRACT

The CD4 gene plays an important role in the immune process by producing CD4 molecules that aid in producing antibodies. IPB-D2 chickens are selected from IPB-D2 chickens based on IgY concentration and ND antibody titer. This study aimed to analyze the polymorphism of the CD4 gene, unravel the mRNA expression of CD4 gene in IPB-D2 chicken related to the immunocompetence index, and detect the CD4 gene pathway. The total samples used were 100 IPB-D2 G2 chickens aged 21 weeks. Blood samples were collected for ELISA test, HI test, sequencing test, and seca tonsil tissue for relative mRNA expression. Polymorphism and association data were analyzed using MEGAX, FinchTV, SNPstat, and DNAsp. The relative mRNA expression analysis was conducted using qRT-PCR. The pathway analysis of the CD4 gene was performed using the Kyoto Encyclopedia of Genes and Genome (KEGG) pathway analysis. The result showed there were 4 SNPs of the CD4 gene in IPB-D2 chicken, i.e., g.7526 C>T, g.7825 C>T, g.8100 C>A, and g.8157 T>A. All CD4 SNPSs showed no association with IgY concentration and ND antibody titers. Relative mRNA expression shows that IPB-D2 chickens with high ND antibody titers have a higher level of expression when compared to IPB-D2 chickens with low ND antibody titers. Furthermore, pathway analysis showed the CD4 gene involved in the T cell receptor (TcR) signaling process. This study concludes that the CD4 gene is polymorphic and involved in the T cell receptor signaling process. This study demonstrated that polymorphisms of the CD4 gene in IPB-D2 chicken might not contribute to the IgY concentration and ND antibody titer but can serve as a reference in the study of CD4 genes in the other chicken breeds related to the other immunocompetence index.

Keywords: CD4 gene; IPB-D2 chicken; IgY concentration; ND antibody titer

INTRODUCTION

The genetic attribute of immunity for disease resistance is complex and complicated. In chickens, genetic variations in how their immune systems react to particular antigens allow for a moderate genetic response to selection for increased and decreased indexes. Various genetic variables have been discovered to influence chicken immunity and disease resistance. Many disease-resistant genes have been found, thanks to recent molecular biology developments. Major Histocompatibility Complex (MHC) genes, Natural Resistance-Associated Macrophage Protein 1 (Nramp1 gene), IFN genes, Myxovirus-Resistance (Mx) genes, Avian Leucosis Virus (anti-ALV) genes, and the Zyxin gene have all been connected to disease resistance in chicken (Dar *et al.*, 2018).

IPB-D2 chicken is one of the candidate chicken lines selected based on disease resistance traits. IPB-D2 chicken is selected from its parents, IPB-D2 chicken with several immunocompetence indexes such as IgY concentration and ND antibody titer. In addition to conventional selection, IPB-D2 chicken selection efforts are also carried out molecularly by analyzing gene diversity and association. Several molecular studies on IPB-D2 G0 chickens have been conducted. Lestari *et al.* (2022) stated that SNPs in the DMA gene are associated with IgY concentrations in IPB-D2 G0 chickens with an average IgY of 10.28±1.77 mg mL-1. In their research, Miraj *et al*. (2022) stated that there were 6 SNPs in the BG1 gene of IPB-D2 chickens and 2 deletions that caused changes in the amino acids formed. In addition, Putri *et al.* (2022) also stated that there were 2 polymorphic SNPs in the Thy1 gene intron.

CD4 (cluster of differentiation 4) is the accessory protein non-covalently bound to the T cell receptor that recognizes an invariant region of MHC class II on antigen-presenting cells (Napolitano *et al.*, 2021). CD4 plays

a role in differentiation, migration, and cytokine expression (Glatzova & Cabecauer, 2019). The CD4 molecule is encoded by the CD4 gene.

In chicken, the CD4 gene is located in chromosome 1, consisting of 10 exons (GenBank Accession Number: NC_006088.4). It is already known that certain variations in the CD4 gene sequence impact certain diseases. According to Oyugi *et al.* (2009), CD4 gene variations can make people more susceptible to contracting HIV. Recent studies have also highlighted an important function for CD4 in immune and production traits, particularly in dairy cows. Zeb *et al.* (2020) found that four SNPs between exons 2 and 4 of the CD4 gene were strongly linked to the number of clinical mastitis cases and the milk produced each year. Napolitano *et al.* (2021) also stated that haplotypes in the CD4 gene are significantly associated with milk and protein yields in Simmental sires.

Controlling the expression of the CD4 gene is critical for optimal T lymphocyte development. CD4 molecules are essential for T-cell antigen recognition because they determine whether a T cell can detect an antigen presented by MHC molecules that acquire their peptide antigens predominantly from extracellular (MHC class II) sources (Morel, 2018). Signals conveyed by the T-cell antigen receptor (TCR) during thymic selection processes are thought to regulate CD4 gene expression during various phases of T-cell development (Sarasofa & Siu, 1999). Expression of CD4 gene promoter in chickens infected with Marek's virus shows a downregulated mechanism (Luo *et al.*, 2011).

Many studies on the diversity and association of the CD4 gene with disease-resistance traits have been carried out in humans, cattle, and pigs. However, no study investigated the association and expression of the CD4 gene with immunocompetence index in chickens, especially in IPB-D2 chicken. This study aimed to analyze the polymorphism of the CD4 gene, to unravel the mRNA expression of the CD4 gene in IPB-D2 chicken related to immunocompetence index, and to detect the CD4 gene pathway.

MATERIALS AND METHODS

Animals and Blood Samples

The total samples used were 100 IPB-D2 G2 (second generation) chickens aged 21 weeks (60 female and 40 male). The chickens were reared in an intensive system and fed twice a day. The fed was given 100% commercial feed for DOC up to 4 weeks old and commercial feed and rice bran with a ratio of 70:30 for 4 to 12 weeks old. Chicken at 12 to 21 weeks old was given commercial and rice bran with a ratio of 60:40. Water was given ad libitum. Chicken kept in a cage with facilities for feeding, drinking water, laying eggs, and husks.

The blood sample was taken from the brachialis vein using a 3 mL syringe as much as 2-2.5 mL. The Brachialis vein was cleaned using 70% alcohol before and after blood collection. The blood was then stored in a syringe to separate the serum and placed in an EDTA tube for CD4 gene diversity analysis. This experimental procedure was approved by Institutional Animal Care and Use Committee (IACUC) at IPB University (approval ID: 224-2021 IPB).

Immunocompetence Index Analysis

IgY concentration measurement. IgY concentration was determined using an indirect ELISA method based on Jiang *et al.* (2018) with some modifications. Chicken serum was isolated from fresh chicken whole blood using centrifugation for 8 minutes at 8000 rpm. The incubation of each step was at 37 °C for an hour, and between each step, the microplate was washed with phosphate-buffered saline containing Tween 20 (PBST-20, pH 7.4) three times. To begin, 96-well plates were coated with goat anti-IgY (SAB3700195 Sigma-Aldrich, 2.5 gmL^{-1}) diluted in bicarbonate buffer (Na_2NO_3) pH 9.6 at 4 °C for one whole night. After that, blocked the 100 µL with 2% BSA at 37 °C for an hour. Each well had serum that had been diluted by a factor of 100 added to it and incubated for 1 hour at 37 °C.

Each well was then given 100 µL of a peroxidase enzyme-conjugated secondary antibody made from IgG rabbit anti-IgY (A9046 Sigma-Aldircj). In a volume of 100 µL, the substrate solution was added to each well. Afterward, H_2SO4 was added to the reaction, and the absorbance was measured at 450 nm using a microplate reader (Bio-Rad, USA).

ND antibody titers measurement. ND antibody titers were determined using Hemagglutination Inhibition (HI) test based on OIE (2012). The HI test was used to evaluate the ND antibody titers, based on the resistance in dilution that can bind antigen at a concentration of 4HAU unit and prevent red blood cell agglutination. The first step was to add 25 µL of PBS to the microplate. Next, 25 µL of the serum was added to the first row of wells and serially diluted to the 11^{th} row. Then, 25 µL of antigen was added to all wells except the 12^{th} row and incubated for 30 minutes at room temperature. After that, 25 µL of 1% RBC was added to all wells. The presence of RBCs and antigen was a positive control in the 11th well, while RBCs alone was a negative control in the 12^{th} well. The RBCs were allowed to settle for 40 minutes at room temperature by gently shaking the microplate. A sharp button formed as a result of the settlement of whole RBCs was noted as a positive test result after the test result was evaluated by tilting the plates. The test's endpoint was the maximum dilution of each sample, and the serum antibody titer was computed from that by measuring the observed result backward.

CD4 Gene Polymorphism Analysis

Genomic DNA samples were taken from the whole fresh blood using the genomic DNA extraction mini kit (GeneaidTM, Taiwan) as directed by the manufacturer. The primers were designed using PrimerStat. Primer base sequence using data from National Center for Biotechnology Information (NCBI) access code

Table 1. Primer sequence of CD4 polymorphism analysis in IPB-D2 chicken

Gene	Primer sequence	PCR product (bp)	Annealing temperature (°C)
CD4	F: 5′ -GATGGGACCTGTACTTGG- 3′ R: 5′ -GAGTCTGTGTGCAAGCTG- 3′	863	58

Table 2. Primer sequence of CD4 and GADPH gene for relative mRNA expression analysis in IPB-D2 chicken

No	Gene	Primer sequence	PCR product (bp)	Temperature (°C)
1	CD4	F: 5′- GTGTCAGACTTGAGCCTGGA -3′	179	62
		R: 5- CTGAGATGGGGTTGTGAGTG -3′		
2	GAPDH	F: 5′- CACTGTCAAGGCTGAGAACG -3′	179	62
		R: 5- GCTTAGCACCACCCTTCAGA -3′		

NC_006088.4 with a target exon 3-5 along 863 bp. The primer sequences are presented in Table 1.

The final PCR volume of the CD4 gene was 25μL (20 pmol μL^{-1}) consisting of DNA samples of 1 μL, 11 μL of DW, forward and reverse primer 0.25 μL each, MyTaqTM HS Red Mix 12.5 μL. Amplification is performed using a thermocycler machine (Applied Biosystem 9700) with the following procedure: pre-denaturation at 95 °C for 1 minute, denaturation at 95 °C for 15 seconds, annealing at 58 °C for 15 seconds, extension at 72 °C for 10 seconds for 30 cycles. Visualization of PCR products using 1% agarose gel in an electrophoresis machine (Mupid Exu). CD4 gene diversity was identified using the direct sequencing services of Macrogen, Korea.

Relative mRNA Expression of CD4 Gene

The sample used in the CD4 gene expression analysis was IPB-D2 chicken seca tonsil tissue based on differences in ND antibody titers. RNA extraction is performed according to the protocol of the kit used (RNeasy Mini Kit, Qiagen). Complementary DNA (cDNA) was synthesized using a First Strand cDNA Kit (Thermo Scientific). Quantification of cDNA is performed using the qRT-PCR method. The primers used in qRT-PCR analysis were designed using PrimerStat (Table 2). The qRT-PCR mix consists of 2 μL of cDNA sample, 3 μL of DW, 0.5 μL of forward primer, 0.5 of reverse primer, and 5 μL of Sbyr Green (Toyobo THUNDERBIRD® SYBR® qPCR Mix). Quantification was performed under conditions of 95 °C for 1 minute, 95 °C for 15 seconds, and 62 °C for 1 minute with 40 repetitions. All samples were analyzed 3 times and the geometric mean of the Ct values will be used to profile the mRNA expression. The geometric mean of the GADPH housekeeping gene will be used to normalize the target gene.

Pathway Analysis

Pathways analysis of the CD4 gene was identified based on Gunawan *et al.* (2018) by KEGG pathway analysis using Cytoscape software.

Statistical Analysis

Polymorphism analysis. Polymorphism of the CD4 gene was analyzed using MEGAX and FinchTv. Allele frequencies, genotype frequencies, and Hardy-Weinberg equilibrium values were calculated using SNPStat (Pan *et al.*, 2019).

Association analysis. Data were standardized by sex and then associated with the immunocompetence index. Association analysis of the CD4 gene related to immunocompetence index analyses was performed using SAS ver 9.2 (SAS Institute Inc., Cary, USA). The genotype mean values were compared with the T-test.

Expression analysis. The difference in CD4 mRNA expression was analyzed with the T-test in Minitab based on delta Ct (ΔCt). Values of $p<0.05$ indicate a statically significant difference. The (ΔCt) calculated based on Silver *et al.* (2006) :

$$\Delta Ct = Ct_{target} - Ct_{housekeepinggene}.$$

RESULTS

Polymorphism of CD4 Gene

The CD4 gene in chickens is located on chromosome 1 with a length of 11468 bp, consisting of a promoter, 10 exons, and 9 introns. The result of CD4 gene amplification produced 863 bp (Figure 1). Based on the alignment results with NCBI data (NC_006088.4), 4 CD4 gene SNPs were found in IPB-D2 chickens. A total of 4 SNPs were found, i.e., g.7526 C>T on intron 3, g.7825 C>T on intron 4, g.8100 C>A, and g.8157 T>A on exon 5 (Figure 2). Genotype frequency, allele frequency, Hardy-Weinberg equilibrium, and heterozygosity of the CD4 gene are presented in Table 3. All SNP were polymorphic. SNPs g.8100 C>A and g.8157 T>A in exon 5 are nonsynonymous SNPs causing amino acid changes.

Association of CD4 gene

The association of SNPs in the CD4 gene with IgY concentration and ND antibody titers in IPB-D2 chicken is presented in Table 4. The SNP with the highest IgY

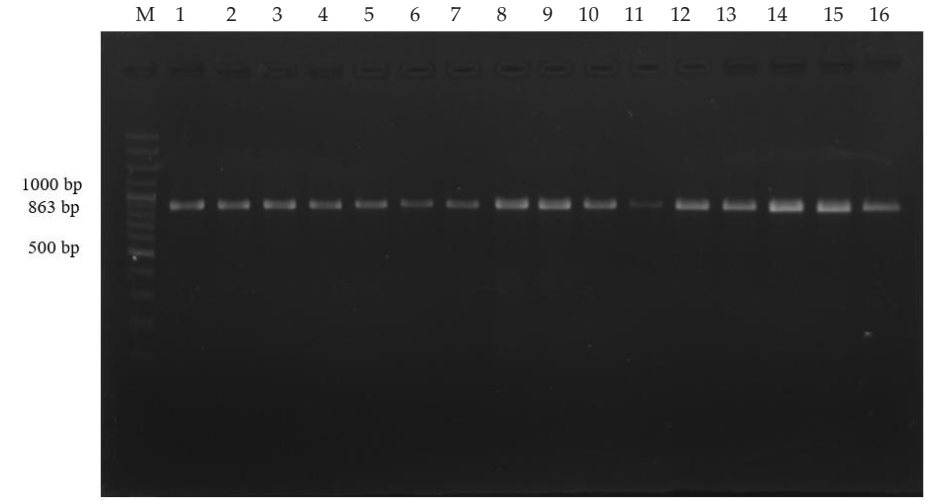

Figure 1. Amplification of CD4 gene in IPB-D2 chicken. M= Marker (100 bp), 1-16= Samples.

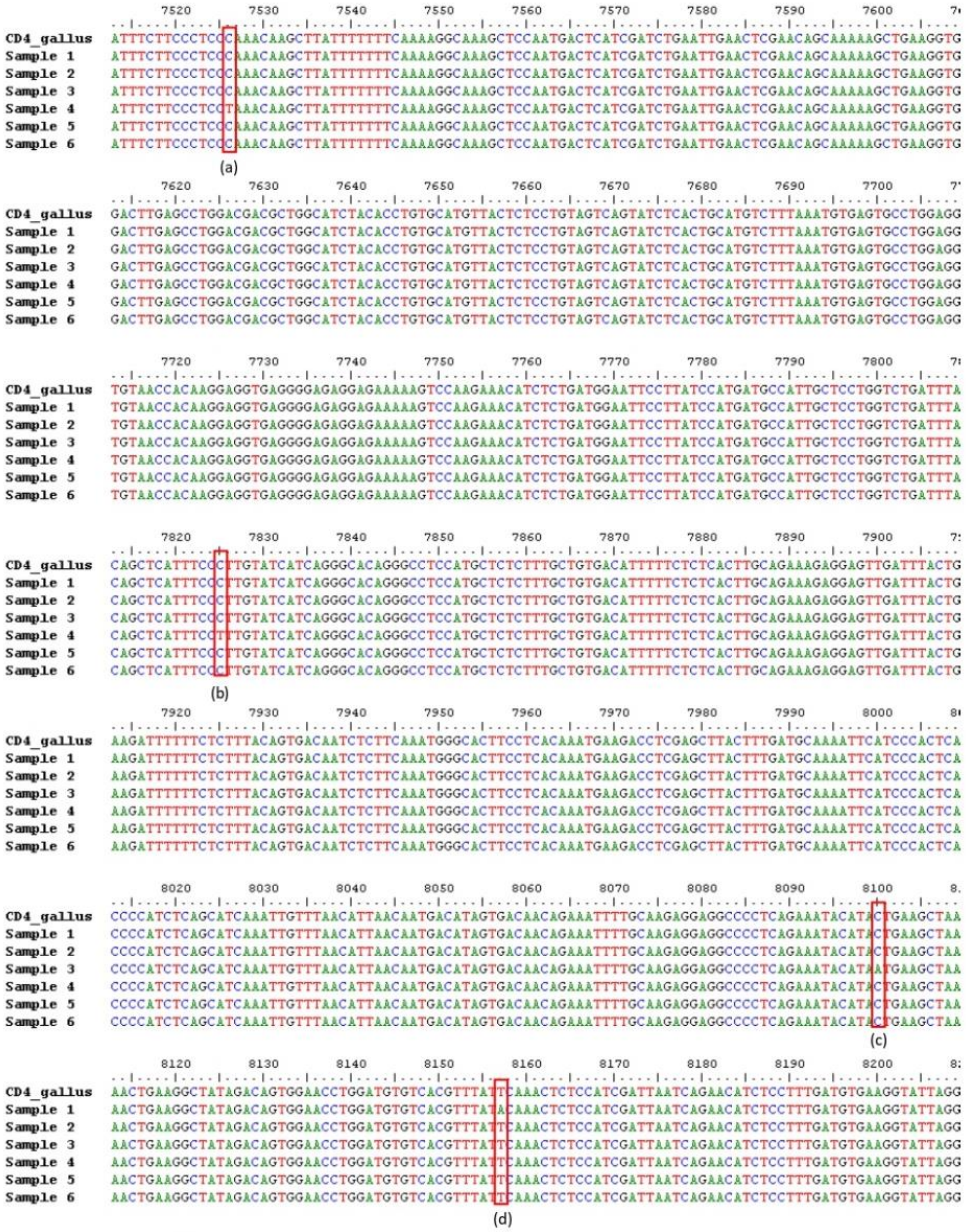

Figure 2. SNPs of CD4 gene in IPB-D2 chicken. Red box indicates SNP (a, b, c, d). Intron 3: 7465 7550, exon 4: 7551-7694, intron 4: 7695-7934, exon 5: 7933-8208.

Table 3. Genotype and allele frequency, Hardy-Weinberg equilibrium and heterozygosity of CD4 gene in IPB-D2 chicken

SNPs	Genotype frequency (n)						Allele frequency			x^2	Ho	He
	CC	CT	TT	TA	CA	AA	C	T	A			
g.7526 C>T	0.94 (66)	0.04 (3)	0.01 (1)				0.96	0.04		0.071	0.043	0.069
g.7825 C>T	0.99 (69)	0.01 (1)					0.99	0.01		1	0.014	0.014
g.8100 C>A	0.99 (69)				0.01 (1)		0.99		0.01	1	0.014	0.014
g.8157 T>A			0.99 (69)			0.01 (1)		0.99	0.01	0.007	0	0.028

Note: n: Number of sample.

Table 4. Association of SNP of CD4 gene with IgY concentration and Nd antibody titers in IPB-D2 chicken

SNPs	Genotypes	IgY concentration (mg mL^{-1}) (n)	ND antibody titers (log_2) (n)
g.7526 C>T	CC	12.41±2.01 (66)	2.30±1.96 (57)
	CT	13.58±0.30 (3)	0.45±0.31 (3)
	TT	12.20 (1)	1.34 (1)
g.7825 C>T	CC	12.47±1.96 (69)	2.21±1.96 (60)
	CT	12.20 (1)	1.34 (1)
	TT		
g.8100 C>A	CC	12.47±1.98 (69)	2.19±1.96 (60)
	CA	12.17 (1)	2.69 (1)
	AA		
g.8157 T>A	TT	12.45±1.97 (69)	2.23±1.94 (60)
	TA		
	AA	13.61 (1)	0 (1)

concentration is g.7526 C>T CT genotype. Based on statistical analysis, the SNP in the CD4 gene did not significantly differ between genotypes for IgY concentrations and ND antibody titers.

Relative mRNA Expression of CD4 Gene

Based on the qRT-PCR results, it can be illustrated that the delta Ct value of the chicken population with low ND titer is higher than that of the chicken population with high ND titer. This indicates that chickens with low ND titers can produce fewer CD4 transcripts than chickens with high ND titers (Figure 3).

Pathway Analysis of CD4 Gene

Pathway analysis showed that the CD4 gene involved in the signaling T cell receptor (TcR) (Figure 4). CD4 molecules play a role in the immune system in the T cell receptor signaling pathway together with class II MHC molecules.

DISCUSSION

Various techniques can be used to characterize genetic information. Recently, it has been common practice to characterize objects using molecular markers that can detect genetic diversity at the DNA level. A total of 4 SNPs were found, i.e., g.7526 C>T on intron 3, g.7825 C>T on intron 4, g.8100 C>A, and g.8157 T>A on exon 5. An imbalance in the population genotype can be determined by comparing the values of Ho and He (Tambasco *et al.* 2003). The SNP of the CD4 gene has a lower Ho value than the He value. This indicates that the SNP of the CD4 gene is not on the Hardy-Weinberg equilibrium. This corresponds to the value of x^2. The Hardy-Weinberg equilibrium test was performed to determine whether or not the population is stable (Tambasco *et al.*, 2003). IPB-D2 chickens are selected based on IPB-D1 chickens with the criteria of high concentration and ND antibody titer. Selection is made by mating individuals from generations that meet the criteria to get the next generation, and this can result in

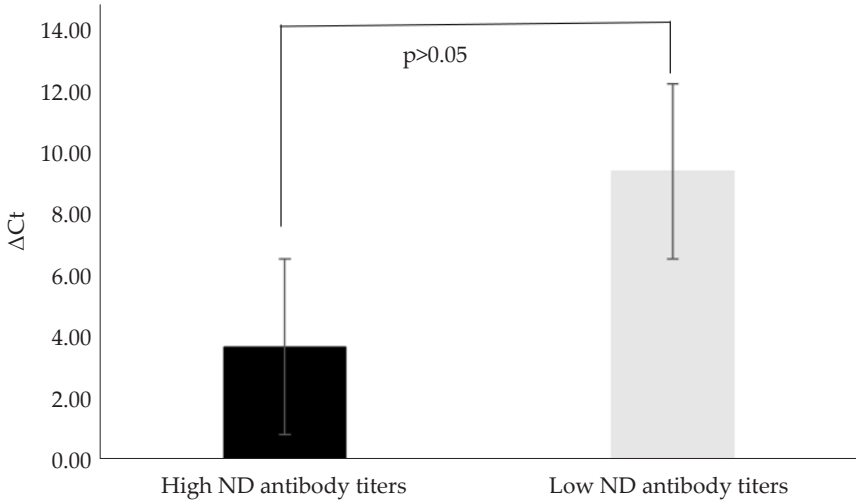

Figure 3. Relative mRNA expression of CD4 gene in IPB-D2 chicken

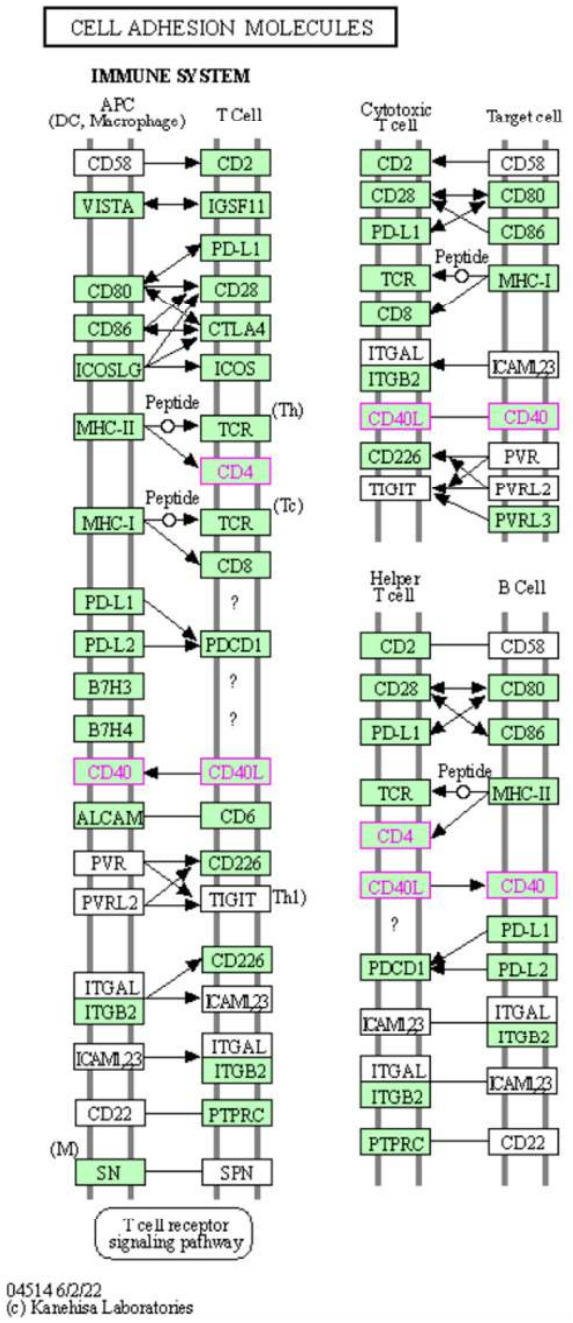

Figure 4. Pathway of CD4 gene in IPB-D2 chicken (KEGG, 2023)

IPB-D2 chickens not being in Hardy-Weinberg equilibrium because there is inbreeding.

There are 2 SNPs that caused amino acid changes, i.e., g.8100 C>A and g.8157 T>A. Changed base C to A at 8100 changed the amino acid leucine into methionine, while the change of T to A at 8157 changed the amino acid serine into threonine. SNPs that can alter the amino acid sequence and alter the structure of proteins can have an impact on how a gene functions (Putri & Wathon, 2018). The changes in leucine and methionine that occur do not have different impacts. Leucine and methionine have the same structure and function, which is a hydrophobic protein. The leucine and methionine side chains are fairly non-reactive and are thus rarely directly involved in protein function. The changes from serine to threonine also have no different impacts. Serine and threonine have the same structure and function. As fairly indifferent amino acids, serine and threonine can reside on the inside or surface of proteins. Serine and threonine are quite commonly found in the functional centers of proteins (Betts & Russell, 2003).

IgY is the main antibody in chicken (Wang *et al.*, 2019). The concentration of total serum IgY in chickens can be used to measure their fitness, health, and nutrition (Sun *et al.*, 2013). The average IgY concentration of IPB-D2 chickens was higher than IPB-D1 chickens (Al-Habib *et al.*, 2020), and the normal concentration was 5-10 mg mL^{-1} (Oberlander *et al.*, 2020). Based on statistical analysis, the SNP in the CD4 gene did not significantly differ between genotypes, for IgY concentrations, SNP g.7526 C>T CT has the highest average IgY concentration of 13.85±0.18 mg mL^{-1}.

The protective immune reaction against poultry Newcastle disease is indicated by serum antibody titers (Rahman *et al.*, 2017). Sarcheshmei *et al.* (2016) state that ND antibody titer > 3 $\log_2$ HI unit is protective against the ND virus. IPB-D2 chickens had a mean ND antibody titer of 1.55±1.4 $\log_2$ HI units. A lower serum antibody titer may be caused by either immunization failure or by maternal antibodies that neutralize the vaccine virus (Rahman *et al.*, 2017). Based on statistical analysis, the SNP in the CD4 gene did not significantly differ between genotypes for ND antibody titer in IPB-D2 chicken. These results may be due to the use of IPB-D2 chickens with almost uniform IgY and ND concentration criteria.

qRT-PCR analysis of the CD4 gene in IPB-D2 chickens using seca tonsil tissue from IPB-D2 chickens. Seca tonsil tissue is included in the GALT (gut-associated lymphoid tissue) category of secondary lymphoid tissues. Tonsil tissue is made up of lymphoid nodules that form lymphoid organs. The tonsils are the largest galit lymphoid organs connecting the cecum and rectum (Hewajuli & Dharmayanti, 2015). The expression of CD4 gene mRNA in the high ND antibody titer category was higher than in the low ND antibody titer category. This is because the CD4 molecule has a role in helping B cells to produce antibodies, so chickens with high ND antibody titers have higher CD4 gene mRNA expression than chickens with low ND antibody titers. Different from Luo *et al.* (2011) study, which stated that the CD4 gene promoter region had a downregulated mechanism when infected with the Marek virus.

T cells are essential for developing an efficient adaptive immune response mediated by cells. Using KEGG pathway analysis, the participation of the CD4 gene in the T cell receptor signaling pathway has been validated and demonstrated (Figure 4). Normal T cell development in the thymus involves T cell receptor (TCR) signaling at a critical developmental phase (Shah *et al.*, 2021). T cell function is controlled by TcR activation and signaling. TcRs interact with small peptides presented by MHC class I or II molecules (MHC class I for CD8 T cells and MHC class II for CD4 T cells). For TcRs to send signals, they need coreceptors like CD4 for Th cells and CD8 for Tc cells. These co-receptors act as cell adhesion molecules that bind to MHC molecules

and keep T cells and MHC cells from getting in each other's way (Kadzierska & Kaoutsakos, 2020).

The utilization of genetic variation in the selection of a trait has been widely done. Although the results of this study did not show significant results, the CD4 gene has the potential in selection related to immunocompetence index, such as IgY concentration and ND antibody titer in local Indonesian chickens, especially IPB-D2 chickens. Additional samples can be done for analysis related to the CD4 gene in the other chickens or next-generation IPB-D2 chickens.

CONCLUSION

In conclusion, there are 4 SNPs in the CD4 gene. Those SNPs are not associated with IgY concentrations and ND antibody titers in IPB-D2 chickens. The CD4 gene expression level in IPB-D2 chickens is upregulated and may involve in the T cell receptors signaling process. This study demonstrated that polymorphisms of the CD4 gene in IPB-D2 chicken might not contribute to the IgY concentration and ND antibody titer but can serve as a reference in the study of CD4 genes in the other chicken breeds related to the other immunocompetence index.

CONFLICT OF INTEREST

A. Gunawan and C. Sumantri serve as editors of the Tropical Animal Science Journal, but have no role in the decision to publish this article. The authors' names are listed to certify that they have no affiliations with or involvement in any organization or entity with any financial or non-financial interest in the materials discussed in this manuscript.

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