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Apolipoprotein E4 Prevalence in Different Canine Breeds

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ABSTRACT

Three common isoforms of apolipoprotein E (APOE) are found in most populations: E2, E3, and E4, the E4 isoform has been proven to be associated with Alzheimer's disease (AD) and coronary heart disease (CHD). In human, attributable risk due to APOE4 varies by region and by race/ethnicity. To gain a better understanding of APOE genetic polymorphism in canines, we aimed to explore the prevalence of apolipoprotein E isoform distribution in five different pure breeds (Thai Ridgeback, Golden Retriever, Labrador Retriever, Bulldog, Shih Tzu) using APOE gene sequencing analysis. Our results showed a very high homology of apolipoprotein E sequences in the examined canines, including human APOE3 and human APOE4, ranging from 71.76-100 and 78.23-100% identity and similarity, respectively. All investigated canine breeds could be identified as APOE4 carriers due to the presence of arginine at the positions 112 and 158, which correspond to APOE4 of human. This indicates that APOE polymorphism was not present in the genome of all examined canine breeds. It is possible that the finding of canine apolipoprotein E4 may be important in predicting an increased risk of pathophysiological development of neurodegenerative diseases and coronary heart disease. Our reconstructed phylogenetic tree showed two well-defined clusters based on the amino acid sequence of apolipoprotein E, including canine and human group. Further studies should explore if these different clusters are associated with diseases.

Key words: Apolipoprotein E, Canine, Polymorphism

INTRODUCTION

Apolipoprotein E (APOE) is a protein synthesized the APOE gene. It plays an important role in lipid metabolism and lipid transport in the brain of human and other mammals (Heffernan et al. 2016; Fernandez et al. 2019). This lipoprotein has been one of the most thoroughly studied genetic polymorphisms, particularly for its effects on genetic risk factor for the neurodegeneration in sporadic Alzheimer's disease (AD) (Roda et al. 2019), cerebrovascular disease (CVD) and coronary heart disease (CHD) (Lv et al. 2020). Apolipoprotein E has been found to exhibit in three isoforms, including APOE2, APOE3 and APOE4. These isoforms are structurally different due to the two amino acid substitutions at positions 112 and 158 in the protein with cysteine/arginine exchange. The most common isoform APOE3 contains cysteine and arginine at these two positions, whereas the APOE2 isoform contains cysteine and the APOE4 isoform contains arginine at both positions (Zhong et al. 2016). Genetic polymorphism in APOE4 of apolipoprotein E gene (APOE) has been documented as an increased chance of developing the development of late-onset Alzheimer's disease (LOAD) along with polymorphism in TREM2 gene, which plays an important role in nourishing normal immune functions in the brain (Wolfe et al. 2018). Although researchers have identified several loci as risk factors for AD, including APOE, ABCA7, APP, CLU, CR1, PICALM, BIN1, MS4A, MEF2C CD33, EPHA1, CD2AP, SORL1, TRIP4 and TREM2 (Ashford 2004; Karch et al. 2012; Mäkelä et al. 2018). Remarkably, the APOE4 polymorphism has been uncovered to be unique in the genome of humans and non-human primates, including baboons (*Papio hamadryas*) and cynomolgus macaque (*Macaca fascicularis*). Moreover, many studies demonstrated that APOE4 carriers are more likely to develop coronary heart disease (CHD), whereas APOE2 carriers are associated with a lower risk of Alzheimer's dementia pathogenesis (Anuurad et al. 2006; Walker and Jucker 2017). As mentioned above, it can be concluded that APOE4 carriers are likely to be a significant genetic risk determinant for AD and CHD pathogenesis. However, there have been no published evidence on the prevalence of the APOE polymorphism in different canine breeds in Thailand. Consequently, we aimed to investigate APOE gene polymorphism in the different canine breeds using a polymerase chain reaction (PCR) and nucleotide

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sequencing approach. The obtained information could serve as potential use of the APOE gene polymorphism as key data for further studies on the relationship between the apolipoprotein E4 expression profile and various dog breeds, which could lead to the subsequent development of biomarkers for early detection of age-related neurodegenerative diseases and coronary heart disease (CHD) in canines.

MATERIALS AND METHODS

Bio-Ethics Approval

In all procedures opted for research, a trained and registered veterinarian was involved. She kept all the bio-ethical instructions and safety precautions needed for procedures.

Sample Collection

Twenty canine blood samples were collected by veterinarians using blood collection tubes (BD Vacutainer®, Becton Dickinson, USA) with K_2EDTA as an anticoagulant. The blood samples were obtained from five different pure breeds (Thai Ridgeback**,** Golden Retriever, Labrador Retriever, Bulldog, Shih Tzu).

DNA Isolation and Polymerase Chain Reaction (PCR)

Genomic DNA of selected dogs were isolated from whole blood using a QIAamp DNA Blood Kits (Qiagen, Hilden, Germany), the isolated DNA samples were measured for their quantity using NanoDrop spectrophotometer. The quality of the extracted DNA samples was electrophoresed on 0.8% agarose gel and then these DNA samples were stained with GelRed® Nucleic Acid Stain 10000X DMSO (Biotium, Inc., USA), followed by observation under UV light. All isolated DNA samples were stored at -20°C until used as a template for the polymerase chain reaction (PCR). Subsequently, APOE gene was amplified from genomic DNA of five different dog breeds. DNA polymerase enzyme (BioTaq DNA Polymeras, Bioman) was used for the amplification of particular target gene. The reaction of 25µL total volume of PCR reaction consists of 20-40ng of DNA template, 0.5 units of DNA polymerase, 2.5pM primers, 1x buffer supplied with enzyme, 1.5mM $MgCl_2$, 0.2 mM dNTPs and sterile distilled water for adjust volume. The PCR was performed in Thermocycler (GeneAmp PCR system 2400, Perkin-Elmer) pre-heated at 94°C done for 3min before starting with 30 cycles of the denature temperature of 94°C for 1min, annealing temperature of 65°C for 1min and extension temperature of 72°C for 2min. After 30 cycles of the amplification were done, the final extension at 72°C was done for 8min. PCR amplicons of APOE gene were analysed on 2% agarose gel.

Cloning of APOE Gene

The PCR products of the canine APOE gene were electrophoresed on 2% agarose gel to confirm the correct amplicon size. All amplicons were stained with Novel Juice DNA Staining Reagent LD001-1000 (Bio-Helix, Taiwan), and then visualised under UV light. After that the amplicon bands were excised and purified from the agarose gel by using QIAquick Gel Extraction kit (Qiagen). The concentration of purified fragments was measured using a NanoDrop spectrophotometer, respectively. Purified fragments were used for construction of APOE - pGEM®-T vector in the ligation reaction. Ligation of purified fragments of APOE gene with the linear cloning vectors, pGEM®-T vector (promega) was done by adding 5mL of total volume of reactions which contain 25ng of pGEM®-T cloning vector, 2µL of purified amplicons and 1x ligation buffer, ligation mixers were subsequently incubated on an ice box for three hours. The ligation products were then transformed to the *DH*5α competent cells to manipulate the APOE gene in the pGEM®-T plasmid. Clones were induced with IPTG and x-gal was used as the substrate analogues. The white colonies with ampicillin resistance property were selected and blue colonies were used as control. The plasmids of transformants were extracted and subsequently used as template for amplification to confirm that transformants carried the inserted gene in the vector. Finally, the positive transformants were sequenced.

DNA Sequencing

Plasmids containing APOE gene were sequenced on both strands using T7 promoter primer and SP6 promoter primer. The DNA sequencing reactions were done by using ABI PRISM® BigDye™ Terminator Cycle V2.0 DNA Sequencing Ready Reaction Kit (Perkin-Elmer Biosystems). Sequencing reactions were performed in a GeneAmp® PCR system each reaction containing 200ng of plasmid DNA, 2µl of premixed reaction, 10 pmole primer and 1x sequencing buffer. The PCR program was performed by starting with 25 cycles of denaturation (96°C for 10sec), annealing (50°C for 5sec) and extension (60°C for 4min). The extension products were precipitated by ethanol-sodium acetate precipitation and subsequently loaded to ABI PRISM™ 310 Sequencer (Perkin-Elmer, USA). This DNA sequencing was carried out by Macrogen, the Republic of Korea.

Nucleotide Sequences Analysis and Phylogenetic Tree Analysis

The sequencing data of individual transformant was analyzed by BLAST program (Altschul et al. 1990) to show the degree of similarity between canine APOE clones and published human APOE sequences from GenBank. The level of percent identity and similarity was analyzed by using the SIAS program (Rech 2008) to show the amino acid sequence homology between five investigated canines and published human APOE sequences from GenBank. All protein sequences were aligned using the ClustalW. Phylogenetic tree was constructed using MEGA X based APOE amino acid sequences (Kumar et al. 2018). The evolutionary history of the examined dogs and human was inferred by using the Neighbour-Joining method (Saitou and Nei 1987). The bootstrap analysis was performed with 2000 replicates.

RESULTS

The amplification of the partial APOE gene coding for canine apolipoprotein E was done. The result showed the expected amplicon size (510 bp) as shown in Fig. 1. The investigated canine APOE, including human carrying APOE3 and APOE4 exhibited a high amino acid sequence homology, ranging from 71.76-100 and 78.23-100% identity

Table 1: Levels (%) of amino acid sequences identity in different canine breeds, including Human APOE3 (GenBank: NM_001302689.2) and Human APOE4 (GenBank:M10065.1)

Human ApoE3	100						
Human ApoE4	99.41	100					
Thai Ridgeback	72.35	72.94	100				
Labrador Retriever	71.76	72.35	99.41	100			
Shih Tzu	72.76	72.35	99.41	100	100		
Golden Retriever	71.76	72.94	100	99.41	99.41	100	
Bulldog	71.76	72.35	99.41	100	100	99.41	100
	Human ApoE3	Human ApoE4	Thai Ridgeback	Labrador Retriever	Shih Tzu	Golden Retriever	Bulldog

Identity Statistics: Min=71.76; Max=100; Mean=86.6085714285714; SD=13.6557883368937.

Table 2: Levels (%) of amino acid sequences similarity in different canine breeds, including Human APOE3 (GenBank: NM_001302689.2) and Human APOE4 (GenBank: M10065.1)

Human ApoE3	100						
Human ApoE4	99.41	100					
Thai Ridgeback	78.23	78.82	100				
Labrador Retriever	78.23	78.82	99.41	100			
Shih Tzu	78.23	78.82	99.41	100	100		
Golden Retriever	78.23	78.82	100	100	100	100	
Bulldog	78.23	78.82	99.41	100	100	100	100
	Human ApoE3	Human ApoE4	Thai Ridgeback	Labrador Retriever	Shih Tzu	Golden Retriever	Bulldog

Similarity Statistics: Min=78.23; Max=100; Mean=91.210612244898; SD=10.5371409994117

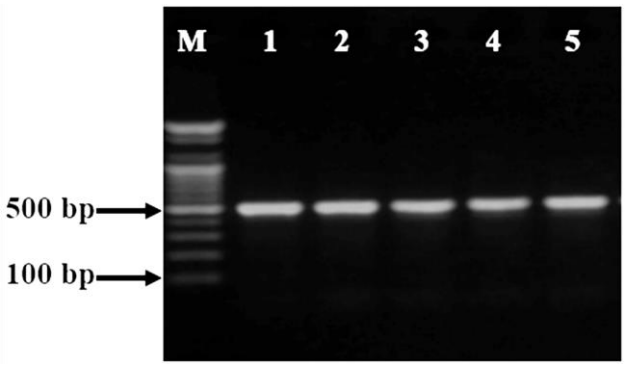

Fig. 1: Amplification amplicon of the partial APOE gene in five canine (dog) breeds. M=100 bp DNA ladder marker, Lane 1=Thai Ridgeback, Lane 2=Golden Retriever, Lane 3=Labrador Retriever, Lane 4=Bulldog and Lane 5=Shih Tzu).

identity and similarity, respectively (Table 1 and 2). The amino acid sequences of canine apolipoprotein E were aligned with the corresponding sequences of the published human APOE3 (GenBank accession No. NM_001302689.2) and human APOE4 (GenBank accession no. M10065.1). The alignment revealed that all canine breeds show arginine at the positions 112 and 158, which correspond to human APOE4 (Fig. 2). In addition, the five different pure dog breeds (Thai Ridgeback, Golden Retriever, Bulldog, Shih Tzu) can be separated as a group from human by several amino acid sequence differences (Fig. 2), which correspond to the phylogenetic relationships between these seven organisms (Fig. 3). Fig. 3 shows a constructed phylogenetic tree based on the partial APOE amino acid sequence alignment of five canine breeds, including human APOE3 human APOE4 from the GenBank. The phylogenetic clustering in the APOE coding region of all selected organisms was distinctly separated into two clusters. Thai Ridgeback, Golden Retriever, Bulldog, Shih Tzu belong to the same cluster while human APOE3 (GenBank accession no. NM_001302689.2) and human APOE4 (GenBank accession no. M10065.1) were grouped closely together.

DISCUSSION

We analyzed APOE amino acid sequences for different pure dog breeds, including Thai Ridgeback**,** Golden Retriever, Labrador Retriever, Bulldog and Shih Tzu. Our results showed a very high homology of APOE in the examined canines, human APOE3 and human APOE4, ranging from 71.76-100 and 78.23-100% identity and similarity, respectively (Table 1 and 2). These results indicate that the APOE gene is conserved in the partial amino acid sequences from site 96 to 265 in different canine breeds. In addition, all investigated canine breeds could be identified as APOE4 due to the presence of arginine at the positions 112 and 158, which correspond to APOE4 of human (Fig. 2). This suggests that APOE gene polymorphism was not present in the genome of these canine breeds. Genetically, APOE4 isoform found in dogs may be associated with neurodegenerative diseases that researchers have identified in dogs over the age of 11. Additionally, these examined canine APOE4 carriers can be initially predicted to be at increased risk of developing coronary artery disease (CHD). In accordance, human apolipoprotein E exhibits a polymorphism consisting of three different isoforms (isoform E2, isoform E3 and isoform E4) that may be associated with different physiological disease processes (Minta et al. 2020). In human, apolipoprotein E4 is associated with very low-density lipoprotein (VLDL), which increases the risk of developing Alzheimer's disease (AD) (Montagne et al. 2021) and coronary heart disease (CHD) (Witoelar et al. 2019), while apolipoprotein E3 associates with high-density lipoprotein (HDL) (Ashiq and Ashiq 2021). A study by Jiang et al. (2008) showed that APOE2 had the most potent effect in promoting the degradation of soluble amyloid beta protein (Aβ), whereas APOE4 was much less

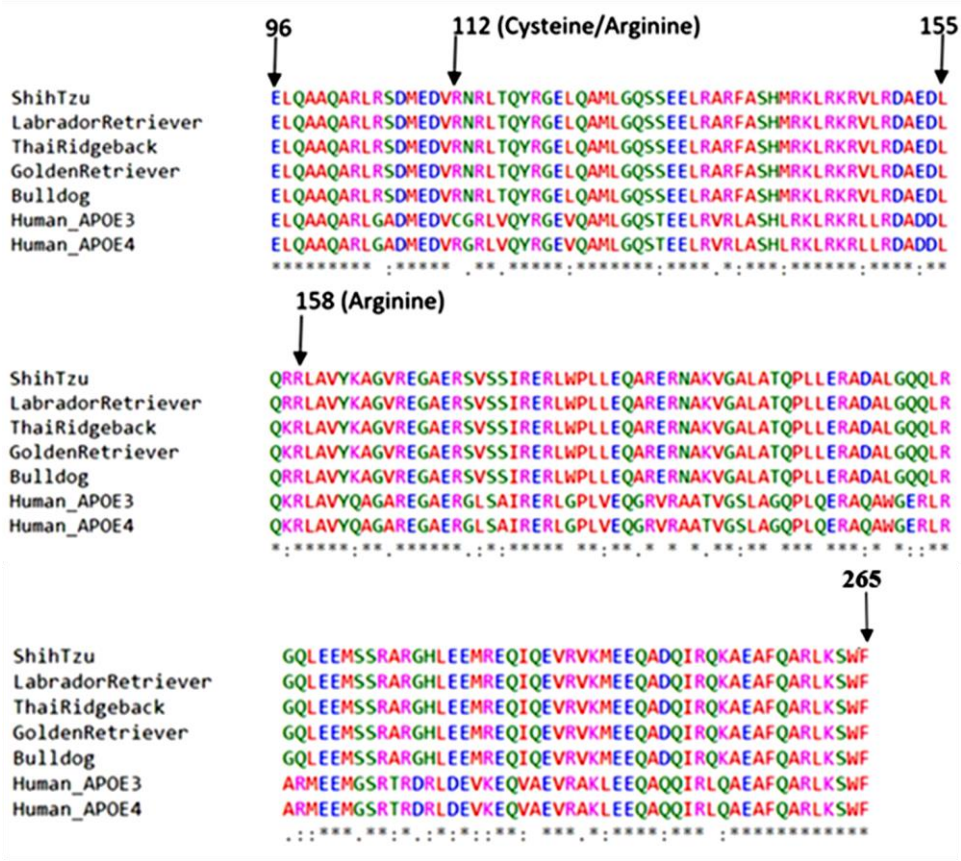

Fig. 2: Multiple sequence alignment analysis. ClustalW was used to align partial amino acid sequences from site 96 to 265 of APOE gene in different canine breeds, including Human APOE3 (GenBank: NM_001302689.2) and Human APOE4 (GenBank: M10065.1).

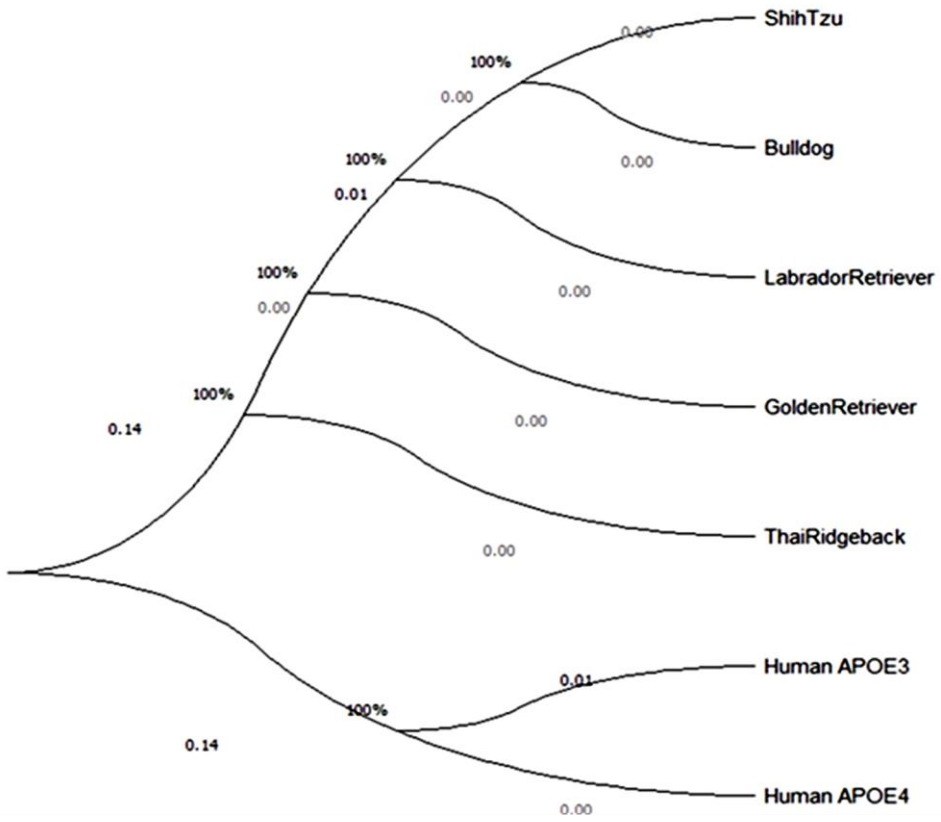

Fig. 3: Phylogenetic tree of different canine breeds, including Human APOE3 (GenBank: NM_001302689.2) and Human APOE4 (GenBank: M10065.1) constructed by MEGA X based on the partial APOE amino acid sequences (Kumar et al. 2018). The evolutionary history was inferred using the Neighbor-Joining method. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (2000 replicates) are shown next to the branches.

effective, leading to the development of Alzheimer's disease. Our results may be consistent with studies of APOE4 in humans, suggesting the investigated canine carrying APOE4 may be less efficient in stimulating the degradation of soluble amyloid beta protein (Aβ) (Lee et al. 2008). Therefore, functional studies of apolipoprotein E4 in various dog breeds should be conducted to confirm whether the results are consistent with human APOE4 function. The evolutionary history inferred that a high level of amino acid sequence differences exists between canine apolipoprotein E and human apolipoprotein E. Furthermore, the phylogenetic analysis demonstrated that the analysis of APOE amino acid sequences provided evidence for the generation of well-defined groups, including canine group and human group. Overall, our findings demonstrated that all five different dog breeds exhibited apolipoprotein E4, this study could be clarified at the genomic level. We hoped that the obtained information from the canine sequence analysis could support the identification of APOE genotypes and further study on apolipoprotein E4 expression for predicting the risk of neurodegenerative diseases and coronary heart disease in various canine breeds.

Conclusion

All canine breeds have the identical amino sequences of the partial amino acid sequences from site 96 to 265 of APOE gene. Furthermore, the canine breeds investigated in this study could be identified as isoform 4 due to the presence of arginine at the positions 112 and 158, which is the same unique amino acid substitution of APOE4 of human. Phylogenetic analysis showed that amino acid sequences of APOE provided evidence for generating two well-defined lineages, including a canine cluster and a human cluster. Collectively, these canine APOE4 carriers might be at high risk of developing neurological diseases and atherosclerosis. The findings could be used as fundamental genetic information for further diagnose disease in canines.

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Author's Contribution

B. Dechawut designed the study, amplified APOE gene, assisted in cloning APOE gene, analyzed all data and wrote the first draft of the manuscript. S. Wilasinee collected blood samples and edited the manuscript. A. Chalothon analyzed data and edited the manuscript. All authors revised the manuscript as well as read and approved the final manuscript.

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