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Expression of *APP*, *CDK5*, and *AKT1* Gene Related to Alzheimer Disease in Brain of Long-tailed Macaques

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ABSTRACT

Amyloid plaques and Neurofibrillary Tangles (NFTs) are known to be key pathological features of Alzheimer disease. To gain a better understanding of this disease, studies were carried out on the Indonesian primates, the long-tailed macaques, using a spontaneous Alzheimer's disease model. Examining and identifying genetic markers involved in plaque formation and NFTs in long-tailed macaques is necessary to reveal their physiological processes. In this study, the expression of genes involved in the development of amyloid plaque (Amyloid Precursor Protein (*APP*)) and those that control the phosphorylation of tau protein (*CDK5* and *AKT1*) was examined in the long-tailed macaque brain. This study showed that *APP, CDK5*, and *AKT1* may potentially be developed as genetic markers of Alzheimer's disease. Long-tailed macaques exhibited the development of amyloid plaque in the aging brain based on the analysis of the gene expression profile of its biomarker. Furthermore, long-tailed macaques can be optimized for neurodegenerative models.

1. Introduction

Alzheimer's is one of the main causes of dementia, which reduces a person's capacity to carry out daily tasks due to cognitive decline and memory loss. WHO and Alzheimer's Disease International (ADI) reported that 35.6 million persons globally had Alzheimer's disease in 2010. This population is estimated to double by 2030 and triple by 2050, reaching an estimated 115 million individuals (World Health Organization 2012). Over one million cases of Alzheimer's were recorded in Indonesia in 2013, and this condition can continue to increase over time as the life expectancy of the Indonesian people increased (Ministry of Health Republic of Indonesia 2019).

Alzheimer's disease has been associated with beta-amyloid plaques, the main proteins in neuritis deposits and neurofibrillary tangles (NFTs). Beta-amyloid plaques resulting from proteolytic cleavage of the precursor protein amyloid glycoprotein (*APP*). The endoplasmic reticulum produces *APP*, which is then transported to the Golgi complex and then transported to the plasma membrane. Beta and gamma secretases cleave mature *APP* on the plasma membrane to make amyloid beta (Chen *et al.* 2017).

The physiological function of *APP* in the hippocampus has been thoroughly investigated in rodents (Del Turco *et al.* 2016), *APP* transgenic mice (Jia *et al.* 2017), and STZ-induced *Macaca fascicularis* (Park *et al.* 2015; Del Turco *et al.* 2016). NFT is a protein that experiences hyperphosphorylation due to changes in kinase or phosphate activity, which causes the formation of NFT (Bhaskar C *et al.* 2018). Amyloid plaques and NFTs can damage nearby healthy cells, resulting in cell death. Meanwhile, *CDK5* and *AKT1* are two genes linked to tau protein and are involved in the control of tau phosphorylation. *CDK5* increases tau phosphorylation, which in turn can lead to neurodegeneration. In addition, the enzyme's activity is controlled by the endogenous activator p35 *CDK5* kinase, which phosphorylates tau protein (Li *et al.* 2020).

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Non-human primates have similar anatomy, pathology, and genetics to humans (Mariya *et al.* 2019; Darusman *et al.* 2021; Higo 2021). Primates are potential animal models to explore the molecular mechanism of amyloid plaque formation and tau protein (Darusman *et al.* 2014a; Park *et al.* 2015; Latimer *et al.* 2019). Older vervet monkeys naturally develop amyloid plaques in the cortex region, and paired helix filaments are discovered that help to generate NFTs. According to histology, amyloid plaques have also been found in the frontal, temporal, and parietal lobes, as well as the hippocampus, in the aged monkey's cerebral cortex (Nakamura *et al.* 1998; Darusman *et al.* 2014a).

To determine the occurrence of a physiological process of Alzheimer's disease, it is important to analyze the molecular mechanism based on genetic marker expression in an *APP*ropriate animal model. This study is an *APP*roach to discovering and understanding the underlying mechanisms of Alzheimer's disease using adult and aged long-tailed monkeys as translation in the human body. This study will also examine the expression of gene *APP*, *CDK5*, and *AKT1* in the adult and aged monkeys at the cortex and hippocampus brain region.

2. Materials and Methods

2.1. Samples Collection

The samples are brain tissue archives, where the location of Alzheimer's disease is linked with memory impairment and cognition of six female long-tailed macaques (*Macaca fascicularis*). These samples are divided into two groups: the adult group, which is sampled from animals 10–12 years old, and the old group, which is >15 years old. The animals are from the Primate Research Center Bogor Agricultural University (PRC IPB), West Java, Indonesia. Brain tissue areas are the cortical and hippocampus areas, which are used as samples and an archive stored in a freezer at -20°C in the IPB PRC Pathology Laboratory. Dental scaling is used to determine age (Darusman *et al.* 2014b). Age parameters are defined as adults (between 7 and 15 years) and aged (beyond 15 years) (Gartland *et al.* 2020). All examination was conducted in duplicate, and ethical clearance was obtained by Primates Research Center IPB University as PRC -19-A012.

2.2. RNA Extraction and cDNA Preparation

Total RNA was extracted from 2 mm^3 of cortical and hippocampus sections of 6 long-tailed monkeys using RNeasy Mini Kits (Qiagen, Hilden, Germany) following company procedures. Brain tissues were lysis with RLT buffer, and ethanol absolute was added. Purification was carried out by spin column and washed using RW and RPE buffer. RNAse Free Water elutes the RNA in the column and its concentration was measured using a Nanodrop 1,000 spectrophotometer (Thermofisher Scientific, USA). For the reverse transcription process, three ng/µL RNA was used as a template. According to company procedures, the cDNA synthesis process was carried out using the reverse transcriptase enzyme (Sensifast cDNA Synthesis Kit, Bioline, Meridian Bioscience, USA). A total amount of 10 ul RNA (3 ng/µL) was added to 4 ul RT buffer, 1 µL RT enzyme, and 5 µL nuclease-free water. The RT-PCR mix was then incubated in a thermocycler following the program at 25°C for 10 minutes, 42°C for 15 minutes, and 85°C for 5 minutes, while the cDNA was stored at 4°C.

2.3. RT-qPCR Amplification

The CFX Opus 96 instrument was used for the PCR amplification process (Biorad, USA). A total of 18 µL of a reaction containing 6 µL of Nucleotide Free Water (NFW), 10 µL of Sensifast Sybr mix (Bioline, Meridian Bioscience, USA), and 1µL (10 µM) of forward and reverse primers of *APP*, *CDK5*, *AKT1*, and beta-actin (ACTB) were used in each reaction (Table 1) (Park *et al.* 2015). The RT-qPCR process was carried out at 95°C for 2 minutes as predenaturation, 95°C for 10 seconds as denaturation, 55°C for 20 seconds as annealing, and 65°C for 10 seconds as extension and data collecting. This process was repeated for 40 cycles.

2.4. Data Analysis

The data from the analysis include the Relative Quantification (RQ) value, which was calculated with a 2-ΔCt formula using the Cycle Threshold (Ct) information from qPCR. This value was calculated to measure the mRNA expression level in fold-change after re-normalizing the ACTB housekeeping gene.

Data analysis adopted SPSS version 26 and Microsoft Excel. The Shapiro-Wilk test and genethe

Table 1. Gene target and primers used in this study modified from Park *et al.* (2015)

Gene symbol	Gene name	Primer Forward (F)/Reverse (R)
ACTB	Beta-actin	F: ACAGAGCCTCGCCTTTGC R: CACGATGGAGGGGAAGAC
CDK5	Cyclin-Dependent Kinase 5	F: CAGTGGCCCTCTATGACCAA R: CGTTCACCAGGGATGTTGTG
APP	Amyloid Precursor Protein	F: GCAAACTGAAACCTGGGAA R: TTCCTTCCCTTGCACAGTCT
AKT1	V-akt Murine Thymoma Viral Oncogene Homolog1	F: CCACGCTACTTCCTCCTCAA R: CGGATGATGAAGGTGTTGG
GAPDH	Glyceraldehyde-3-Phosphate Dehydrogenase	F: CAACAGCCTCAAGATCGTCAG R: ACTGTGGT/CATGAGTCCTTCC

Levene test were used to determine the normality and homogeneity of data, respectively. An independent t-test was then performed on the data to check for variations across areas and age groups.

3. Results

Analysis of the RT-qPCR-based relative mRNA levels of *APP*, *CDK5*, and *AKT1* gene in the cortex and hippocampus brain region of adult and aged long-tailed monkeys are presented in Figure 1. *APP* gene is implicated in the formation of beta-amyloid, while *CDK5* and *AKT1* contribute to the production of tau protein.

3.1. Expression Analysis of APP mRNA Gene in the Long-tailed Macaques Brain

The cortical region of the brain of aged monkeys compared to the adults in a quantitative test of *APP* gene expression, and the results revealed a 33-fold increase in gene expression, which was statistically significant at $p<0.05$. Although, not statistically different, expression of the *APP* gene in the hippocampal region of the adult monkeys' brain was 2.03 fold change higher than that of the aged monkeys. The result also showed that expression of the *APP* gene was 2.45-fold higher in the cortical region of the aged monkeys' brains than in the hippocampus region, but it is not statistically significant. However, expression of the *APP* gene was 26 times higher in the hippocampus region of the adult monkey brain than in the cortical region, which is significantly different, as indicated by $p<0.05$ (Figure 2).

3.2. Expression Analysis of CDK5 mRNA Gene in the Long-tailed Macaques Brain

CDK5 expression gene in both groups of monkeys was compared, and the result showed that the expression of *CDK5* gene in the adult monkeys' hippocampus area was 2.7 times higher. This result is statistically significantly different, as indicated by $p<0.05$. Meanwhile, expression of the *CDK5* gene in the cortical region of the brain of adult monkeys was 2.4 foldchange higher than the old cortex region, but not statistically significant.

3.3. Expression Analysis of AKT1 mRNA Gene in the Long-tailed Macaques Brain

The examination of *AKT1* gene expression results in the cortex region of adult and aged monkeys showed almost the same gene expression values. Meanwhile, gene expression in the hippocampal region of aged monkeys showed a 1.9 foldchange higher than adult monkeys but not significantly different.

4. Discussion

There were two significant results findings in this study, namely gene expression of the *APP* gene and expression of the *CDK5* gene. *APP* gene is related to the formation of peptide amyloid beta, and the significance found is in the cortex region, showing that gene expression in the cortex region is higher in aged monkeys. The results' significance statistically indicates a link between this gene's expression and the formation of senile plaques. These amyloid plaques form earlier in the cortex region, indicating that these formations occur in age monkeys.

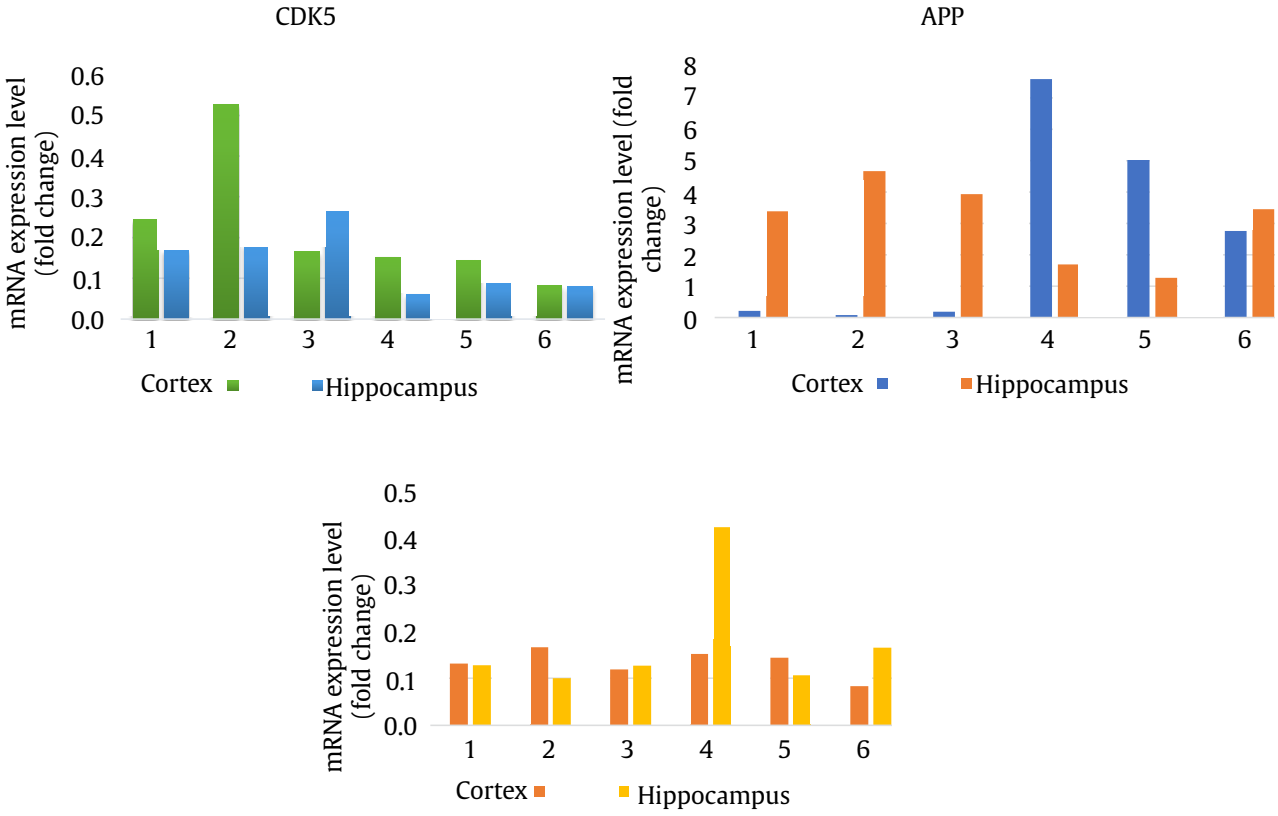

Figure 1. A representation of mRNA level expression of APP, CDK5, and AKT1 of the cortex and hippocampus brain region on adult and aged long-tailed macaques. Quantification data for all genes were normalized using appropriate reference gene ACTB and relative fold changes. Data are expressed as means ± SD

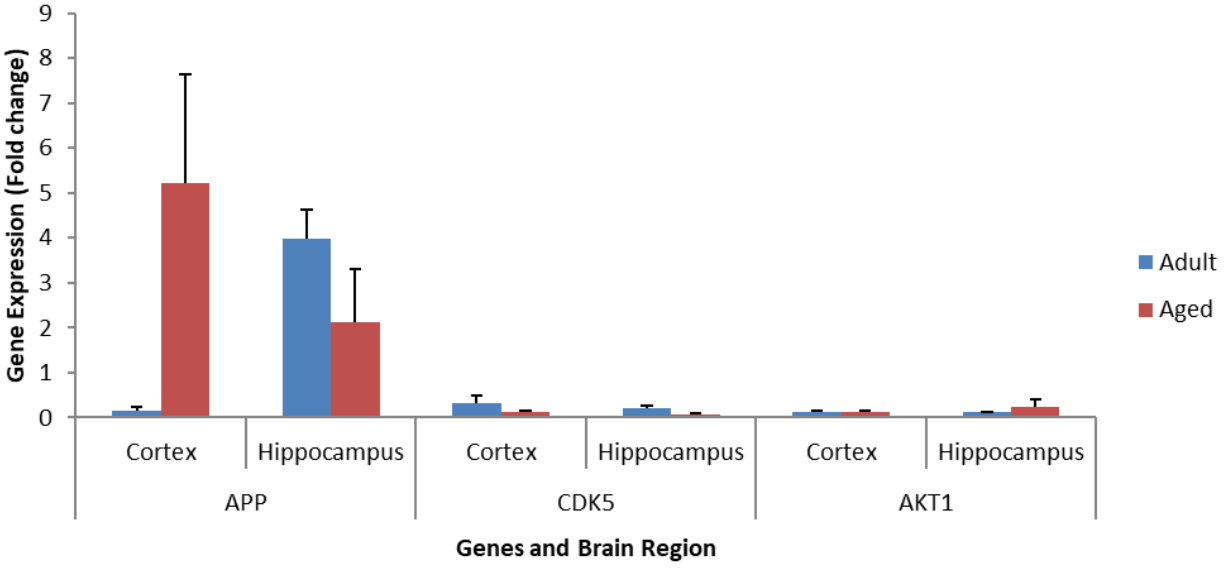

Figure 2. Evaluation of mRNA gene expression related to amyloid plaque and neurofibrillary tangle development of the cortex and hippocampus brain region on adult and aged long-tailed macaques. The histogram displays expression of APP, CDK5, and AKT1, mRNA gene normalize to beta-actin housekeeping gene and measure in foldchange. Data are expressed as means, and error bars = SD. * indicates a significantly different expression

The second finding was statistically significant ($p<0.005$) *CDK5* gene expression in the hippocampal region. *CDK5* gene is related to pTau formation and is statistically significant in adults. Hence, the process of pTau formation has started in adults, or another possibility is that pre- and post-synaptic signaling events in neurons modulate memory formation. *CDK5* neuronal protein kinase phosphorylates various synaptic substrates. It is involved in memory formation (Guan *et al.* 2011), but in old monkeys, the process of memory formation decreases, resulting in lower *CDK5* gene expression or *CDK5* begins to lose its function.

APP is one gene responsible for synthesizing amyloid beta peptide or amyloid plaque. Toxic plaque or amyloid peptide kills neuronal cells, resulting in gradual cognitive impairment. In this study, aged monkeys had a 33-fold higher expression of *APP* gene in the cortical region than adult monkeys. These results are consistent with a study by (Park *et al.* 2015) in long-tailed macaques induced by STZ, in which the frontal cortex showed the highest levels of *APP* gene expression. Additionally, spontaneous *APP*/A-immunoreactive (ir) plaques were discovered in the neocortex and hippocampus areas of 55-year-old female gorillas (Perez *et al.* 2013). In *Macaca fascicularis*, the intracellular amyloid beta was detected by immunohistochemistry in the cortical region at different ages, and the overall level of amyloid beta increased with the aging (Nakamura *et al.* 1996, 1998; Kimura *et al.* 2005). Other studies revealed that the prefrontal brain of aged monkeys exhibits a higher amyloid beta staining (Jester *et al.* 2022). Neuronal synapses are created and repaired in part by *APP*. However, excessive *APP* expression may raise the risk of Alzheimer's disease through decreased long-term potentiation and increasing sensitivity to ischemic brain damage (Zhang *et al.* 1997; Matsuyama *et al.* 2007).

The results of this study are consistent with the report of a previous investigation that the critical region expression of aged monkeys of the *APP* gene was higher than that of adult monkeys. Amyloid beta, which causes the development of senile plaques, can occur due to variations in gene expression levels and noticeably varied outcomes. More functional studies are required to fully comprehend the impact of elevated cortical *APP* expression levels in old monkeys. Furthermore, when *APP* is overexpressed in mouse embryonic neural precursor cells, it speeds up the migration of cells to the cortex, and *APP* contributes quantitatively to the precise location (Young-Pearse *et al.* 2007).

According to the result of this study, *APP* expression in the brain of adult monkeys was 2.03 times higher in the hippocampal region than in the aged monkeys. This can be attributed to one of the roles of *APP*, which is to support neuronal healing and differentiation in the adult monkeys' hippocampal region. (Anand and Dhikav 2012) claim that learning, memory, and spatial navigation occur in the hippocampus in humans at a young age. Still, memory and processing speed skills deteriorate with aging (Reuben *et al.* 2011).

In individuals with Down syndrome, the hippocampus is the primary site of *APP* expression (Del Turco *et al.* 2016) and was markedly expressed in the hippocampus of *APP* transgenic mice (Jia *et al.* 2017). At the early stages of disease progression, the afflicted hippocampus is where memory, learning, and formation take place (Braak and Braak 1991). Expression of *APP* gene in the hippocampal region was higher in adults than in the aged monkeys, which is related to the development and learning of these brain regions. Phosphorylation of *APP* at Thr668 dramatically increased the amount of Ab build-up in the hippocampus of Alzheimer's patients (Lee *et al.* 2003). Another idea is that the development of amyloid plaques via -secretase and -secretase from *APP* is similarly connected to the enhanced gene expression in the hippocampus at a young age.

CDK5 is a serine/threonine kinase protein that plays a role in cell proliferation (Allnutt *et al.* 2020). According to (Cruz *et al.* 2003), *CDK5* is mostly found in postmitotic neurons, which are critical for brain development, neuronal survival, synaptic plasticity, microtubule regulation, and pain signaling (Lopes and Agostinho 2011). The monomeric form of *CDK5* is not enzymatically active, but it functions as an activator and causes an increase in tau phosphorylation and neurodegeneration. In this study, *CDK5* was analyzed using RT-qPCR to examine the potential for tau protein formation in groups of aged and adult long-tailed monkeys in the cortex and hippocampus. While the cortical region did not differ substantially, expression of *CDK5* mRNA in the adult hippocampus

region was 2.7 foldchange higher than that of the old animals. This result is statistically different, as indicated by $p<0.05$. A previous study by Oikawa *et al.* (2010) discovered the production of Paired Helical Filament (PHF), a fibril unit of NFTs, in the hippocampus of the brain of cynomolgus monkeys. According to (Abid *et al.* 2019), examining the p25 gene's expression as a *CDK5* activator implicated in tau hyperphosphorylation in mice revealed that tau pathology worsens with advancing age. This contradicts the result of this study, indicating that the expression of the *CDK5* gene in the hippocampal area of adult monkeys was higher than in the aged monkeys. This is due to the possibility that neurons' pre- and post-synaptic signaling activities influence memory formation. To establish memories, the neuronal protein kinase *CDK5* phosphorylates a variety of synaptic substrates (Guan *et al.* 2011). However, in old monkeys, the memory creation process is slowed, resulting in decreased *CDK5* gene expression or loss of *CDK5* function. Aging affects brain function in the hippocampal region and can result in memory loss. Hence, expression of the *CDK5* gene in the cortex of old monkeys diminishes. Expression of the *CDK5* gene is 2.4 times higher in the cortical region of adults than in old monkeys.

Cruz *et al.* (2003) showed that mice's cortical and hippocampal regions displayed aberrant *CDK5* activity caused by the accumulation of p25 inducing the formation of endogenous tau filaments. The cortical tissue of old primates showed an increase in phosphorylation exposed to Pb, leading to the activation of kinases and activators (Bihaqi and Zawia 2013). According to (Hisanaga and Endo 2010), the *CDK5* gene contains unique activators called p25, p35, and p39 that can create a p35-*CDK5* or a p25-*CDK5* complex and phosphorylate serine/threonine kinase. In humans, the p25-*CDK5* complex has a substantially greater capacity to phosphorylate au than the p35-*CDK5* complex (Hashiguchi *et al.* 2002).

AKT1 gene, often referred to as protein kinase B, is a component of several signaling pathways, and its unique function is believed to be a crucial aspect of various disease processes, such as cancer and diabetes (Curtis and Bandyopadhyay 2021). *AKT1* participates in signaling pathways, resulting in phosphorylated GSK3β, which is essential in controlling the activity of glycogen synthase kinase 3-beta (GSK3-beta) (Sen *et al.* 2020). *AKT1* regulates cell development, proliferation, and metabolism under physiological settings and participates in synaptic plasticity. According to (Levenga *et al.* 2017), it is crucial to the serine/threonine kinase present in nearly all cell types throughout the body. In this study, the hippocampus region of old monkeys had an *AKT1* gene that was 1.9 times higher than that of adult monkeys, but this difference was not statistically significant. *AKT1* gene's expression level in the cortical region was nearly the same in adults and the aged.

This study examined *APP*, *CDK5*, and *AKT1* gene expression in the cortex and hippocampus of two groups of adult and aged monkeys. The results showed that the expression of the *APP* gene was higher in the cortical region of aged monkeys compared to adults, while the *CDK5* gene was higher in the adult hippocampus compared to that of the aged. Furthermore, there was no significant increase in the expression of the *AKT1* gene in either group. According to this study, *APP* gene expression related to amyloid plaques in the cortex brain region of aging cynomolgus monkeys resembles a human phenomenon. Further studies are needed to examine the mechanism of the formation of amyloid beta protein by analyzing other gene expression markers, as well as in older animals (more than 20 years) or longitudinal studies. This is needed to determine the expression of genes at the protein level in monkeys with poor memory and biomarkers of neurogenerative disease of Alzheimer's type.

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