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RESEARCH ARTICLE

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Gen Expression of Bax and Bcl-2 in Cynomolgus Monkeys (*Macaca fascicularis*) as Animal Model for Papillomavirus Study

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Abstract
Cervical cancer is one of the fourth cancers in the world that occurs in women. Papillomavirus infection has been reported as one of the causative agents of cervical cancer. The apoptotic mechanism is one of the signs of cancer. Cynomolgus monkeys have been widely used as animal models in biomedical research because they are similar to humans in terms of genetics, anatomy, and physiology compared to other animals. Previous studies reported that cynomolgus monkeys have undergone spontaneous infection by papillomavirus, but this infection did not show the phenomenon of cervical cancer. This study aimed to evaluate the prediction of papillomavirus-infected cervical cancer through apoptotic mechanisms regulated by Bax and Bcl-2 genes. Gene expression was performed in this study using Real-Time PCR technique. The results showed an increase in Bax and Bcl-2 gene expression in positive group cynomolgus monkeys with papillomavirus infection. Bcl-2 as anti-apoptotic gene expression increased significantly higher than Bax as pro-apoptotic gene. Bax and Bcl-2 have potential as biomarkers to predict the phenomenon of cervical cancer in cynomolgus monkeys with papillomavirus infection.
Key words: Apoptotic, Bax, Bcl-2, Cynomolgus monkeys, Papillomavirus

1. Introduction

Cervical cancer occupies the fourth position in the world that occurs in women based on the World Health Organization (WHO) report in 2020. Figo World Congress (2019) reported that about 90% of deaths in women living in low- and middle-income countries are caused by cervical cancer. Most of them will not have had access to the key cervical cancer services. Genetic factors are one of the factors that cause cervical cancer (Ramchandran and Dörk 2021). The mechanism of cervical cancer is programmed cell death or apoptosis (Hernawati 2014). Apoptosis is influenced by signaling pathways whose regulation is divided into two pathways based on the type of ligand, namely intrinsic and extrinsic (Wong 2011). Viral infections have been reported as one of the many factors that cause cancer. The virus that can induce cervical cancer in humans is Human Papillomavirus (HPV).

HPV virus is the main factor causing persistent infection in cervical cancer. Human papillomavirus (HPV) is the most common sexually transmitted pathogen in women or men (Okunade 2020). Scientists report that most cervical cancers are caused by HPV infection. Cervical cancer occurs due to an abnormal system in cells located in the cervix or due to growth in cervical epithelial tissue. HPV viruses have no envelope, are relatively small in size, and belong to the papillomaviridae family. More than 200 types of HPV viruses have been characterized and as many as 30-40 of them can infect the epithelial and anogenital layers of the mucosal area in the human body. The level of DNA homogeneity determines the

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division of HPV virus classifications found to date. HPV viruses will infect cervical epithelial cells due to injury to epithelial tissue or abrasion. Abrasion is an increase in maturation of a previously infected host (Kane and Golovkina 2010).

Nonhuman primates (NHPs) have been widely used as research animal models because they are similar to humans in terms of genetics, anatomy, and physiology compared to other animals (Pijoh *et al*. 2015, Mariya 2019). Cynomolgus monkeys (*Macaca fascicularis*) is one of the nonhuman primates that have good relevance as an HPV study to find solutions in the treatment of HPV virus infections. Cynomolgus monkeys naturally can be infected with papillomavirus type-3 but the infection has not been able to establish a vaccine-inducible immune response (Sari *et al*. 2014) Papillomavirus type 3 is phylogenetically and phenotypically similar to HPV 16 so it can be further identified by making the long-tailed monkey an ideal research model (Ragonnaud 2017). This study aimed to evaluate the expression of genes involved in the apoptotic process, which are *Bax* as a pro-apoptotic and *Bcl-2* as an anti-apoptotic gene in cynomolgus monkeys spontaneously infected with papillomavirus type-3 to early detect of cervical cancer symptoms.

2. Materials and Methods

2.1. Animal Ethics Approval

The animal husbandry and all procedures has been approved by the the Institutional Animal Care and use Committee of the Primate Research Center (PRC) IPB which has been published in Addenum›s ethical approval letter Number IPBPRC-19- A012, August 4, 2020. In addition, sampling method procedures were done following the Guide for the Care and Use of Laboratory Animals Guidelines.

2.2. Sample Collection

All animals in this study were from the animal breeding facility of the Primate Research Center of IPB. This study used 27 archive vaginal swabs samples from cynomolgus monkeys. A total of 18 vaginal swabs cynomolgus monkeys spontaneously infected with Papillomavirus (positive-PV), and 9 vaginal swabs were from healthy cynomolgus monkeys (negative-PV). The vaginal swabs were collected by cytobrush (OneMed, Indonesia) and dipped into Tris EDTA-NaCL(TEN) buffer media (Sigma Aldrich, Germany) (2 mL Tris HCl 1M pH 7.5; 0.2 mL EDTA 0.5 M; 0.2 mL 5 M NaCl; and 97.6 mL distilled water). The collected swabs were stored in a 4°C TEN buffer.

2.3. RNA Extraction and Synthesis cDNA

Extraction of total RNA derived from vaginal swabs of cynomolgus monkeys was carried out using the Quick-RNA™ Miniprep Kit (Zymo Research, USA) following the manufacturer›s protocols. The quantity and purity of the extracted RNA was evaluated by Nanodrop® OneC Spectrophotometer.

The mRNA 5 ng/µl concentration was used for cDNA synthesis. The cDNA synthesis was carried out using the SensiFAST™ cDNA Synthesis Kit (Meridian Bioscience, Bioline, USA) following the manufacturer›s procedures. The PCR conditions were set at 25°C for 10 min, 42°C for 15 min, 85°C for 5 min, and 4°C for 2 min.

2.4. Quantitative Polimerase Chain Reaction (RT-qPCR)

PCR reaction using forward and reverse primers of each gene. The *ACTB* as a reference gene was used to normalized the mRNA expression level analysis. The sequences of forward and reverse primer are shown in Table 1. mRNA expression level amplification using

Table 1. Primer sequences apoptosis related genes

Gene	Nucleotide Sequences (5'-3')	References
ACTB Forward *ACTB* Reverse	AGA GCT ACG AGC TGC CTG AC AGC ACT GTG TTG GCG TAC AG	Patil *et al*. 2021
Bax Forward *Bax* Reverse	CCC GAG AGG TCT TTT TCC GAG CCA GCC CAT GAT GGT TCT GAT	Borhani *et al*. 2015
BCL-2 Forward *BCL-2* Reverse	GCT CTA AAA TCC ATC CAG CCT CTC CAT CAT CAA CTT	Nohara *et al*. 2007

sybr was performed with SensiFAST™ SYBR ® Lo-ROX Kit and carried out in a CFX OPUS 96 Real-Time PCR (Bio-Rad). The reactions were performed in duplicate under the following conditions: 95°C for 2 min as the polymerase activation step, 40 cycles of 95°C for 10 sec for denaturation, and 56°C for 30 sec for primer annealing. Each run includes negative control.

2.5. Data Analysis

Analysis of gene expression level data was performed with descriptive statistics. Relative Quantitative (RQ) analysis were calculated using the $2^{-\Delta Ct}$ method (Livak dan Schmittgen 2001).

3. Results

Based on our results of the *Bax* gene expression identified by PCR in cynomolgus monkeys positive-PV and negative-PV are shown in Figure 1a. We used 18 cynomolgus monkeys spontaneously infected with papillomavirus and 9 cynomolgus monkeys not infected with papillomavirus. The histogram of the *Bax* gene expression in cynomolgus monkeys positive-PV is 1.57 times higher than cynomolgus monkeys negative-PV.

The results of the analysis of Bcl-2 gene expression in positive-PV and negative-PV showed that cynomolgus monkeys positive-PV was 10.26 times higher than cynomolgus monkeys negative-PV (Figure 1b).

4. Discussion

The expression of genes that related to apoptosis were measured by Real-Time PCR. This study can identify the occurrence of cervical cancer through the mechanism of apoptosis. Changes in the expression levels of apoptosis-related genes such as *Bax* and *Bcl-2,* also *ACTB* as reference genes, this research is important for the future development of cervical cancer treatment strategies. The expression of *Bax* and *Bcl-2* genes in cynomolgus monkeys positive-PV was higher than negative-PV. The increase in *Bcl-2* gene expression was higher than Bax gene expression. This results evaluated *Bax* and *Bcl-2* gene expression to predict cervical cancer in cynomolgus monkeys positive-PV through disruption of apoptotic mechanisms in long-tailed macaques. This result is in line to our previous studies. Previous studies in Darusman *et al*. (2022) showed that increased expression of mRNA level of *KI67* genes as biomarker oncogenesis of papillomavirus was significantly higher in the positive-PV group than negative-PV.

The expression level of Bax in PV-positive cynomolgus monkeys was 1.52 times higher than that of PV-negative cynomolgus monkeys. The apoptotic pathway is generally divided into the mitochondrial (intrinsic) and the death receptor (extrinsic) pathways.. The process of apoptosis is determined by the balance between pro-apoptotic and anti-apoptotic proteins (Wong 2011). *Bax* gene is a pro-apoptotic protein that regulates the balance of cell life and death. The appearance of the *Bax* gene in the instrinsic pathway

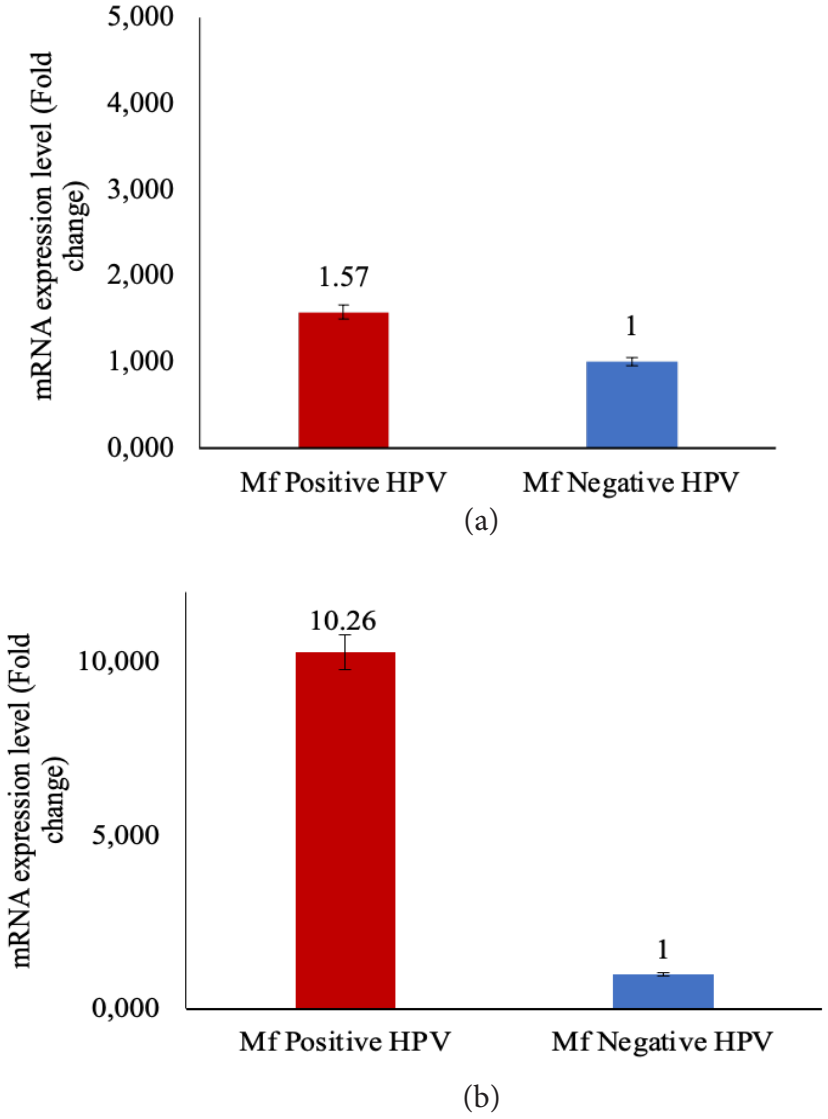

Figure 1. The mRNA expression levels for *Bax* (a) and *Bcl-2* (b) genes. Expression levels of upregulated mRNAs in the PV-positive for and PV-negative groups. The expression of *Bax* and *Bcl-2* both tended to be higher in the positive-PV group.

of apoptosis indicates mitochondrial dysfunction that will break down cellular substrates that cause changes in cell morphology and biochemistry as part of the characteristics of apoptosis. The breakdown of cellular substrates occurs due to the presence of Caspase which has previously been activated (Mcllwain *et al*. 2013).

The results of *Bcl-2* (anti-apoptosis) expression in cynomolgus monkeys positive-PV were ten times higher than those of the negative-PV cynomolgus monkeys group. One of the characteristics of cancer cells is to inhibit apoptosis (Putra *et al*. 2015). Inhibition of apoptosis can aid oncogenic transformation by aiding continued tumor growth, resulting in the stability of cell survival during the metastatic process and potentially causing resistance to therapy. Increased expression of the pro-survival *Bcl-2* protein is found in various cancers. This regulation can occur through various mechanisms including, chromosomal translocation, gene amplification, and increased gene expression or translation (Perini *et al*. 2018).

The increase in *Bcl-2* gene expression was significantly higher than that of *Bax* in PV-positive cynomolgus monkeys. The ratio of *Bax*/*Bcl-2* gene

expression was 1.57/10.26. The high expression of the *Bcl-2* gene compared to the Bax gene expression indicates an indication of cervical cancer in long-tailed monkeys through papillomavirus infection.This is due to the high anti-apoptotic gene and low pro-apoptotic regulator, which is *Bax*. The increase in *Bcl-2* gene expression is probably in line with the increase in *Bcl-2* protein found in various cancers. The process of apoptosis is determined by the balance between pro-apoptotic and anti-apoptotic proteins. In the extrinsic pathway, apoptosis is triggered by the activation of extracellular ligand-induced death receptors that lead to the activation of the Caspase group of proteins. The intrinsic or mitochondrial pathway is initiated within the cell and is strongly regulated by the *Bcl-2* family, which leads to the activation of the Caspase family of proteins (Juan *et al*. 2012).

These apoptotic mechanisms can be biomarkers to predict the incidence of cervical cancer in long-tailed monkeys so that by evaluating the expression of *Bax* and *Bcl-2* genes, the incidence of cancer in long-tailed monkeys infected with papillomavirus can be prevented.

In Conclusion, this study proved that in cynomolgus monkeys spontaneously infected with papillomavirus induced upregulation of anti-apoptotic proteins and downregulation of pro-apoptotic proteins. This is potentially developing cervical cancer by papillomavirus-infected. Future research is needed to evaluate other genetic markers related to apoptosis,.Further research is important to validate the mechanism of papillomavirus infection that causes cervical cancer.

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