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E6/E7 Oncogenes Mutation of Human Papillomavirus Type 16 Associated with P16 Protein Expression in Cervical Cancer

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ABSTRACT

The genetic composition of the E6 and E7 oncogenes is very susceptible to mutation. Mutations occur due to interactions between the viral genome and the host. Changes in one nucleotide oncogenes E6 and E7 can affect the function of these oncogenes so that they can trigger the persistence of Human Papilloma Virus (HPV) infection and cervical cancer progression in several intratypic variants of HPV type 16 and alteration p16 expression in cervical cancer cases. This study was conducted on cervical cancer women first diagnosed from May 2021 to November 2021 who had not received surgery, chemotherapy, or radiation therapy. Willing to participate in the study after signing the informed consent. Cervical tissue samples with a positive test result for HPV 16 were then grouped based on the mutation sequencing of E6 and E7 into a wild-type group and a mutant group. Furthermore, the immunohistochemical examination was carried out to assess the expression of p16 protein in paraffin blocks. The results of this study showed that there was no association between mutations in the E6 and E7 oncogenes of HPV Type 16 with p16 expression (c= 0.048 and p value 0.78). The expression of p16 was stronger in the mutant group with the median percentage of cells from p16 immunohistochemistry staining which was 60.5% (range 3-73%) in the mutant group and 53% (range 2-65%) in the wild type of group. However, the correlation coefficient interval between HPV type 16 and E6 oncogene mutations with p16 protein expression is very weak.

Keywords: cervical cancer, E6, E7, HPV, oncogene mutation, protein expression, relationship.

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I. Introduction

Cervical cancer is a significant public health problem for women worldwide and is the fourth most common malignancy after breast, colorectal, and lung cancer. Cervical cancer is closely related to persistent infection caused by the high-risk type of Human Papilloma Virus (HPV) [1].

Studies on risk factors that can increase cervical cancer progression are urgently needed as part of secondary prevention. These risk factors include internal factors in the form of genetic factors and external factors such as smoking habits, multiple partners, alcohol consumption, and several children. Genetic factors are currently a concern because they are sporadic and cannot be modified like external factors. One of the genetic factors that can influence the pathogenesis of cervical cancer is a mutation in the E6 and E7 oncogenes of HPV 16 [2], [3].

E6 and E7 are early genes in the region coding for the functional oncogenic HPV genome. Cross-interaction of E6 and E7 oncogenes with various pathways play a crucial role in cervical cancer pathogenesis [4]. Transcription of oncogenes E6 and E7 is always found in all cases of cervical cancer and is the main factor that triggers HPV-mediated carcinogenesis in cervical tissue [5]. Oncogenes E6 and E7 in HPV will cause dysregulation of cell proliferation and

apoptotic mechanisms through binding to tumor suppressor proteins, one of which is p16 protein [6].

P16 is a cyclin-dependent kinase inhibitor that regulates the activity of cyclin-dependent kinases 4 and 6 (CDK4/6). Oncogene E7 will trigger a cellular defense response called oncogene-induced senescence (OIS), which is mediated by tumor suppressor protein p16 (p16) and retinoblastoma protein (pRb) [7]. In normal cells, the polycomb repressive complex inhibited p16 expression at the gene level. High expression of p16 is an excellent biomarker for cancers associated with high-risk HPV infection. E7 inhibits cell cycle arrest and cell senescence in cervical cancer via proteasomal degradation of pRB. Induction of p16 in the absence of pRb will cause changes in p16 expression [8].

The genetic composition of the E6 and E7 oncogenes is very susceptible to mutation. Mutations occur due to interactions between the viral genome and the host. Changes in one nucleotide oncogenes E6 and E7 can affect the function of these oncogenes so that they can trigger the persistence of HPV infection and the progression of cervical cancer in several intratympanic variants of HPV type 16 [9], [10]. Mutations of the E6 and E7 oncogenes and their effects on cell cycle proteins have an essential role in determining differences in the biological properties of viruses that affect their infectivity and pathogenicity [11].

Thus, analysis of mutations in the E6 and E7 oncogenes of HPV 16 and the relationship between these oncogenes and cell cycle proliferation regulatory proteins such as p16 is expected to be one approach to deepen understanding between the interactions of E6 and E7 oncogenes with host cells and their effects on cervical cancer development. Until now, no research has examined the effect of mutations in the E6 and E7 HPV type 16 oncogenes on p16 protein expression; therefore, this study is expected to provide an initial description of the mutation pattern and its effect on the expression of the p16 protein.

II. Materials and Methods

A. Study Designs

This cross-sectional study was conducted on 31 cervical cancer women first diagnosed at the Obstetrics and Gynecology Polyclinic, Sanglah Hospital, Denpasar (Bali, Indonesia). Data were taken from hospital patients between May 2021 to November 2021. Patients were diagnosed and categorized into the mutated E6 HPV type 16 oncogene and the E7 HPV type 16 oncogene mutation. The patients were further classified into two groups of wild type and mutant group. Other exclusion criteria were women with cervical cancer who were first diagnosed and had not received surgery, chemotherapy, and radiation therapy.

B. Ethical Considerations

This study was approved by the Research Faculty of Medicine, Udayana University/Sanglah Hospital Number 815/UN14.2.2.VII.14/LT/2021. This research obtained a Research Permit from the Education and Research Section of Sanglah Hospital Number LB.02.01/XIV.2.2.1/17375. Informed consent was obtained from all participants in writing after receiving an explanation of the study objectives and procedures.

C. Methods

Paraffin blocks of cervical cancer tissue were taken from 31 women with cervical cancer who visited the Obstetrics and Gynecology Polyclinic, FK UNUD/Sanglah Hospital. Demographic and clinical data included age, parity, histopathology, and clinical stage, which were determined based on the history, physical examination, and supporting examinations as indicated.

D. Viral DNA Extraction and PCR Amplification

According to the product protocol, DNA was isolated using the DNA Extraction Kit, Roche® extraction kit. After extraction, PCR was performed to determine positive and negative HPV with primary targets at My09 (5'- CGT CCM ARR GGA WAC TGA TC-3') and My11 (5'- GCM CAG GGW CAT AAY AAT GG-3'). PCR for genes E6 and E7 was performed using specific primers designed according to GenBank's K02718/HPV16R gene sequence. The primary sequences are as follows: upstream, 5'AAG GGC GTA ACC GAA AT3'; downstream, 5'TCC ATT ACA TCC CGT ACC CTC3'(Primary 2 OD; 1 OD= 33 g) which are both primary targets. Primer was dissolved up to 100 mmol/L at a concentration of 10 M (M=mol/L). The E6/E7 gene PCR reaction mixture (25 l) was as follows: 1 l DNA template (1:20), 1 l upstream primer (10 M), 1µl downstream primer (10 M), 12.5 l of 2x Taq PCR MasterMix, and double-distilled water. The PCR program was as follows: predenaturation at 94°C for 5 minutes; 30 cycles of denaturation at 94°C for 30 seconds, annealing at 55°C for 30 seconds, extension at 72°C for 60 seconds; and final extension at 72°C for 5 min. After the PCR was completed, 5 ml of the sample was analyzed on 1.5% agarose gel for electrophoresis.

E. Direct Sequencing and Phylogenetic Analysis

Sequencing of genes E6 and E7 using the MEGA6 software program. The HPV 16 prototype (HPv16. P, GenBank access code: NC_001526) was selected from the European variant for the nucleotide sequence matching of the E6 and E7 genes using the BLAST software program (http://blast.ncbi.nlm.nih.gov/Blast) [12]. Immunohistochemical staining using the DakoEnVision®+ Dual Link System-HRP (DAB+) kit (Dako, Denmark) and monoclonal anti-mouse p16 antibody (F12, sc-1661, Santa Cruz 1:200). Other materials use materials from Sigma-Aldrich (USA). The tissue was cut 3–5 m thick using a microtome. Place the slide in the oven at 60 °C for 20 min. Before painting, the tissue slides will go through a process of deparaffinization and rehydration, including immersion in a solution of xylene 2×5 minutes, ethanol (2×3 minutes in 95% ethanol and 2×3 minutes in 70% ethanol), and distilled water for 30 seconds.

Furthermore, antigen retrieval was carried out by immersing the slide in citrate buffer with pH 6.0 for 50 minutes, while being heated in an oven at 95–100 °C for 30 minutes and left at room temperature for 20 minutes, followed by rinsing the TBS buffer for 5 minutes. They were then immersed in 3% hydrogen peroxide (peroxidase blocking reagent) for 5-10 minutes at room temperature, followed by rinsing the TBS buffer for 5 minutes. The slides were then incubated with primary antibodies; Close the slide,

which has been dripped with 200 L of primary antibody, and leave for 30 minutes at room temperature, followed by rinsing the TBS buffer for 5 minutes. Drop 200 L of polymer reagent conjugated with horseradish peroxidase and anti-Fab antibody, cover with a cover glass, and incubate for 30 minutes, followed by rinsing of TBS buffer 2×5 minutes. Add 200 L of DAB (2.2′ diaminobenzidine) on the slide for 10 min. Rinse with distilled water—counterstain with hematoxylin for 5 minutes in a hematoxylin bath. Rinse with running water than distilled water.

F. Statistical Analysis

Statistical analysis was performed using SPSS version 22.0 (IBM Corp.). The continuous variables were presented as Middle and Close range (IQR), while the categorical variables were presented as number (n) and percentage (%) of patients. The proportion comparison test used the Chi-square test with P<0.05 was considered to indicate a statistically significant difference. The correlation analysis test of 2 correlated variables uses the Contingency Coefficient correlation test. This test, if c = 0 then it means that the variable has no correlation or no relationship, if c = 1 means that there is a positive relationship between the independent and dependent variables.

III. Results

Of the 31 samples with positive HPV 16, there were 19 samples with wild-type E6 and E7 oncogenes and 12 samples with E6 and E7 oncogene mutants. In the wild type of group of E6 and E7 oncogenes, the majority had a median age of 50 years (range 33-61 years), a mean BMI of 23.7 kg/m2 (range 18.4-36.1 kg/m^2), parity was dominated by less or less frequency. equal to twice (73.7%), the histological type of squamous cell carcinoma (89.4%) and stage II (57.9%). In the group of mutant oncogenes E6 and E7, the majority had a median age of 54.5 years (range 21-58 years); mean BMI 22.1 kg/m^2 (range 19.5-28.2 kg/m^2), parity with a balanced frequency of more or less equal to twice, histological type of squamous cell carcinoma (91.7%) and stage II (58.3%). Analysis of significant differences in characteristics between the mutant and wild type groups using the chi-square test showed that there were no significant differences in characteristics between the mutant and wild type groups (p value> 0.05).

The characteristics of mutations in the E6 and E7 oncogenes in the study subjects are summarized in Table II. The proportion of E6 mutations was dominant in the study subjects with 25.8% (8/31), while the proportion of E7 mutations was only 12.9% (4/31). Point mutations with a change in the position of the 27th nucleotide (T → C) was the most common type of E6 mutation, namely in five samples (16.1%), followed by point mutations with a change in the position of the 360th nucleotide (A → G) in two samples (6.4%) and one sample (3.2%) had a point mutation at the 371st nucleotide (G → A). Most E6 oncogene mutations were synonymous mutations, and E6 G371A/R124K was the only non-synonymous mutation. In the E7 oncogene, there were four types of point mutations with the same proportion of 3.2% for each point mutation. Most mutations in the E7 oncogene are non-synonymous mutations, which are 9, 6% (3/31) (N29T; N29S; R77C).

The relationship between mutations in the E6 and E7 HPV Type 16 oncogenes with p16 expression in research subjects. There was no association between mutations in the E6 and E7 HPV Type 16 oncogenes with p16 expression (c= 0.048 and p value 0.78). When viewed from the interval, the correlation coefficient between the mutations of the E6 and E7 oncogenes of HPV type 16 with p16 protein expression is very weak.

IV. Discussion

High expression of the cyclin dependent kinase inhibitor p16 is strongly correlated with activity of the high-risk HPV E7 oncogene and is a major marker of impaired function of the tumor suppressor protein pRB. In replicating cells, E2F transcription is regulated by phosphorylation of pRB mediated by CDK4/CDK6 and controlled by kinase inhibitors (INKs) [13]. The presence of the E7 oncogene will inhibit the binding between pRB-E2F and will trigger an increase in the expression of p16 which is a CDK inhibitor, but the release of E2F is not mediated by phosphorylation of pRB so that there is no counter-regulatory effect of p16 on the cell cycle [14]. Overexpression of p16 was found in all cases of cervical cancer and as much as 73.33% was detected in cases of squamous cell carcinoma type and in all cases of adenocarcinoma type [15].

Until now, not many studies have analyzed the effect of oncogene variants E6 and E7 HPV type 16 on p16 expression [16]–[19]. The effect of tumerogenesis by the Asian-American (AA) HPV type 16 variant which showed a significantly higher cell percentage of p16 expression in HPV type 16 variant AA compared to HPV type 16 variant European prototype (E) [20]. Where the AA variant has a higher risk of causing cervical cancer than the E variant and is more significant in causing cervical cancer at a younger age this is associated with the presence of mutation variants that are often found in the AA variant, namely non-synonymous mutations that have an impact on changes in the Q14H amino acid, H78Y and L83V in oncogene E6 [21]. In addition, the AA variant has significantly higher E6 and E7 expression than the E variant. The E7 oncogene has three regions that have different functions, where the E7-pRB binding site is located at amino acids (aa) 22-26 which has an LxCxE arrangement in the Conserved Region II (CR II), where studies have shown that changes in the oncogenic potential of E7 are centered on the E7 oncogene. pRB binding site region. Preliminary analyzes of this region suggest that mutations in C24G, E26G and D21G can affect oncogenic activity and binding to pRB [22]-[24].

E7 oncogene mutations are located in the N29S, N29T, R77C and S95S amino acids which are outside the pRB binding site region, proving that the N29S, N29H and R77S variants have lower E7 levels in cells compared to wild type E7 and impaired amino acid residues. proximity to the LxCxE (aa 22-26), CK II (aa 31-32) and CxxC (aa 58-61 and aa 91-94) regions can disrupt the structure of E7 and reduce its oncogenic potential [25]. While the E6 mutation in this study was dominated by non-synonymous mutations that did not cause changes in amino acids and did not change the function

of the protein at the codon so that it did not have the potential to change the oncogenic potential of E6, in contrast to previous studies which had non-synonymous mutation variants in Q14H, H78Y and L83V which has a higher oncogenic potential and has a higher p16 level than other variants [26]-[29]. Non-synonymous mutation variants cause changes in the R124K amino acid which is located in the nuclear localization sequence 3 (NLS 3) region, this region containing the C-terminal amino acid arginine (R) binding to mediate the E6 oncogene nuclear import to the host cell through the Kap β2 pathway family. Changes in the amino acid arginine can reduced the nuclear import activity of the HPV16 E6 oncogene, the R124G mutation may causes a decrease in the number of E6 nuclei import in the host cells compared to the wild type and reduces the effectiveness of E6 in causing epithelial cell immortality [30]-[32].

CONFLICT OF INTEREST

Authors declare that they do not have any conflict of interest.

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