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Association Between E6 and E7 Human Papilloma Virus Type 16 Oncogen Mutations and P21 Protein Expression in Cervical Cancer

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ABSTRACT

Cervical cancer is a disease characterized by the growth of abnormal cells in the cervical tissue. Cervical cancer is mostly caused by Human Papillomavirus (HPV) types 16 and 18. The role of genetic factors in the development of cervical malignancy is mediated by the presence of a mutation in the HPV 16 oncogene, especially oncogenes E6 and E7. Oncogenic proteins E6 and E7 in HPV initiate dysregulation of cellular proliferation and apoptotic mechanisms by targeting tumor suppressor proteins, such as the p21 protein. The purpose of this study was to assess the association between mutations in the E6 and E7 oncogenes of HPV-HR Type 16 and the pattern of p21 protein expression. This cross-sectional study was conducted at the Obstetrics and Gynecology Polyclinic of Prof. Dr. I.G.N.G. Ngoerah Hospital, from September 2020 to September 2021. The material taken was cervical cancer tissue from cervical cancer patients and then put into a preservative solution and then put in a cooler. DNA isolation was performed, and PCR was performed to determine positive and negative HPV. The amplification of the E6 and E7 genes was carried out before the sequencing and analysis of the E6 and E7 gene mutations was carried out. Then, immunohistochemical staining of p21 was carried out, followed by data analysis using SPSS for windows version 22.0. There were no significant differences in characteristics between the two groups. There was no association between mutations in the E6 and E7 HPV Type 16 oncogenes with p21 protein expression in cervical cancer cases (p-value 0.22).

Keywords: Cervical Cancer, Human Papilloma Virus, E6 and E7 oncogenes.

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I. Introduction

Cervical cancer still is a malignancy gynecology that has become a problem worldwide, including in Indonesia. Various studies for understanding abnormality underlying biomolecular have been done, until analysis mutation expected genetics could give innovative and more strategic management plans specific. Gene mutations involved in various complexity abnormality proliferation cells have been researched. One of which is still a little discussed and studied in Indonesia is a mutation of E6 and E7 oncogenes involved in the virulence of the Human Papilloma Virus (HPV) as the most important etiology in the pathogenesis of cancer cervical. Likewise, understanding of the influence of mutation oncogenes E6 and E7 against activity cellular and phenotype cervical cancer is still very limited.

Cervical cancer is a marked disease with the existing growth of abnormal cells in the cervical tissue. Cervical cancer part big caused by Human Papillomavirus (HPV) types 16 and 18 [1], [2]. Based on results Global Cancer Statistics (GLOBOCAN) 2018, malignancy this occupy rating fourth as cancer common in women worldwide. Incident a new cases cervical cancer in the 2012 GLOBOCAN survey experienced enhancement from 527,000 to 570,000 women in 2018, and mortality increased to 265,700 in 2012 to 311,000 deaths in 2018 [3]. Nearly 90% of deaths because cervical cancer occurs in the population Public with the low economy, where to access screening and

prevention cervical cancer is very limited [4]. Cervical cancer in Indonesia occupies order second cancer highest in women in Indonesia, with a case new of 32,469 (incidence 17.2%) [3]. Estimation amount of woman sufferer of new cervical cancer range between 90-100 cases per 100,000 population, and the number of cases per year is estimated to reach 30 to 40 thousand cases [2]. In Bali, the prevalence of HPV-16 occupies order highest that is 15% of entire group HPV oncogenic risk high. The incidence rate of cervical cancer at Prof. Dr. I.G.N.G. Ngoerah Hospital Denpasar from 2018 to 2019 was obtained among 649 cervical cancer patients. The highest proportion was with stage IIB, with as many as 201 cases (31%) and staged IIIB with 169 cases (26%) [5].

Studies on risk factors that increase the susceptibility of cervical cancer development are increasingly needed as part of secondary prevention. These risk factors include internal factors in the form of genetic factors and external factors (smoking habits, multiple partners, alcohol consumption, number of children) [6]. Currently, genetic factors are increasingly attracting attention because they are sporadic and cannot be modified like external factors. Genetic factors in the development of cervical malignancy are mediated by the presence of a mutation in the oncogene Human Papilloma Virus (HPV) 16, especially oncogenes E6 and E7.

E6 and E7 are early genes in the region coding for the functional oncogenic HPV genome [7]. Cross-interaction of E6 and E7 oncogenes with various pathways plays a key role in cervical carcinogenesis [7]. Transcription of the E6 and E7 oncogenes has always been reported in cervical carcinoma and is the first indication of a major role in pathology mechanism—viral genes in HPV-mediated cervical cancer. Oncogenic proteins E6 and E7 in HPV initiate dysregulation of cellular proliferation and apoptotic mechanisms by targeting tumor suppressor proteins of which are p21 proteins [8].

The p21 protein is a protein that plays an important role in the regulation mechanism of cell proliferation. Inhibiting p21 protein activity by oncoproteins E6 and E7 may facilitate malignant transformation. A case-control study in Brazil involving 132 women with HPV positive status and cervical lesions (cases) and 154 women with HPV negative and without cervical lesions (controls) examined the association of single nucleotide polymorphisms (SNPs) p21 protein on susceptibility to cervical lesion progression. The study concluded an association between p21 protein polymorphisms and susceptibility to cervical lesion development (P=0.0009). Single nucleotide polymorphism in the protein p21 (C>A) at codon 31 (p21 Ser31Arg; rs1801270) will produce an amino acid substitution of arginine for serine, which results in changes at the protein level. Mutations in these tumor suppressor genes have been reported to play a role in human tumor progression [6].

A study on HOX Antisense further strengthened the evidence for the role of p21 protein in cervical cancer pathogenesis. Overexpression Intergenic RNA (HOTAIR) in 25 cervical cancer tissues. The study found that increased HOTAIR expression was associated with the downregulation of p21. Protein in cervical cancer cells (P<0.01). Increased HOTAIR could induce radio-resistance by inhibiting p21 protein in HeLa cells (P<0.0001) while decreasing HOTAIR, on the other hand, upregulated p21 protein, thereby improving the radio-sensitivity of C33A cells [9]. Another study on the role of micro-RNA-921 (miR-92a), a short non-coding RNA that plays a role in cell proliferation, showed that downregulation of the p21 protein would lead to increased expression of miR-92a, which contributed to the increased role of miR-92a in the promotion of proliferation. or cervical cancer cell growth [10].

The mechanism of the E6 and E7 oncogenes in regulating the p21 protein was reported. This study reported that the expression of E6 and E7 HPV16/18 was associated with the expression of TMPOP2 in CaSki and HeLa cervical cancer cells. Indirectly, increased expression of TMPOP2 was accompanied by downregulation of p53 and p21. The repressive activity of the p53-p21-DREAM protein complex was also inhibited by oncogenes E6 and E7 [11], which affects the next gene target [12]. A study in transgenic mice found that the p21Cip1 gene functions as a tumor suppressor in cervical carcinogenesis and that inactivation of the p21Cip1 protein by E7 HPV-16 partially contributes to the role of E7 in cervical carcinogenesis [13].

The genetic sequences of the E6 and E7 oncogenes are very susceptible to mutation. Mutations occur due to interactions between the viral genome and the host. Changes in one nucleotide of oncogenes E6 and E7 can affect the function of E6 and E7 proteins. There are differences in infection persistence and cervical cancer progression in several intertypic variants of HPV HR type 16 [14]. Mutations on the E6 and E7 oncogenes of the HPV16 variant and the effect of these mutations on the interaction pattern between E6 and E7 oncoproteins and cell cycle proteins are important factors that can determine differences in the biological properties of viruses that affect their infectivity and pathogenicity [15].

Thus, biomolecular analysis of mutations in the E6 and E7 HPV-HR Type 16 genes is an important approach to understanding the relationship between HPV-HR Type 16 infection and cervical cancer development, particularly in the female population with cervical cancer in Bali. A comprehensive analysis of the association between the E6 and E7 mutation patterns of HPV-HR type 16 oncogenes and the expression patterns of proteins that play a vital role in proliferation, apoptosis, and cell cycle regulation, such as the tumor suppressor p21 is also a very important approach to understanding between mutational interactions. Oncogenes E6 and E7 with host cells and their effect on cervical cancer development. There has been no research that examines this in the population of women with cervical cancer in Bali. Given the enormous benefits of understanding the relationship between mutations in the E6 and E7 HPV-HR Type 16 oncogenes and the p21 protein expression pattern, this research is very important.

II. Discussion

This study is analytic with a cross-sectional design, which sample in this study uses tissue paraffin block with cervical Cancer taken from a woman with cervical Cancer who visited the Obstetrics and Gynecology Clinics, FK UNUD/ Prof. Dr. I.G.N.G. Ngoerah Hospital In the year 2020-2021, selected by consecutive sampling from the population after classified in inclusion and exclusion criteria. Inclusion criteria this is a study of a Woman with cervical Cancer diagnosed at the

Obstetrics and Diseases Polyclinic The content of Prof. Dr. I.G.N.G. Ngoerah Hospital, Denpasar during 2020-2021 and not yet get therapy surgery/chemotherapy/radiation, readily follow study after

signing informed consent. Criteria exclusion research is Cervical Cancer is not caused by HPV-HR type 16 and samples network cervical cancer damaged or no representative for DNA PCR examination and immunohistochemistry.

Informed consent, history, and examination record medical for knowing identity, especially age and parity subject research, General Physical Examination, Gynecology Examination for getting sample network cervical cancer performed on patients who are willing Become sample on research this. Patients with cervical cancer were recruited from the Obstetrics and Gynecology Polyclinic of Prof. Dr. I.G.N.G. Ngoerah Hospital during the study period. The patient had never received surgery, radiotherapy, or chemotherapy prior to cervical cancer tissue sampling. Demographic and clinical data, including age, parity, and clinical stage, were determined based on anamnesis, physical examination, and supporting examinations. Next material is taken in the form of network cervical cancer from sufferer cervical cancer then entered to in solution conservative then entered to in cooler until conducted extraction more continued. Procedure taking sample network cervical cancer by complete yet confirmed the place storage/container sample in condition sterile. Two-tube, the tube I (contains 1X/NaCl 0.9% PBS buffer): for PCR examination in the Microbiology Lab made two tubes. Tube II (contains PBS Formalin buffer): for IHC examination in the Histology Lab created for one tube. Brought to the laboratory with use icebox (in condition permanent cold). Sample for PCR (2 pieces Tube I) and IHC (1 piece Tube II) no can until swapped. Next inspected to find out the consequence of the interaction between the viral genome and the host. Mutations in both oncogenes this cause a change arrangement of the E6 and E7 oncoprotein amino acids, which in turn could influence pattern interaction Among oncoproteins E6 and E7 and protein p21.

After That, all the data that has been obtained and analyzed used with the software Statistical Product and Service Solutions (SPSS) program for Windows version 22.0. All data collected conducted analysis Test the normality of the distribution of numerical variables with the Shapiro Wilk test, which aims to find the distribution value on the characteristics of the sample, whether it is normally distributed or not. Next is the correlation analysis test of 2 correlated variables where there are variables that influence, and there are variables that use the Contingency Coefficient correlation test. Study this already get agreement appropriateness ethics from Research Ethics Commission Medical Faculty of University Udayana/Prof. Dr. I.G.N.G. Ngoerah Hospital dated 19 March 2021 number 823/UN.14.2.2.VII.14/LT/2021. get Permission Study from the Education and Research Section dated 24 May 2021, Number LB.02.01/XIV.2.2.1/17374

This study uses a cross-sectional design to know the association between mutation oncogenes E6 and E7 High-Risk Human Papilloma Virus (HPV-HR) type 16 with an expression of protein 21 in the case of cervical cancer. The study was conducted against 31 women with cervical cancer group mutant and wild-type.

From 31 samples with HPV16 positive, there were 19 samples with wild-type E6 and E7 oncogenes and 12 samples with mutant oncogenes E6 and E7. The characteristics subject study is summarized in Table 1.

TABLE I: CHARACTERISTICS OF RESEARCH SUBJECTS

	Mutant	Wildtype	P-value
Age (years) , median	54.5 (21-58)	50 (33-61)	0.72
Parity , n(%)			0.18
2	6(50)	14(73,7)	
>2	6(50)	5(26.3)	
BMI (kg/m^2), median	22.1 (19.5-28.2)	23.7(18.4-36.1)	0.34
Histological Type, n (%)			0.63
Squamous cell carcinoma	11(91,7)	17 (89.4)	
Adecarcinoma	1 (8.3)	1 (5,3)	
Neuroendocrine carcinoma	0	1 (5,3)	
FIGO stage, n (%)			0.87
Stage I	1 (8.3)	2(10,5)	
IA1	0	1 (5,3)	
IB1	1 (5,3)	1 (5,3)	
Stage II	7(58.3)	11(5 7,9)	
IIA1	0	2 (10,5)	
IIA2	1 (5,3)	0	
IIB	6 (50)	9 (47.4)	
Stage III	4 (33.3)	6 (31.6)	
IIIB	4 (33.3)	6 (31.6)	

BMI: body mass index; kg: kilograms; m2 : square meter.

In this study, we analyse mutation to sequence HPV HR Type 16 E6 and E7 oncogenes and their relationship to the expression of protein 21 (p21) in patients with cancer cervical. This study found that the median age of the patient with cervical cancer wild type is 50 years with a span age of 33-61 years. In the E6 and E7 oncogene mutant groups, most have 54.5 years (range 21-58 years). This is similar to GLOBOCAN research in 2020 that describes population cancer as highest at the age of 30 to 49 years (90% of cases found at the age this), so that recommended for conducted screening routine at age the with visual inspection using sour acetate or Papanicolaou test [16].

In the case of HPV16 E6 and E7 mutants, the most histology type in this study is squamous cell carcinoma (91.7%). This is different from studies conducted in China in 2017 by Jiang *et al*. That case has the most mutations was found in cervical cancer is non-squamous cell carcinomas (SCCs) [17].

This study shows that the stage of cervical cancer that experienced the most mutation is stage II (58.3%), followed by stage III (33.3%). In patients with cervical cancer, most wildtype stage II (47.9%) followed by stage III (31.6%). This result was similar to research by Yang *et al*. in 2013. they found that cervical cancer is more often at an advanced stage based on FIGO, namely at stage IIA to stage IV (55.1%). In this study, it was also found that the older was dominant at more than 35 years [18].

The characteristics of mutations in the E6 and E7 oncogenes in this study subjects are summarized in Table 2. The proportion of E6 mutations is dominant in this study subjects at 25.8% (8/31), while the proportion of E7 mutations is only 12.9% (4/31). Point mutations with changes in the position of the 27th nucleotide (T→C) was the most

TABLE II: PROPORTIONS AND CHARACTERISTICS E6 AND E7 MUTATIONS IN SUBJECT STUDY

Position Nucleotide	Prototype	Variant	N (%)	Position amino acids	Prototype	Variant	N (%)
E6							
27	T	C	5(16,1)	9	F	F	-
360	A	G	2(6,4)	120	E	E	-
371	G	A	1(3,2)	124	R	K	1 (3,2)
E7							
86	A	C	1(3,2)	29	N	T	1 (3,2)
86	A	G	1(3,2)	29	N	S	1 (3,2)
229	C	T	1(3,2)	77	R	C	1 (3,2)
285	T	C	1(3,2)	95	S	S	-

common type of E6 mutation, namely in five samples (16.1%), followed by point mutations with changes 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 mutations in the E6 oncogene are synonymous mutations, and E6 G371A/R124K is 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 were non-synonymous mutations, namely 9.6% (3/31) (N29T; N29S; R77C).

There are three different regions in the HPV genome: early gene coding region (E), late gene coding region (L), and control region length (LCR). Early genes (E1, E2, E4-E7) encode for viral replication and regulatory proteins, three of which, E5, E6, and E7, are oncogenic. Depending on function specifically, early genes are expressed in various Step cycle live viruses. The final gene coding two structural proteins involved viral information capsid, while the LCR involved element regulator controlling viral DNA replication and transcription [19].

Based on type mutation, similar to the results obtained in the previous study, this study finds part big occurs in mutation non-synonymous. However, in this study, the evaluation type of different mutations, namely in the G12, G13, and Q61 mutations, was partially found in exon 2.

In this study, the proportion of E6 mutants was 25.8% (8/31), and E7 was 12.9% (4/31). Though thereby, only 1 of 8 samples mutant E6 and 3 of 4 samples mutant E7 experienced non-synonymous mutation, whereas the rest experience mutation synonymous.

Synonymous mutations are point mutations, which means they are incorrectly copied DNA nucleotides that change only one base pair in the RNA copy of DNA. Codons in RNA are a set of three nucleotides that code for a specific amino acid. Most amino acids have multiple RNA codons translated into specific amino acids. Mutation point mutations are synonymous because the mutated codon has the same meaning as the original codon and therefore does not change the amino acid. If the amino acids are not changed, the protein is not affected either [20].

Non - synonymous mutations have a much greater effect on the individual than synonymous mutations. In non - synonymous mutations, there is usually an insertion or deletion of one nucleotide in the sequence during transcription when the messenger RNA copies the DNA. This single missing or added nucleotide causes a frameshift mutation that changes the entire reading frame of the amino acid and codon sequences. This will affect the encoded amino acid and alter the resulting expressed protein. The severity of this mutation depends on how early the amino acid sequence was. The earlier it occurs, the more many altered proteins.

Another way a non-synonymous mutation occurs is when a point mutation converts a single nucleotide into a codon that does not translate into the same amino acid. A single amino acid change often does not affect the protein and can persist. However, if this occurs at the beginning of the sequence and the codon is changed to translate into a stop signal, the protein is not formed. Mutation non - synonymous no always character negative but also can positive [20].

This study found the proportion of E6 mutants at 25.8 % (8/31) and E7 at 12.9% (4/31). Based on a review study previously found that mutation, the most common E6 and E7 genetics researched is HPV-16, 33, and 58. A study in China on 138 cases lesson cervix and SCC with HPV-16 infection shows that there is four mercy a missense mutation was found in the E6 gene; locus with frequency mutation highest are T350G (36/75, 48%) and T178G (19/75, 25.3%). In the E7 gene, the. The locus with the frequency mutation highest is the A647G (18/75, 24%). Virus mutations can cause substitution amino acids in the protein coded accordingly, which can change the characteristics of the biologic and immunogenicity of the virus and affect the ability of HPV16 carcinogenic [21].

The relationship between E6 and E7 HPV Type 16 oncogene mutations with p21 protein expression in patients with cervical cancer is summarized in Table III. There was no association between HPV Type 16 E6/E7 oncogene mutations and retinoblastoma protein expression (c = 0.216 and p-value = 0.22). The coefficient interval correlation level shows that the connection mutation HPV type 16 E6 and E7 oncogenes with p21 protein expression is weak.

TABLE III: ASSOCIATION BETWEEN E6 AND E7 HPV TYPE 16 MUTATIONS WITH EXPRESSION OF PROTEIN 21 IN THE PATIENTS WITH CERVICAL CANCER

	p21 expression			
	Weak	Strong	C-Value	P-value
Mutants E6 and E7	1	11	0.216	0.22
Wild type E6 and E7	5	14		

There is study investigate the association between different HPV genotypes and p53, p21 and p27 expression in carcinoma cervical. Research results show that p53 and p27 expression is not related to HPV genotype, but in carcinoma cervix HPV-18 positive, p21 expression found to decrease by significant or same very nothing [22]. Research with use mice find that p21 on cell's mice working as a tumor suppressor were found in mice that were given estrogen for six months, mice that did not there is p21 showing happening cervical cancer compared with p21 containing mice. However, linkages between E7 and p21 are still a paradox. On one side,

E7 causes enhancement of p21 levels in cells/tissues in humans and mice.

On the other hand, the E7 can disable p21. In this study, observe E7 's ability to disable p21 via CDK2 and phospho-CDK2 activation. Observations on the network mice are consistent with observations previously that E7 can induce proliferation cells because of the ability to disable p21 in cell humans. This shows that induction of p21 by E7 is not missing from E7 's ability to disable p21 [23]. Following results of this study where p21 expression does not relate to mutation oncogenes E6 and E7 in HPV type 16 infection.

Studies conducted stated that p21 expression does not relate to HPV status. This possibility caused the difficulty of detecting low viral levels in the population's studies. Difficulty in differentiating fresh samples and preserved specimens with paraffin also being one possible factor played a role in the results of this study. This is also the constraint in a study where several fresh samples and samples have been Preserved in paraffin before being sent together for analysis. Limitations potential other is lack of analysis continuity live, so fail to determine score prognostic HPV infection and regulatory proteins cycle cells/apoptosis [24].

There is study analyzed 132 women HPV positive with lesson cervical and 154 controls to polymorphism p21 and p27. The study's results in the state polymorphism of the two proteins do not relate to lesion grade cervical. Not related connection p21 polymorphism with lesson cervix possibility played by existence genetic heterogeneity due to different ethnicity and/or difference distribution and frequency alleles in the study population [6].

Another research also states no difference in mean p21 genotype frequency in the group cases and controls. women with HPV-HR infection do not find an existing connection means p21 genotype with cancer cervical. Although they put forward several limitations in studies conducted, such as small amount sample and study the hospital-based, however a small possibility that there are biased results as a result of the selection process because group case and control chosen prospective during the study period [25].

The result of this study is the same as previous study results. This is what shows no existence difference in significant p21 expression between E6 and E7 groups mutant and wildtype. In research on this mutation, The E7 oncogene is located in the amino acids N29S, N29T, R77C and S95S, which are outside from pRB binding site region, other than that study proved that the N29S, N29H and R77S variants have higher levels of E7 low in the cell compared with wild type E7 and interference residue adjacent amino acids with region LxCxE (aa 22-26), CK II (aa 31-32) and CxxC (aa 58-61 and aa 91-94) can disturb E7 structure as well as reduce potential oncogenic. Whereas E6 mutation in research this more dominated by non-synonymous mutations that do not cause change which and not amino acids change the function of the protein at the codon the so that no potential change potency E6 oncogenic, different with study the previous variant non-synonymous mutations in Q14H, H78Y and L83V that have potential oncogenic higher and have higher levels of high levels of p21 compared another variant [26].

In this study, dominated by the E6 mutation compared to the E7 mutation, the presence of different mutations was found compared with the previous study. There is no difference in mean percentage p21 expression in both groups. The E7 oncogene has three regions that have different functions, where the binding site of E7-pRB is at amino acid (aa) 22-26, which has arrangement LxCxE in the Conserved Region II (CR II), where studies have proven that potential change region - centre E7 oncogenic binding site pRB. Preliminary analysis of the region shows that C24G, E26G, and D21G mutations can influence activity oncogenic and binding with pRB [27].

In this study, variant non-synonymous mutations were found to cause a change in R124K amino acid, which is located in the nuclear localization sequence 3 (NLS 3) region; NLS 3 is the region containing the C-terminal bond of the amino acid arginine (R) to mediate E6 oncogene enters into the cell host through plot Kap 2 family. Change in the amino acid arginine could disturb the mechanism of entry oncogene E6 into a cell thing, where The R124G mutation causes a declining number of E6 cores in cell host compared with wild type and reduced effectiveness of E6 in cause immortality cell epithelium.

III. Conclusion

Based on the data and discussion above, it can be concluded that there is no association between mutations in the E6 HPV Type 16 oncogene and p21 protein expression. In the case cancer cervical cancer and there is no association between mutations in the E7 HPV Type 16 oncogene and p21—protein expression in the case of cancer cervical.

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