RESEARCH ARTICLE

HPV18 E6/E7 Mutation and Their Association with The Expression Level of Tumor Suppressor Proteins p53 and pRb among Indonesian Women with Cervical Cancer

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Abstract

BACKGROUND: The E6/E7 mutation contributes to the intra-typic variant of HPV18 which may differ in their oncogenic potential. E6 and E7 target the tumour suppressor protein p53 and pRb, respectively, and their degradation play a crucial role in cervical carcinogenesis. However, the prevalence of HPV18 E6/E7 variants among Indonesian women with cervical cancer has not been elucidated. Therefore, this study was conducted to characterize the HPV18 variants among Indonesian women with cervical cancer and their association with tumor suppressor protein p53 and pRb.
METHODS: A hundred Indonesian women with pathologically proven cervical cancer were consecutively recruited into the study. Polymerase chain reaction (PCR) was performed to detect HPV18 DNA E6 and E7 oncogenes using specific primers and the variants was determined through nucleotide sequencing. Expressions of p53 and pRb were analyzed through immunohistochemistry by using specific antibodies targeting p53 and pRB.
RESULTS: The rate of HPV18 positivity was 24%. The rate of E6 and E7 mutation was 45.4% and 59.1%, respectively. Those with E6 mutation had significantly higher expression of p53 and pRb as compared to those with wildtype E6 (p<0.05). Subjects with E7 mutation only had higher expression of pRb (p<0.05). Phylogenetic analysis revealed that 54.5% subjects had genetic sequences closely related to Asian lineages, particularly A1, A4, and A5 sublineage. Interestingly, 3 subjects had genetic sequences closely related to MK813921, a newly identified sequences. However, 45.5% subjects had distinct genetic sequences that did not related to the reference sequence used in this study.
CONCLUSION: E6 and E7 mutation was common among Indonesian women with HPV18 cervical cancer and associated with the level of tissue p53 and pRb expression.
KEYWORDS: HPV18 E6/E7, mutation, epidemiology, Indonesian women

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Introduction

Cervical cancer remains as one of the most significant health problem for women all over the world. In 2020, approximately 604,127 new cases of cervical cancer and 341,831 related deaths were reported worldwide, with an age-standardized incidence rate of 13.3 cases per 100,000 woman-years and a mortality rate of 7.2 deaths per 100,000 woman-years.(1) The incidence of cervical cancer in Indonesia is 100 cases per 100,000 women.(2) It is well established that persistent infection with high-risk human papillomavirus (HPV-HR) is the primary cause of cervical cancer. HPV18 is the one of the most common genotypes that causes cervical cancer.(3,4) It contributes to around 12% of cervical squamous cell carcinoma (SCC) cases and

37% of adenocarcinoma (ADC) cases.(5) In Indonesia, the overall prevalence of HPV18 infection was reported to be 16.1%, which was the third commonest HPV-HR after HPV52 (23.2%) and HPV16 (18%).(6)

As in other HPV-HR genotypes, E6 and E7 also play vital role in driving carcinogenesis after persistent HPV18 infection. E6 oncoprotein acts specifically in driving carcinogenesis by inducing the proteasomal degradation of p53, while E7 induce pRb inactivation.(7) Primarily a transcription factor, the p53 protein controls a multitude of processes, including cell cycle arrest, DNA repair, cell apoptosis, autophagy, and metabolism, and it also decides whether cells die under stress. By recruiting transcriptional corepressors and/or chromatin remodelling protein factors, such as Histone deacetylase (HDAC), Swi-independent (Sin3), C-terminal binding proteins (CtBP), and SWItch/Sucrose Non-Fermentable (SWI/SNF), to promoter regions, pRB suppresses the transcription of the E2F-target gene, thereby halting cell cycle progression.(7) A recent study reported that inactivation of HPV18 E6/E7 oncogene by Clustered Regularly Interspaced Short Palindromic Repeats/CRISPR-associated protein 9 (CRISPR/Cas9) vector increase the p53 and p21 protein levels, inhibit cell proliferation, induced apoptosis and subsequently, inhibit tumour growth.(8) Another study demonstrated that knockout of HPV18 E7 expression resulted in decreased E6 expression with activation of pRb/p21 pathway.(9) However, the genetic sequences of HPV18 E6/E7 oncogenes are prone to mutation which result from the interaction between HPV genome and the host cells.(10) A study in Korea had identified 15 new nucleotides substitution within the E6, E7 and L1 oncogene, and 6 of those mutations resulted in amino acid changes.(11) While a study in China reported that 81.1% HPV18 variants that were identified were novel variants.(12) An international study involving HPV18-positive cervical samples from 39 countries had identified a total of 209 unique HPV18 sequence variants which form three distinct phylogenetic lineages.(13)

The E6/E7 mutation contributes to the intra-typic variant of HPV18 which may differ in their oncogenic potential. One study reported the association between certain HPV18 variant lineages and the risk for higher grade cervical lesions. Non-European variants were more strongly associated with risk of high-grade cervical lesions as compared to European variants.(14) On the contrary, several studies had reported that HPV18 variants had no significant effect on the degree and progression of the cervical lesions.(11,13,15) A study conducted in Japan found no significant difference in the prevalence of specific HPV18 sub-lineages between cervical cancer/precancer cases and controls, nor between cases of SCC and ADC.(16) However, one study showed that Non-European HPV18 variants were commonly seen in ADC.(17) These conflicting findings may indicate that HPV18 genetic diversity exists within the context of geographic or ethnical variation. Thus, knowledge of the HPV18 variation on certain geographic or ethnic population is important and serves as a potential tool in developing strategies of combating cervical cancer for targeted populations. In Indonesia, data about the prevalence and pattern of HPV18 variants based on E6 and E7 gene sequence is still lacking. Moreover, their association with the target tumor suppressor protein p53 and pRb is a novel and interesting topic to study. Thus, this study was conducted to characterize the HPV18 variants among Indonesian women with cervical cancer and their association with the tumor suppressor protein p53 and pRb.

Methods

Study Design and Subject Recruitment

A cross-sectional study was conducted involving 100 Indonesian women with pathologically proven cervical cancer who attended the Gynecologic Oncology Outpatient Clinic, Prof. dr. I G. N. G. Ngoerah General Hospital, Denpasar, Bali, Indonesia during June 2019 to December 2020. Tissue sample from cervical cancer was taken to analyse the HPV18 E6/E7 variants and the expression of p53 and pRb protein. The inclusion criteria were women with pathologically proven cervical cancer and HPV18 positive with clear quality of sample. The exclusion criteria were women who had underwent any form of treatment (surgery, radiation, or chemotherapy) before the commencement of this study. Before the study, all subjects or their legal surrogate signed a written informed consent to participate in this study. With the prevalence of 0.25 (16), the the adequate sample size in this study was calculated following the formula as mentioned in previous publication (18), and the total number of samples required was 22. This study was approved by the Ethical Committee of Faculty of Medicine, Udayana University/Prof. dr. I G. N. G. Ngoerah General Hospital, Denpasar, Bali, Indonesia (No. 2381/UN14.2.2.VII.14/LT/2022).

Clinical Characteristics

Data about the clinical characteristics of the study population (age, parity, histologic type, and stage) were all obtained from the medical records. Stages were classified according

to 2018 FIGO Classification.(19) Histologic types were classified according to WHO Classification, *i.e.*, squamous carcinoma and adenocarcinoma of the cervix.(20)

DNA Isolation

Cervical cancer tissue was biopsied from the study participants and embedded in PBS 1X/NaCl 0.9% solution. DNA isolation was performed using High Pure PCR Template Preparation kit (Cat.No. 11796828001; Roche, Basel, Switzerland). A 25-50 mg tissue was placed into a mortar and 200 µL tissue lysis buffer was added before grinding. The tissue was then transferred into 1.5 mL centrifuge tube and incubated at 55°C for 60 minutes. A 200 µL lysis buffer and 40 µL proteinase K were added to inactivate the DNAse and the tissue was incubated subsequently at 70°C for 10 minutes. A 100 µL isopropanol was added to precipitate the DNA. The sample was then transferred into a filter tube and centrifuged at 8000 rpm for 1 minute. A 500 µL inhibitor removal buffer was added to remove all the residues. After centrifugation, a 500 µL wash buffer was added. The sample was centrifuged again at 8000 rpm for 1 minutes. Elution was added before another round of centrifugation at 8000 rpm for 1 minute. The solution of sample was then used for further analysis.

Polymerase Chain Reaction (PCR)

Before proceeding with HPV18 detection, identification of the universal HPV DNA in collected samples were performed. The sample which were positive for universal HPV were then considered eligible for HPV18 detection. One hundred cervical cancer tissue sample were screened and the positivity rate for HPV18 was found to be 24%. Subsequently, the sample that was positive for HPV18 was included into the study population. PCR was used to detect HPV DNA with primers My09 (5'-CGT CCM ARR GGA WAC TGA TC-3') and My11 (5'-GCM CAG GGW CAT AAY AAT GG-3').(21) The PCR protocol included an initial denaturation at 95°C for 3 minutes, followed by 35 cycles of denaturation at 95°C for 1 minute, annealing at 55°C for 1 minute, extension at 72°C for 1 minute, and a final extension at 72°C for 5 minutes. HPV18 DNA was amplified using specific primer for E1 gene, as the surrogate gene.(22) (Forward: 5'-ATA GCA ATT TTG ATT TGT C-3'; nucleotide position: 1989–2007; Reverse: 5'-AAA CTC ATT CCA AAA TAT G-3'; reverse primers position: 2385–2403; amplified DNA size 415 bp). The PCR protocol included an initial denaturation at 95°C for 3 minutes, followed by 35 cycles of denaturation at 95°C for 1 minute, annealing at 45°C for 1 minute, extension at 72°C for 1 minute, and a final extension at 72°C for 5 minutes. The 25 µL reaction mixture consisted of 2.5 µL DNA template, 1.25 µM of each primer, 12.5 µL Go2Green Master Mix (Promega, Madison, WI, USA), and 7.5 µL of water. DNA amplification was performed using a MiniAmp Thermal Cycler (Applied BioSystems, Waltham, MA, USA).

E6 and E7 Gene Amplification

HPV18 E6 and E7 oncogenes were amplified using specific primers as follows: HPV18-E6 F: 5′-AGA AAC ACA CCA CAA TAC TAT GGC G-3′; HPV18-E6 R : 5'-GTC GGG CTG GTA AAT GTT GAT-3'; HPV18-E7 F: 5'-CGACAGGAACGACTCCAACGA-3'; HPV18-E7 R: 5'-ATA AAA CCA GCC GTT ACA ACC CGT G-3'.(11) The 25 µL amplification mixture included 2.5 µL DNA template, 2 µM of each primer, 12.5 µL Go2Green Master Mix (Promega), and 6 µL of water. DNA was amplified on a MiniAmp Thermal Cycler (Applied BioSystems) using the following settings: initial denaturation at 95°C for 3 minutes, 35 cycles of denaturation at 95°C for 1 minute, annealing at 57°C for 1 minute, extension at 72°C for 1 minute, and a final extension at 72°C for 5 minutes. After PCR, samples were analyzed by electrophoresis on a 1.5% agarose gel.

E6 and E7 Gene Sequencing

The amplified E6 and E7 sequences were aligned with the prototype sequence from NCBI (http://blast.ncbi.nlm.nih.gov/Blast) to create alignments. The HPV 18 prototype E6/E7 gene sequence (GenBank: NC_001357) served as the reference standard for comparisons.(23) Sequencing was performed using software program MEGA10 (Mega Software, Pennsylvania State University, University Park, PA, USA). The sequence of nucleotide and amino acid was aligned using software program BioEdit (Informer Technologies, Los Angeles, CA, USA). Phylogenetic analysis was performed using MEGA10 software to classify the HPV variants into their respective lineages (A, B and C) and sublineages (A1-8, B1-3 and C).The distinction into lineages and sublineages was based on their rate of nucleotide variations, *i.e.*, the lineages and sublineages vary by 1–10% and 0.5–1%, respectively. The accession numbers used for phylogenetic analysis were as follows. A1 sublineage: EF202143-EF202145, A2 sublineage: EF202146, A3 sublineage: EF202147- EF202149, A4 sublineage: EF202150-EF202151, A5 sublineage: GQ180787, A6 sublineage: KY457833- KY457836, A7 sublineage: KY45737-KY45840, A8 sublineage: KY457826-KY457827; B1 sublineage: EF202153-

EF202155, B2 sublineage: KC470224-KC470225, B3 sublineage: EF202152; and C lineage: KC470229-KC470230. (11)

Immunohistochemistry (IHC)

Cervical cancer tissue was immersed in a 10% phosphate-buffered formalin solution for 24 hours. It was then soaked in graded alcohol successively (30%, 40%, 50%, 70%, 80%, and 96%) 3 times each for 25 minutes. The tissue was placed in xylene clearing agent for 1 hour each 3 times until transparent. After infiltrating 3 times for 1 hour each with pure paraffin, the tissue was embedded in liquid paraffin, left to form blocks (± 3 hours) so that it could be easily sliced with a microtome. The tissue was then cut using a microtome for 5 µm thick, and attached to a glass object that has been coated with an adhesive such as Poly-Lysine, then incubated at 60°C for 2 hours.

p53 staining was performed using rabbit polyclonal antibody that target nuclear p53 protein (Cat. No. bs-0033R; Bioss, Woburn, MA, USA) with the concentration of 1 µg/µL, and dilution 1: 50 in PBS pH 7.4, following the protocol stated by the manufacturer. pRb staining was performed using rabbit polyclonal antibody Rb/P105 RB (Ser807 + Ser811) that targets nuclear pRb protein (Cat. No. bs-3380R; Bioss) with the concentration of 1 µg/µL and dilution 1: 100 in PBS pH 7.4, with the same protocol as p53 staining. The expression of p53 and pRB was examined in one preparation and one area was selected randomly. The expression was examined in high field magnification (40x Objective). The immune-reactive score (IRS), which was based on the proportion of positive cells and the staining intensity, was used to evaluate the IHC staining of p53 and pRb. The cells were considered to be positive if an intranuclear DAB staining was observed (brown hue with mild, moderate, and intense staining). The labeling index was used to calculate the proportion of positive cells (Labeling Index = Number of IHC Positive Cells × 100/Total Number of Cells Observed). The IRS score, which ranged from 0 to 12 and represented groupings of p53/pRb expression with ≤6 low and >6 high, was calculated by multiplying the two scores (Table 1). Two observers completed the counting, with the final count being determined by taking the mean.(24)

Results

Baseline Characteristics

The baseline characteristics of the study population are summarized in Table 2. There was no difference in age, FIGO stage, and histologic subtype of cervical cancer among wildtype and mutant E6/E7 group.

Mutation of HPV18 E6 and E7 Oncogene

One hundred cervical cancer samples during the study period were collected. The rate of HPV18 positivity was 24%. Among 24 samples which were HPV18 positive, 22 were eligible for E6/E7 gene sequencing and subsequent mutation analysis. Two subjects were not eligible for gene sequencing due to fragmentation of DNA material before the analysis. The length of E6 gene and amino acids that had been successfully sequenced were 477 base pairs and 158 amino acids, respectively. Meanwhile, the length of E7 gene and amino acids that had been successfully sequenced were 318 base pairs and 105 amino acids, respectively.

The rate of E6 mutation was 36.4% (8/22 subjects) (Table 3). E6 C445A, which was a synonymous mutation, was the most prevalent mutation (8/22 subjects; 36.4%). The rate of non-synonymous mutation was only 18.2% (4/22 subjects). Two subjects (9.1%) present with more than one type of E6 mutation (C28T or T10C co-exist with C33A+C445A).

The rate of E7 mutation was 59.1% (13/22 subjects) (Table 3). E7 C162T, which was a synonymous mutation, was the most prevalent mutation (6/22 subjects; 27.3%). The rate of non-synonymous mutation was 40.9% (9/22 subjects). Two subjects (9.1%) present with more than one type of E7 mutation (G241A+C276G and C162T+G312C). Eight subjects (36.4%) had co-existing E6 and E7 mutation.

Table 1. The Immunoreactive score.

A (Percentage of Positive Cells)	B (Intensity of Staining)	IRS Score (Multiplication of A and B)
0 : no positive cells	0 : no color reaction	0-1 : negative
1 : <10% positive cells	1 : mild reaction	2-3 : mild
2 : 10-50% positive cells	2 : moderate reaction	4-8 : moderate
3 : 51-80% positive cells	3 : intense reaction	9-12 : strongly positive
4 : >80% positive cells	Final IRS score (AxB) : 0-12	

Table 2. Baseline characteristics of the study population (n=22).

Variable	All Subjects	Mutant E6	Wildtype E6	*p*-value
HPV18 E6 gene				
Age (years), mean±SD	49.7±11.8	49.0±15.4	50.1±9.7	0.833[a]
Stage, n (%)				
Early	8 (36.4)	3 (37.5)	5 (35.7)	0.642[b]
LACC	14 (63.6)	5 (62.5)	9 (64.3)	
Histologic subtype, n (%)				
Squamous	14 (63.6)	6 (75)	8 (57.1)	0.649[b]
Non-squamous	8 (36.4)	2 (25)	6 (42.9)	
HPV18 E7 gene				
Age (years), mean±SD	49.7±11.8	48.7±13.4	51.2±9.3	0.632[a]
Stage, n (%)				
Early	8 (36.4)	4 (30.7)	4 (44.4)	0.662[b]
LACC	14 (63.6)	9 (69.3)	5 (55.6)	
Histologic subtype, n (%)				
Squamous	14 (63.6)	9 (69.3)	5 (55.6)	0.662[b]
Non-squamous	8 (36.4)	4 (30.7)	4 (44.4)	

[a]t-test was used to obtain p-value, [b]chi square test was used to obtain *p*-value.

Mutation of E6 C445A/E7 C162T, which was synonymous mutation, was the most prevalent co-existing mutation (6/8 subjects; 75%). Two subjects present with non-synonymous co-existing mutation E6 T449G/E7 T269G, which translates into amino acid changes E6 L150R/E7 F90C (Table 3).

Expression of p53 and pRb

All subjects had low expression of p53 and pRB (<5% positive cells) (Figure 1). However, those with E6 mutation had significantly higher expression of p53 and pRb as compared to those with wildtype E6 ($p<0.05$). Subjects with E7 mutation only had higher expression of pRb ($p<0.05$) (Table 4).

Phylogenetic Analysis

The phylogenetic tree analysis of the 22 samples were summarized in Figure 2. These variants formed 5 major cluster. Four samples in the first cluster had close similarity to sublineage A4 Two samples in the second cluster had distinct genetic sequences that did not match any reference sequences. Two samples in the third cluster were close to sublineage A5. Three samples in the fourth cluster had close similarity to MK813921. The fifth cluster was diverged into two sub-cluster: the first subcluster had close similarity to sublineage A1; the second sub-cluster had distinct genetic sequence that did not resemble the reference sequence. Three samples also had distinct genetic

Table 3. Mutation of HPV18 E6 and E7 gene.

Nucleotide Position	Prototype	Variant	N(%)*	Amino Acid Position	Prototype	Variant	N (%)
E6							
10	T	C	1	4	F	S	1
28	C	T	1	10	R	#	1
33	C	A	2	11	P	P	-
432	A	C	2	144	R	R	-
445	C	A	8	149	R	R	-
449	T	G	2	150	L	R	2
E7							
158	G	A	4	53	R	C	4
162	C	T	6	54	A	A	-
241	G	A	1	81	D	N	1
269	T	G	2	90	F	C	2
276	C	G	1	92	N	K	1
312	G	C	1	104	C	H	1

*Mutation rate was among all subjects with HPV-18 positive cervical cancer, [#]unidentified amino acid.

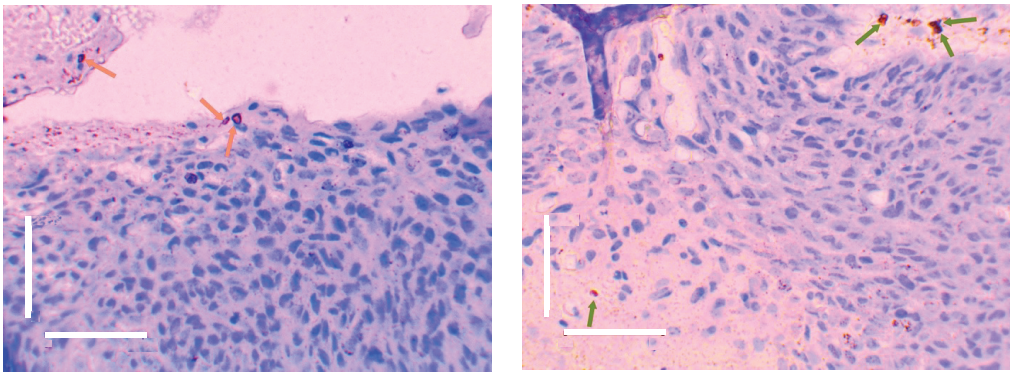

Figure 1. Immunohistochemical staining for p53 (left) and pRB (right), showing positive cells stained brown. Orange arrow: p53 positive cells; Green arrow: pRB positive cells. White bar: 50 µm.

Discussion

In this study, the rate of HPV18 positivity among cervical cancer samples was 24%. The rate of HPV18 positivity in Indonesia range from 24% to 40%.(7) This is in accordance to the rate reported from other South East Asian countries. (25) However, the rate was lower in European countries such as Portugal (4.8%), UK (19.5%) or in Africa (18%). (26-28) These findings indicate that HPV18 positivity in cervical cancer was higher in South-East Asian region as compared to Europe and Africa. Among asymptomatic Indonesian women, a population-based study reported the prevalence of HPV18 infection was 16.1%.(6)

HPV18 genome is relatively conserved and the occurrence of variants is considered a rare event.(29) Among Indonesian women with Balinese ethnicity, as much as 36.4% and 59.1% rate of HPV18 E6 and E7 mutation were identified in this study, respectively. A study involving Indonesian, Surinamese, and Dutch women found the rate of HPV 18 variants was 18.2%.(30) In a study involving 10 HPV18 positive cervical cancer samples from Southwest Chinese women, the rate of HPV18 E6 and E7 mutation was 30% and 10%, respectively.(23) E6 C287G was the most frequent mutation of HPV18 found among Chinese women.(31,32) A study in Mexico reported the rate of HPV18 variants based on LCR gene sequences in cervical cancer samples was 15.5%.(33) In another study conducted in Portugal, based on E6 and LCR gene sequences, the rate of HPV18 variants was 37.2%.(34) None of the nucleotide changes found in studies involving Chinese women was similar with nucleotide changes observes in current study. (23,31,32) A study involving 12 HPV18 positive cervical cancer samples from Korean women also identified nucleotides changes that differed than our results (E6: C287G, T485C, C549A and C554T; E7:C751T).(11) A study involving 25 Taiwanese women with cervical cancer identified C183G was the most common HPV18 E6 mutation.(35) Thus, mutations found in our study may be unique to Indonesian population. To our knowledge, the current study is the first study to identify HPV18 mutations based on E6 and E7 gene sequence among Indonesian women. The mutation patterns were also different as compared to mutation pattern found in other studies.

HPV18 variants were initially categorized into European (E), Asian-Amerindian (AA), or African (AFR) lineages based on the E6-E7, L1, and/or LCR sequences. (13) In this study, we found that 54.5% (12/22) subjects

Table 4. Expression of p53 and pRb among subjects with mutant and wildtype E6/E7.

IRS Score	All Subjects	Mutant E6	Wildtype E6	*p*-value
p53	1.4±0.8	1.9±0.7	1.1±0.8	0.044*[a]
pRb	1.1 (0.7-1.6)	1.4 (1.1-1.9)	0.9 (0.5-1.1)	0.026*[b]
	All Subjects	**Mutant E7**	**Wildtype E7**	***p*-value**
p53	1.4±0.8	1.6±0.7	1.1±0.9	0.256[a]
pRb	1.1 (0.7-1.6)	1.4 (0.9-1.7)	1.0 (0.5-1.1)	0.030*[b]

[a]t-test was used to obtain p-value, [b]Mann-Whitney test was used to obtain *p*-value. *$p<0.05$ is considered significant.

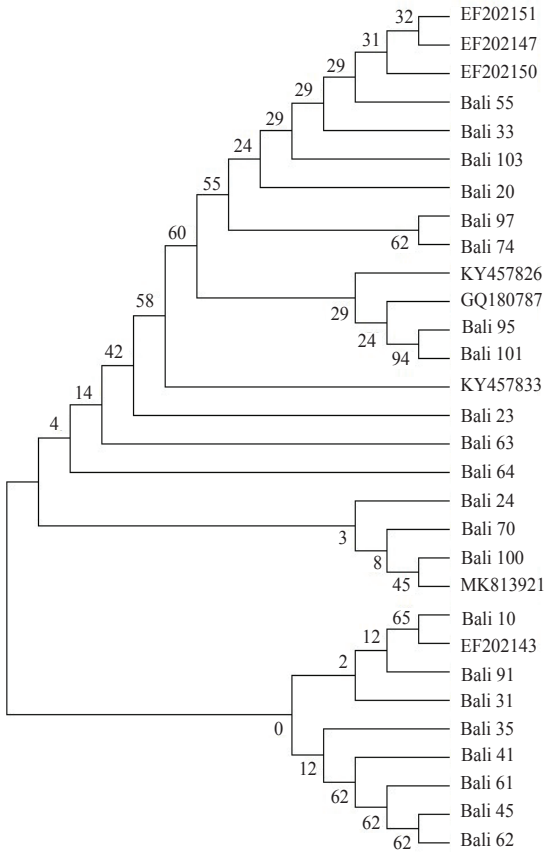

Figure 2. Phylogenetic tree of the HPV18 variants.

had genetic sequences closely related to Asian lineages, particularly A1 (3 subjects), A4 (4 subjects), and A5 sublineage (2 subjects). Interestingly, 3 subjects had genetic sequences closely related to MK813921, a newly identified sequences in one Korean study.(11) However, 45.5% (10/22) subjects had distinct genetic sequences that did not related to the reference sequence used in this study. One study reported that HPV18 variants among Indonesian sample were classified into Asian Amerindian and European phylogenetic branches.(30) The distribution of HPV18 variants have been found to be varied geographically, with a predominance of the A lineage in most regions except sub-Saharan Africa.(13) However, studies investigating the association between HPV18 variants and the progression of cervical lesions reported conflicting results. The non-European HPV variants are associated with HPV persistence and progression of intraepithelial abnormalities.(36) Another study report that HPV18 variants did not associate with the risk of cervical cancer.(13)

In this study, subject with mutant E6 had higher level of p53 expression as compared to wildtype E6. One study in Mexico also demonstrated that E6 variants distinctively affects p53 levels.(37) pRb expression was also significantly higher among patients with mutant E6 and E7 as compared to their wildtype counterparts. No study has ever demonstrated the association between mutation of E6/E7 and pRb expression in cervical cancer. We hypothesize that E6/E7 variants will differ in their capacity to degrade p53/pRb, as compared to the wild-type E6/E7. Furthermore, the results of this study indicate that mutation of E6 and E7 gene could affect the expression of p53 and pRb. It was observed that subjects with E6 mutation had higher p53 and pRb expression while subjects with E7 mutation had higher pRb expression. E6 directly affects the expression of p53 while E7 affects the expression of pRB.(38) It's logical to think that altered expression of E6 and E7 due to mutation will also affect the expression pattern of p53 and pRb.

However, studies investigating mutation or variants of HPV18 E6 and E7 and their association with the biologic behaviour of cervical cancer cells are still lacking. We have been aware that HPV variants can be classified into 3 lineages (A, B and C), and sublineages (A1-8, B1-3 and C) which differ in their rate of nucleotide variations. The nucleotide sequences of HPV intratype variant lineages and sublineages vary by 1–10% and 0.5–1%, respectively. HPV18 variants from A lineage has been reported to exhibit more oncogenic potential than the B lineage. Immortalized primary human keratinocytes (PHK) with E6/E7 from A1 lineage (PHK18A1) showed significantly faster immortalization, generated more colonies in both monolayer and 3D cultures, exhibited enhanced invasion capabilities, and demonstrated increased resistance to apoptosis triggered by actinomycin-D, as compared to cells infected with E6/E7 from B1 lineage (PHK18B1).(39)

Number of subjects recruited in this study is relatively small and only include Indonesian women of Balinese ethnicity. Future study with larger size of participants and multi-centre approach involving various ethnic group to enhance the power of the study and to fully understand the nature of HPV18 E6/E7 mutation in Indonesia is suggested. In this study, semi-quantitative methods of detection for p53 and pRb activity was used, which limits the accuracy. For this issue, employing the use of quantitative methods to detect protein or mRNA expression level for p53 and pRb, such as enzyme-linked immunosorbent assay (ELISA) or reverse transcription-polymerase chain reaction (RT-PCR) should be further studied. Since this study is a pilot study using cross-sectional design, future study with prospective design to evaluate the temporal association between HPV18 E6/E7 mutation with the clinical behaviour of cervical cancer, such as progression, metastasis, recurrence, or response to treatment are also suggested.

Conclusion

In conclusion, we demonstrate that mutation of HPV18 E6 and E7 genes are common among Indonesian women, with higher prevalence of E7 mutation as compared to E6 mutation. Mutation of E6 and E7 also affect the expression of tumour suppressor protein p53 and pRb.

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Authors Contribution

INBM and PKAP were involved in the conception and design of the study. PKAP and IBNPD were involved in the acquisition of data. INBM, PKAP and IBNPD analyzed and interpretated the data. PKAP drafted the manuscript, while INBM and PKAP revised the manuscript critically for important intellectual content. All authors have given approval of the final version of manuscript to be published.

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