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Molecular Variation of E5 Gene Human papillomavirus (HPV) From Cervical Cancer.

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ABSTRACT

Human papillomavirus (HPV) is cervical epithelial infectious agent. Epidemiology proved that one of the major cause of cervical cancer was HPV type 18. Whereas the proteins that give a high risk to cervical cancer case were E5, E6, and E7. The studies about E5 is limited. Several studies stated the expression of E5 protein can induced cervical cancer. The purpose of this studies were to find molecular variation of Human papillomavirus sequence and to determine the genetic relationship of E5 gene HPV type 18 isolated from cervical cancer patient. Sample collected from various sources in West Sumatera and Riau, and it was Andalas University HPV center research collection approved by Andalas University ethical team. This study working with biological sample, so didn't need any ethical repetition. 15 sample of type 18 HPV were isolated and amplified by Polymerase Chain Reaction (PCR). The amplcons then following sequencing process. The sequences were analyzed using Bioedit program, NCBI BLAST, CLUSTAL X 2.1 version to find molecular variation and genetic relationship done by MEGA program 6.06 version using sequence reference NC_001357 type 18 to compare it. E5 gene of type 18 HPV detected on 9 of 15 sample (60%). Molecular variation is not found in this study. The genetic relationship shows that E5gene type 18 HPV close to 8 standard reference from NCBI, 100% close to Asian and Asian-America isolate.

Keywords: Cervical cancer, E5type 18 HPV, molecular variation, genetic relationship.

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INTRODUCTION

One of health problem that take the world attention is cervical cancer caused by Human Papilloma Virus (HPV). Based on WHO 2012, cervical cancer placed the forth position as the most common cancer attack woman. About 85% cases happened in undeveloped area, where it is caused almost 12% of all cancer [3]. International Agency for Research on Cancer (IARC) estimated at 2050 population of woman at age above 15 having cervical cancer around the world reach 3 billion people. In Indonesia new cases of cervical cancer per year stated at 2012 is 20.928 cases and 9.498 of them ended by death [3].

Human papillomavirus (HPV) is an infectious agent on cervic epithelial. Four types of HPV that often found in cervical cancer cells are type 16, 18, 31, and 45 [21]. Viral genom consist of double helix DNA with the amount of bases about 7.200-8.000bp. Functional genom divided on 3 areas. First area is noncoding consist of 400-1.000 bp that also called long control region (LCR). Second area consist of early protein open reading frame (OFR), E1, E2, E4, E5 E6, and E7 they play role on viral replication. The third area is the late protein [20].

Epidemiology study shows that the main agent in cervical cancer world wide is the pathogenesis of high risk Human papillomavirus such as type 16 and 18. Those two type of HPV play an important role on development abnormal cells and causing cervix intraepithelial neoplasia and invasive cervical cancer [8]. Whereas the high risk carcinogens protein that recently on study were E5, E6, and E7 genes [18]. Specially for E5 gene contribute in two carcinogenesis phase, that are promotion and cell development. E5 gene encode E5 protein which have a low transformation potency and also help cellular transformation by interaction with cell membrane growth factor receptor (EGFR). However, just 40% from all cases revealed E5 correlated to severity of cervical cancer [12].

E5 protein mostly found in the early stage of HPV infection and missing in cervical carcinoma stage [9]. Some studes also claim that E5 expression can induced cervical cancer alone and also synergy with E6 and E7 to induced a more advance cervical cancer. Beside that, E5 together with E7 could increase diversity and tumor size. Still contribution of E5 lose when oncogene disclose [12].

The study about E5 gene type 18 HPV is limited. Every mutation or change on nucleotide sequence can induced biological functional alteration from protein encode by this gene. The different of molecular variation need to be learned because the genetic variation dissimilar from one to the other region [2]. Modification in genetic line of type 18 HPV can interrupt viral oncogene potency and not known yet if amino acid modification from viral capsid affected to vaccine efficacy [6]. Study about sequence variation especially E5 gene type 18 HPV is needed to give a useful information. E5 gene type 18 HPV might become an important therapy target to prevent cervical pre-cancer lesion transformation and vaccine development. Screening and vaccination were effective methods to control cervical cancer cases. Molecular variation on HPV cause the effectiveness of HPV vaccine outstanding world wide questionable [13, 22].

Early detection still become a necessary option in cervical cancer research. Because of that, intensive screening to find sensitive biomarker (gene or protein) that could detected early or late stage of cancer progression is the main target from new technology. Genomic and protemics progress in cervical cancer related by HPV infection will help in the most accurate identification for early detection biomarker [4].

Since HPV genom research completely available and filogenic analysis also applied on HPV study, genetic line variation could determined by the different between genom of HPV about 1.0% and the same varian for sublineages divergence about 0.5-1.0% between genetic line. HPV genetic line distribution related with geographic or ethnic group [6].

The purpose of this studies were to find molecular variation of Human papillomavirus sequence and to determine the genetic relationship of E5 gene HPV type 18 isolated from cervical cancer patient.

MATERIALS AND METHOD

This research was conducted in Faculty of Medicine Andalas University, molecular biology laboratory, microbiology section, HPV Cancer research Andalas University Padang from October 2015- February 2016. Sample taken from various source in West Sumatera and Riau and sample was Andalas University collection

approved by Andalas University ethical team. This study working with biological sample, so didn't need any ethical repetition. 15 sample of type 18 HPV were isolated and amplified by Polymerase Chain Reaction (PCR).

E5 GENE TYPE 18 HPV DETECTION

Positif sample of type 18 HPV detected with specific primer for E5 gene type 18 HPV (R4264). Each PCR tube added by 3 µL DNA template, primer HPV 18 Gen E5 Foward (5'TGC GGC ACG GTG GGA TAC CAT ACT -3'), HPV 18 Gen E5 Reverse (5'CAA ATA TTG GTG GGA TAC ATG AC-3') 1 µL each , Platinium® PCR Super Mix 45 µL. The mixture then spindown and amplified using Thermocycler. Thermocycler setting for initial denaturation 94 °C (5 minutes), denaturation 95 °C (30 seconds), annealing 57 °C (30 seconds), extensi 72 °C (60 second) dan final extensi 72 °C (10 minutes) for 35 cycle. The amplicon analysis in electrophoresis process on 1.5% agarose gel staining with SYBR safe for 30 minutes for 100 volt. Amplicon sent to Singapore for sequencing process.

Data Analysis

The sequences were analyzed using BioEdit program, NCBI BLAST, CLUSTAL X 2.1 version to find molecular variation and genetic relationship done by MEGA program 6.06 version using sequence reference NC_001357 type 18 to compare it.

RESULT AND DISCUSSION

About 9 of 15 (60%) posistif sampel being used shown in figure 2.

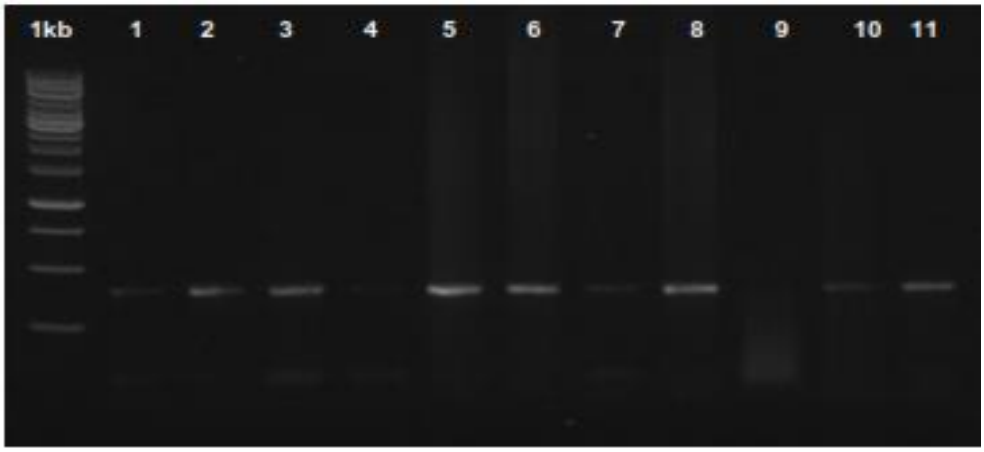

Figure 1. Amplification Result of PCR Sequencing Gen E5 HPV type 18

Figure Information: : 1kb DNA Ladder (bp): 250, 500, 750, 1000, 1500, 2000, 2500, 3000, 3500, 4000, 5000, 6000, 8000, 10000, : Leder, 1 : HPV sample MA-2, 2 : HPV sample MA-3, 3 : HPV sample MA-4, 4 : HPV sample MA-5, 5 : HPV sample MA-7, 6 : HPV sample MA-8, 7 : HPV sample MA-9, 8 : HPV sample MA-10, 9 : HPV sample MA-12, 10 : HPV sample MA-13, 11 : Sampel HPV sample MA-15. E5 gene type 18 HPV detected on 9 of 15 sample.

Based on sequencing result above, just 9 sample adequate to be sequence. Meanwhile the other 6 sample undetected with E5 gene type 18 HPV primer. Consentration of all sample on range 1.8-2.0. Sample purity < 1.8, it means there still protein or reagen contamination in DNA isolation process [19]. The purity of DNA isolate also affected the result of PCR process. When DNA contaminated, the result could be unspesific [15]. DNA concentration about 10-100 ng for each µl template solution alredy include in a good category fro PCR, but the most important is DNA should be free from uimpurities such as proteins or other waste in every process [16]. The other possibility comes from the gene DNA it self have been damaged or their molecular variation that make the working primer not optimum [1, 11].

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Figure 2. Sequencing result Shown in electrophoregram and nucleotides base sequence

Sequencing result shown as DNA electrophoregram and forward-reverese base sequence in Ab1 (figure 3). Because of some overlapping DNA part in result by Ab1, so the analysis continue using contiq and the result shows the bases sequence consensus. The E5 type 18 nucleotide sequence of 9 sample compared with sequence database from Gene Bank using NCBI BLAST on nucleotide level and accessible in http://blast.ncbi.nlm.nih.gov/blast.cgi website. reference sequence HPV 18 NC_001357, Accession number : EF202147.1, EF202150.1, EF202151.1, GQ180786.1, KC470213.1, KC470209.1, X05015.1, AY262282.1, EF202145.1, GQ180784.1, GQ180792.1, KC470211.1, GQ180787.1, KC470230.1, KC470228.1, KC470226.1, KC470224.1, KC470223.1, EF202155.1, KC470215.1) [8]. . the result on this study shown the homolog between sample used and reference sequence HPV 18 NC_001357 100% classified as variant, whereast 16 “comparison” have 98-100% homolog and classified as variant and 4 “comparison” sequence homolog 97% classified as subtype.

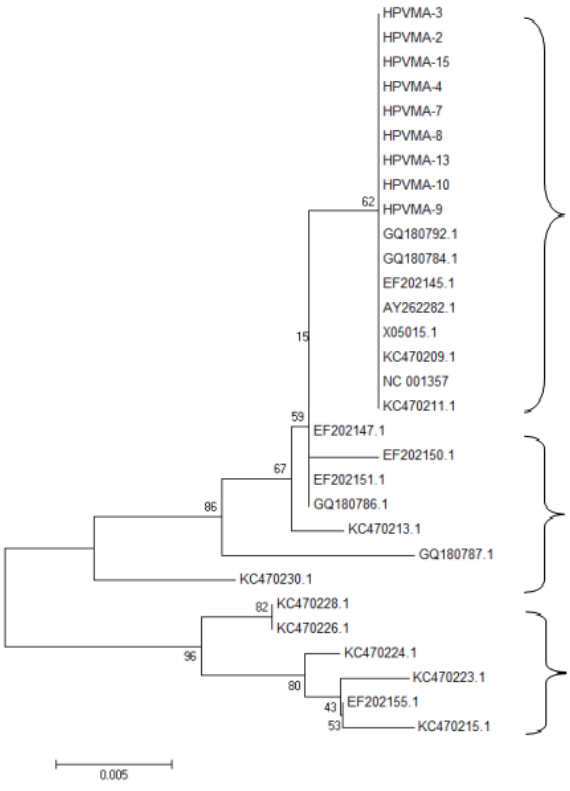

Figure 3. Phylogeny tree

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Table I. Data Sequence of base pairs and Single Polimorfisme Nucleotide (SNPs) Gen E5 HPV Type 18.

NO	Sample Code	The Base Sequence															Number of Bases	Type SNPs		
		56	59	64	80	92	176	202	236	267	268	271	287	316	317	366				
1	**NC_001357**	**T**	**T**	**T**	**T**	**A**	**G**	**C**	**T**	**T**	**A**	**G**	**T**	**T**	**T**	**A**	382	**Insertion**	**Subtitution**	**Deletions**
2	**EF202147.1**	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	382	-	-	-
3	**EF202150.1**	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	381	-	1	-
4	**EF202151.1**	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	382	-	-	-
5	**GQ180786.1**	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	382	-	-	-
6	**KC470213.1**	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	381	-	1	-
7	**KC470209.1**	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	382	-	-	-
8	**X05015.1**	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	382	-	-	-
9	**AY262282.1**	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	382	-	-	-
10	**EF202145.1**	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	382	-	-	-
11	**GQ180784.1**	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	382	-	-	-
12	**GQ180792.1**	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	382	-	-	-
13	**HPVMA-9**	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	380	-	-	-
14	**HPVMA-10**	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	380	-	-	-
15	**HPVMA-13**	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	380	-	-	-
16	**HPVMA-8**	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	380	-	-	-
17	**HPVMA-7**	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	380	-	-	-
18	**HPVMA-4**	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	380	-	-	-
19	**HPVMA-15**	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	380	-	-	-
20	**HPVMA-3**	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	380	-	-	-
21	**HPVMA-2**	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	380	-	-	-
22	**KC470211.1**	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	382	-	-	-
23	**GQ180787.1**	.	.	.	.	T	.	T	.	C	.	.	.	G	.	.	377	-	4	-
24	**KC470230.1**	.	C	.	G	T	.	.	.	.	.	.	.	.	.	T	378	-	4	-
25	**KC470228.1**	.	.	.	.	T	A	.	C	.	C	A	.	.	.	T	375	-	6	-
26	**KC470226.1**	.	.	.	.	T	A	.	C	.	C	A	.	.	.	T	375	-	6	-
27	**KC470224.1**	.	.	G	.	T	A	.	C	C	.	A	.	.	.	T	375	-	7	-
28	**KC470223.1**	.	.	G	.	T	A	.	C	C	.	A	G	.	.	T	374	-	8	-
29	**EF202155.1**	.	.	.	.	T	A	.	C	C	.	A	G	.	.	T	375	-	7	-
30	**KC470215.1**	G	.	.	.	T	A	.	C	C	.	A	G	.	.	T	374	-	8	-

Based on sequencing analysis, on E5 gene type 18 HPV didn't show any Single Nucleotide Polimorphism (SNPs) in DNA sequence. All nucleotide base sequence suitable toreference sequence of HPV 18 NC_001357 so that not even single amino acid change on sample (table 1). The result tell us that E5 gene have a high stability level in protein synthesis process. The whole datas comparison (9 sample of E5 gene type 18 HPV), 20 homolog sequence tpe 18 HPV NC_001357) total conserved of all datas with reference sequencing type 18 HPV NC_001357 contained 382 bases with 16 variable (V) consist of 9 Piarsimoni base (Pi) and 7 Single tone base (S). Kinship analysis done by building a phylogenetic tree. Phylogeny tree build using the Neighbor-Joining Tree with test phylogeny Bloostop Method 1000X and mode / method using the Kimura 2 parameter. Neighbor-joining tree method is suitable when the average evolution of the lineage separation is under consideration of different (fig. 3).

Some epidemiology studies about genom variant of type 18 HPV point to 3 main genetik line, European (E), African (Af), and Asian-American [5]. Based on the yield of phylogeny tree analysis and genetic gap compared with reference sequencing type 18 HPV NC_001357 obtained 3 lineage. The first lineage (A) consist of 9 E5 gene type 18 HPV data that geneticly close to Asian isolate (GQ180792.1, GQ180784.1, EF202145.1) and Asia-Amerika (AY262282.1, X05015.1, KC470209.1, NC_001357 dan KC470211.1) with genetic distance 100%. The second lineage (B) consist of Asian-American isolate sample (EF202147.1 and GQ180786.1) and European (EF202150.1, EF202151.1, KC470213.1, GQ180787.1) with genetic distance compare with reference sequencing value 0,0034 (0,34%). Whilst KC470230.1 grouped as new variation and close to GQ180787.1 reference with genetic distance 0,018 (1.8%). The third lineage (C) consist of African sample isolate (KC470228.1, KC470226.1, KC470224.1, KC470223.1, EF202155.1, KC470215.1) with genetic distance about 0.03 (3%).

Heinzel's research denoted a minor correlation between HPV variant with geography and etnical, because of viral mobility a long evolution still unknown. The other study by Danielewski in Australia also shown HPV isolate with unlimited distribution in various geography. Sichero and Villa have a different result and divided HPV isolate in to nomenclature based on geography (i.e isolate from China and Japan only exist in Asian). This research is not suitable with the previous study that claimed geographical distribution have a phylogeny proximity to HPV variant. Based on phylogeny tree analysis result in this research can be concluded that E5 gene type 18 HPV variation can be ensure have a relatives proximity by geography.

CONCLUSIONS

Molecular variation is not found in this research, so that there were no change in amino acid. Based on phylogeny tree result analysis on E5 gene type 18 HPV the relatives proximity can be confirmed by geographical location to Asian and Asian-American variant with genetic distance 100%.

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