The change of cell biometric and its nucleus on cervical-squamous-epithelial-cell with GA genotype of Fas-promoter-670 gene, high risk human papilloma virus and *Candida species* infection: A case report

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Abstract.

Cervical samples of patients with human papilloma virus (HPV) infections show positive results in HPV DNA testing. HPV infection can alter cervical squamous epithelial cells (CSECs) to be abnormal. Epigenetically, CSECs that change to be abnormal are affected by Fas-promoter-670 gene polymorphism. High risk HPV (hr-HPV) patients can be infected by other microbes, for example *Candida species (Candida sp.).* The purpose of the present study was to show CSEC biometrics based on epigenetics of the Fas-promoter-670 gene polymorphism in Indonesian women with hr-HPV and *Candida sp.* infection. Biometric quantification was performed based on the following analysis of CSECs. Indonesian hr-HPV women at the age of 28 years, with *Candida sp.* infection, underwent a Pap smear examination on April 21th, 2016 using the ThinPrep method and a blood test was also performed using the cubital vein (*vena cubiti*). Blood and ThinPrep samples were examined for the Fas-promoter-670 gene polymorphism. Epigenetically, the subjects had the GA genotype of the Fas-promoter-670 gene in the blood and ThinPrep samples. Patients with *Candida sp.* infection in the early stages were characterized by the appearance of polymorphonuclear leukocytes (PMN), whereas those in advanced stages presented without PMN on the hyphae. CSEC biometric measurements were performed quantitatively using mononuclear CSECs (mn-CSECs) and binucleated CSECs (bn-CSECs). Biometric measurements of the CSECs were performed quantitatively and assessed the length, width, area and perimeter of the cell and its nuclei. In the case investigated, cell length, cell width, nucleus area, nucleus perimeter and nucleus length index were significantly different between the mn-CSECs, the 1st nucleus of bn-CSECs and 2nd nucleus of bn-CSECs ($P<0.05$).

Key words

Epigeneticy, Fas promoter-670 gene, *Candida sp*., cervical-squamous-epithelial-cells, cell biometrics

Introduction

The ThinPrep Pap test can be used to identify precancerous lesions in patients so as to facilitate early detection for the management of further development of cervical cancer. ThinPrep Pap tests are currently the gold standard in liquid-based cytology testing, and their use is greatly improved compared with conventional Pap technology (1). It has been demonstrated that the ThinPrep Pap test has a high sensitivity and high specificity in screening for cervical cancer (2).

Precancerous cervical lesions are strongly associated with high risk human papilloma virus (hr-HPV) infection. It has been demonstrated that persistent infection with high risk types of HPV is the most significant factor for cervical cancer and precancerous lesion manifestation (3). A previous study has reported that incidence and mortality of cervical cancer and their relationship with the human development index in 185 countries in the world. Futhermore, screening methods include cytology (Papanicolaou test) and HPV testing, alone or in combination (4). However, HPV infection is not the only factor for the development of cervical cancer (5); genetic and environmental factors are also associated with the development of cervical cancer.

APO-1/Fas (CD95/TNFRSF6) receptor is a type I transmembrane glycoprotein that belongs to the tumor necrosis factor (TNF) or neuronal growth factor receptor superfamily (6). The human Fas-promoter-670 gene has been mapped, and is located at chromosome 10q24.1 (7) or 10.23 (8). This gene consists of 9 exons and 8 introns. Exon 1 comprises the 5'-untranslated region (UTR) and DNA sequence for the first 10 amino acids. Exons 2-5 encode the extracellular region, while exon 6 encodes the transmembrane region. Exons 7 and 8 encode the membrane proximal cytoplasmic 36 amino acids of the receptor. Exon 9 encodes the remaining 109 amino acids, including the 'death domain' and the 3'-UTR (6).

The Fas-promoter-670 gene plays a central role in the physiological regulation of programmed cell death (also termed apoptosis). Failure of this gene's regulation produces a death signal, which is reported in several types of cancer (9-10). It has been reported that a single nucleotide polymorphism at site 670 in the enhancer region of the Fas gene is associated with tumorigenesis (11). A study has demonstrated an association between Fas-promoter-670 gene polymorphism and cervical cancer. A statistically significant correlation was identified between the susceptibility for cervical cancer and the GA and combined GA+GG genotypes in a study that focused on an Indian population (12). An association between Fas-promoter-670 gene polymorphism and HPV infection has also been shown in cervical cancer in Japan (13). Another study in Asia has also demonstrated a positive association between Fas-promoter-670 gene polymorphism and cervical cancer (14). Additionally, a previous study has reported a case of a high risk Indonesian mother, whose Fas-promoter-670 gene in normal CSECs was mutated from AA to GA, whereas in the lymphocyte cells the genotype was AA (15).

A previous study has reported that the *Candida species (Candida sp.)* is of great concern due to its infection of human hosts, particularly patients with cancer (16). Invasive candidiasis is a serious infection that predominantly affects critically ill and immunocompromised patients. In general, *Candida albicans* is a species that is involved in this infection (17). Furthermore, it has been stated that cancer patients have a greater risk for

serious infections, such as *Candida sp.* Patients with candidemia have a serious problem due to high mortality, particularly if is caused by resistant *Candida spp*. (18).

Binucleation of CSECs in patients with *Candida sp.* infection has been reported. It has been stated that binucleation is a reactive cellular change in Pap smears due to *Candida* infection (19). A study result previously indicated that there is an effect of HPV infection on the number of nuclei in CSECs. Following HPV infection, the appearance of CSECs on the ThinPrep Pap slide demonstrate a different number of nuclei, exhibiting ≥1 nuclei. In the cells with >1 nucleus, if the nuclei pressed against each they were defined as positive compression, while the nuclei contacting but not pressing against each other were defined as negative compression (20). It has been reported that positive compression of binucleated cells may be present as a result of hr-HPV infection, which is caused due to inflammation in intraepithelial lesions or malignancy cases infected with *Candida sp.* (19). The present report evaluates the performance of CSEC biometrics and its nuclei with a GA genotype of the Fas-promoter-670 gene, and who has hr-HPV and *Candida sp.* infection.

Case report

A 28-year-old Indonesian women living with hr-HPV and *Candida sp.* infection on April 21th, 2016 underwent a Pap smear examination and a blood test. Before participating in this study, the participant provided informed consent. Ethical approval for the study was obtained from the *"Komisi Etik Riset Fakultas Kedokteran, Universitas Trisakti, Indonesia"* (approval no. 60/KER/FK/05/2013). Informed written consent was obtained from participants before data collection. The subject concerned was included in the community of Indonesian women living with hr-HPV based on evidence of laboratory examinations. In this case, the subject first has coitus at the age of 16 years, had free sex and had also never received HPV vaccination.

Blood samples (~10 µl) were taken from a cubital vein (*vena cubiti*) are inserted into an ethylene diamine tetra acetate (EDTA) tube. The Pap smear examination was performed according to the ThinPrep method to collect cytological material. Whole blood in the EDTA tube and cytological material in the ThinPrep solution were analyzed for Fas-promoter-670 gene polymorphisms by restriction fragment length polymorphism (RFLP) with *Bacillus stearothermophilus* N1 (BstN1) enzyme. DNA extraction from the blood and ThinPrep samples was performed using chloroform. In addition, the forward primer, 5'CTACCTAAGAGCTATCTACCGTTC3' and reverse primer, 5'GGCTGTCCATGTTGTGGCTGC3' were used. Epigenetically, the subject in this case had

the GA genotype of the Fas-promoter-670 gene both in the blood and ThinPrep samples (Fig. 1).

In addition to analyzing the Fas-promoter-670 gene polymorphism, cytological material in the ThinPrep solution also analyzed cytologically. ThinPrep 2000 was used to creates slides automatically based on liquid-based cervical cytology. Optilab Advance Plus and Image Raster 3 programs (PT MICONOS, Daerah Istimewa Yogyakarta, Indonesia; available at https://miconos.ac.id/new/support/download) were used by three observers. The presence of *Candida sp.* was based on the observation of hyphae under the microscope. *Candida sp.* infection can be divided into two stages, termed the early and advanced stages. *Candida sp.* infection in the early stage is characterized by the appearance of polymorphonuclear leukocytes (PMNs), whereas at the advanced stage no PMNs are observed on the hyphae of *Candida sp*. The spores of *Candida sp.* were observed qualitatively (Fig. 2). The biometric measurements of CSECs were performed quantitatively, focusing on the length, width, area and perimeter of the cell and its nuclei. Optilab plus and Image Raster 3 programs were used to analyze the biometric measurements by three observers. The measurements of the mononucleated-CSECs (mn-CSECs) (Fig. 3) and binucleated-CSECs (bn-CSECs) (Fig. 4) were as follows: Cell length (CL), cell width (CW), cell area (CA), cell perimeter (CP), nucleus length (NL), nucleus width (NW), nucleus area (NA), nucleus perimeter (NP), nucleus length index (NLI), nucleus width index (NWI), nucleus area index (NAI) and nucleus perimeter index (NPI). Based on the results of biometric measurements, CSECs in these cases were divided into three groups: i) mn-CSEC; 1^{st} nucleus of bn-CSEC (1^{st}-bn-CSEC); and iii) 2^{nd} nucleus of bn-CSEC (2^{nd}-bn-CSEC), which are presented in Table I. The CL, CW, NA, NP and NLI were not significantly different between the mn-CSECs, 1^{st}-bn-CSECs and 2^{nd}-bn-CSECs ($P>0.05$); however, CA, CP, NL, NW, NAI, NPI and NWI were significantly different ($P<0.05$). Multiple comparisons between the mn-CSECs, 1^{st}-bn-CSECs and 2^{nd}-bn-CSECs are presented in Table II. The CA and CP in mn-CSECs, 1^{st}-bn-CSECs and 2^{nd}-bn-CSECs were significantly different ($P<0.05$); however, 1^{st}-bn-CSECs was not significantly different compared with 2^{nd}-bn-CSECs ($P>0.05$). The NL and NW of mn-CSECs were significantly different compared with those of the 1^{st}-bn-CSECs, as well as for 1^{st}-bn-CSECs compared with 2^{nd}-bn-CSECs ($P<0.05$), while mn-CSECs were not significantly different compared with 2^{nd}-bn-CSECs ($P>0.05$). The NAI and NPI were significantly different between the mn-CSECs and 1^{st}-bn-CSECs or 2^{nd}-bn-CSECs ($P<0.05$); however, they were not significantly different for the 1^{st}-bn-CSECs compared with the 2^{nd}n-bn-CSECs ($P>0.05$). The NWI of mn-CSECs was significantly different compared with that of the 1^{st}-bn-CSECs, and the NWI of 1^{st}-bn-CSECs

was significantly different compared with that of the 2nd-bn-CSECs (P<0.05), while mn-CSECs did not significantly differ compared with 2nd-bn-CSECs in terms of NWI (P>0.05).

Discussion

DNA extraction from CSECs and leukocytes is useful for the genotyping of the Fas-promoter-670 gene, as well as for electrophoresis optimization. Genotyping of the Fas-promoter-670 gene in CSECs and leukocytes reveals that both have a GA genotype. To evaluate the exposure of HPV infection on the Fas-promoter-670 gene in CSECs, genotyping is necessary for both CSECs and leukocytes. The genotyping result of the Fas-promoter-670 gene in both CSECs and leucocytes of the present case demonstrated the same result, which suggests that the Fas-promoter-670 gene in CSECs does not mutate due to HPV infection. This is in contrast to a previous case, in which the Fas-promoter-670 gene in the CSECs had a GA genotype, whereas had an AA genotype in the leukocytes, therefore the Fas-promoter-670 gene in CSECs had mutated (15). This demonstrates that the Fas-promoter-670 gene in CSECs mutates due to the effect of HPV infection (local infection). We know that HPV infection is a local infection that is in the cervix.

The results of previous study indicated that persistent HPV infection is necessary for the development of cervical cancer. Furthermore, stated that genetic and epigenetic alterations in host cell genes are crucial for the progression of cervical precancerous lesions to invasive cancer (21). A recent study has reported that samples collected from patients with cervical cancer, tested positive for HPV DNA (22). Additionally, a study in Western Kenya also demonstrated associations between vaginal infections and potential high risk and hr-HPV genotypes. In detail, of the free sex workers analyzed, 33.3% had HIV and 57.7% harbored a potential hr-HPV and hr-HPV genotype (23). Based on numerous studies worldwide, the epidemiology of HPV infection and oncogenic properties of HPV type are due to different HPV genotypes. However, there are still many countries where population-based prevalence has not yet been identified. Furthermore, cervical cancer screening strategies are different between countries (24).

The results presented in the present case report are consistent with the results of recent research in Indonesia, which shows that HPV vaccination is not yet a priority for adolescents. This is due to a lack of education and the cost of HPV vaccination. Therefore a program is required to provide accurate information regarding HPV vaccination to the public, particularly teenagers (25).

This case is consistent with studies of the Japanese population, which demonstrated that a GG genotype is associated with an increased risk for the development of cervical cancer, with an odd ratio (OR) of 2.56 compared than the AA genotype. Furthermore, the G allele in the GA or GG genotype also increased the risk of cervical cancer, with an OR of 1.60 (26). Other studies investigating females in Northern India demonstrated that a GA genotype and a combination of GA and GG genotype significantly increased the risk of cervical cancer compared with an AA genotype (12). In addition, a study of Brazilian females <48 years old also reported that a GA genotype increases the risk of cervical cancer 5-fold compared with an AA genotype (27).

Research of a Greek population demonstrated that there is no significant association between EVER1/2 polymorphisms (rs2290907 and rs16970849) and cervical cancer. However, the study did provide additional data that also suggested no association between the FAS polymorphism (rs1800682) and the susceptibility to persistent precancerous lesions and cervical cancer. Current literature for EVER1/2 polymorphisms and cervical cancer is very limited worldwide therefore, prospective studies are needed to further clarify this point (28).

Candida sp., the most common *Candida albicans,* plays an important role in secondary infections of the vaginal and vulvar epithelium. *Candida sp.* is part of the normal flora in women and is often asymptomatic (29). In a study based in Brazil, which investigated 633 pregnant women, 158 specimens (24.1%) exhibited pathogenic infections, while 22.9% were infected with *Candida spp.* (30). A subsequent study in Brazil demonstrated that of the 263 patients analyzed, *Candida spp.* was isolated in 27%, and >60% of the isolates were identified as *Candida albicans.* In addition, *Candida non-albicans* was isolated at a rate of 8.6% in symptomatic patients and 14.3% in asymptomatic patients (31). Another study in Ethiopia reported a high prevalence rate of vulvovaginal candidiasis and *Candida non-albicans*. Therefore it is important to conduct continuous epidemiological surveys to measure changes in species distribution from *C. albicans* to *Candida non-albicans* (32). A study in West Kenya demonstrated that bacterial vaginosis was the most common infection (48.3%), followed by *Trichomonas vaginalis* (31.4%) and *Candida spp.* (19.9%). Significant associations between bacterial vaginosis and HPV 58, and between *Candida spp.* and HPV 16 and HPV 53 suggest the need for sexually transmitted disease management in a cervical cancer prevention program (23).

The appearance of PMN on hyphae of *Candida sp.* in the early stage indicate an infection and allergic reaction. By contrast, in the advanced stage of the infection the hyphae of *Candida sp.* do not present with PMN. It has also been reported that *Candida sp.* infection

produces a peptide toxin called candidalysin. The peptide toxin is produced by hyphae of *Candida albicans* and is characteristic of fungal pathogenesis. In addition, candidalysin is important for *C. albicans* mucosal infections. Candidalysin is known to activate epithelial cells to induce downstream innate immune responses that are associated with protection during vaginal infections. It has been reported that candidalysin plays an importance role in stimulating a strong pro-inflammatory response by neutrophil recruitment. Conversely, if candidalysin is not present, the inflammatory response decreases due to a lack of neutrophil recruitment (33).

Previous studies have shown that *Candida sp.* infects humans, particularly patients with cancer. The present study demonstrated that of the 68 blood samples, 5 (7.35%) were positive for the presence of *Candida spp,* 2 (40%) identified to be positive for *Candida albicans* and 3 (60%) were contained *Candida non-albicans* (16). The results of another study demonstrated that 8.7% of 150 samples were infected with *Candida sp.* (34).

A recent study reported that ThinPrep cervical cytology samples can be used in cervical cancer screening. There have also been reports of Indonesian mothers with hr-HPV and the Fas-promoter-670 gene in CSECs, which is mutated from AA to GA, and had normal CSEC characteristics (15). Based on the appearance of mn-CSEC and bn-CSEC, mn-CSEC characteristics in this case was normal, while bn-CSECs were abnormal. This is consistent with the fact that abnormality of bn-CSECs is due to cytokinesis failure. Futhermore, cytokinesis failure has potential of proliferation (35). Based on the results of cell biometric measurements (Tables I and II), there was a change in the size and shape of CSECs. The CA, CP, NL, NW, NAI, NPI, NWI measurements between mn-CSECs, 1^{st}-bn-CSECs and 2^{nd}-bn-CSECs were significantly different ($P<0.05$); however, CL, CW, NA, NP and NLI were not significantly different ($P>0.05$). The presents results and recent studies suggest that the growth rate of precancerous cells is significantly faster compared with normal cervical cells. However, the proliferation capacity of precancerous cells is similar to cervical cancer cells at the molecular level (36).

In the Pap smear method, the determination of CSEC characteristics can be used as a reference in the diagnosis of cervical cancer. A light microscope and Image Raster 3 are used to analyze CSEC morphology and assess measurements of the cell and its nuclei. Most hospitals in Indonesia have facilities, such as light microscope, the Image Raster 3 program is easy to obtain. This method may be simplest; however, cervical cancer can also be detected using a more sophisticated tool. The present study, which focused epigenetically on the Fas-promoter-670 gene and two contrasting pathogens HPV and *Candida sp.,* may hopefully

improve early screening methods of cervical cancer. Furthermore, the principles of CSEC measurements in this study can be used for the development of cervical cytopathology examinations based on 'biometric artificial intelligence'.

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Availability of data and materials

The datasets generated and analyzed in the present study are included in this published article.

Authors' contributions

Mauritius Lambertus Edy Parwanto, Raditya Wratsangka, Assangga Guyansyah and Reza Aditya Digambiro designed the study, collected the samples, carried out the ThinPrep, genetic and cell imaging analysis, participated in the collecting and interpretation of data, wrote the manuscript, and gave the final approval of the version to be published. David David, Hanslavina Arkeman, Kirana Anggraeni, Haryo Ganeca Widyatama, Hosea Jaya Edy, Yosua Jaya Edy and collected and analyzed the data, performed the literature review and wrote the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The authors declare that they have obtained written informed consent for the publication of the details relating to the patient in this report. All possible steps have been taken to safeguard the identity of the patient. This study has ethical clearance by the 'Komisi Etik Riset Fakultas Kedokteran, Universitas Trisakti, Indonesia'.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Table I. Comparison of cell biometric and its nuclei between mn-CSEC and bn-CSEC in Indonesian women living with highrisk HPV and *Candida sp.* infection

Variable	mn-CSEC mean ± SD (n cell=27)	1st nucleus of bn-CSEC mean ± SD (n cell=6)	2nd nucleus of mn-CSEC mean ± SD (n cell=6)	P value
CL (µm)	5.44±0.31	5.72±0.61	5.73±0.26	0.900
CW (µm)	4.49±0.29	4.47±0.28	4.76±0.13	0.930
CA (μm^2)	20.45±1.23	25.45±2.78	25.61±0.71	0.000
CP (µm)	16.67±1.09	18.64±1.72	19.30±0.96	0.000
NA (μm^2)	0.47±0.05	0.45±0.04	0.45±0.03	0.403
NP (µm)	2.50±0.26	2.44±0.29	2.46±0.10	0.835
NL (µm)	0.83±0.08	0.76±0.09	0.89±0.05	0.034
NW(µm)	0.63±0.06	0.72±0.05	0.62±0.09	0.014
NAI	2.31±0.24	1.79±0.14	1.74±0.11	0.000
NPI	15.05±1.78	13.11±1.28	12.77±1.03	0.003
NLI	15.36±1.83	13.50±2.26	15.59±1.08	0.074
NWI	14.20±1.68	16.17±2.12	12.98±1.92	0.012

Abbreviations: mn-CSEC, mono nucleated cervical-squamous-epithelial-cell; bn-CSEC, binucleated cervical-squamous-epithelial-cell; 1th nucleus of bn-CSEC, first nucleus of binucleated-cervical squamous ephitelial cell; 2nd nucleus of bn-CSEC, second nucleus of binucleated cervical-squamous-epithelial-cell; SD, standard of deviation; n, sample size; p, significant level; CL, cell length (the longest cell diagonal that passes through the nucleus); CW, cell width (cell diagonal perpendicular to the diagonal of CL); CA, cell area; CP, cell perimeter; NA, nucleus area; NP, nucleus perimeter; NL, nucleus length (the longest cell nucleus diagonal); NW, nucleus width (cell nucleus diagonal perpendicular to the diagonal of NL); NAI, nucleus area index (nucleus area:cell area)x100; NPI, nucleus perimeter index (nucleus perimeter:cell perimeter)x100; NLI, nucleus length index (nucleus length:cell length)x100; NWI, nucleus width index (nucleus width:cell width)x100; µm, milli-micron; μm^2, milli-micron square.

Table II. Multiple comparison between mn-CSEC, 1st n-bn-CSEC and 2nd n-bn-CSEC in Indonesian women living with highrisk HPV and *Candida sp.* infection

Variable	Group	p value
CA (μm^2)	mn-CSEC - 1st n-bn-CSEC	0.000
	mn-CSEC - 2nd n-bn-CSEC	0.000
	1st n-bn-CSEC - 2nd n-bn-CSEC	0.848
CP (µm)	mn-CSEC - 1st n-bn-CSEC	0.001
	mn-CSEC - 2nd n-bn-CSEC	0.000
	1st n-bn-CSEC - 2nd n-bn-CSEC	0.336
NL(µm)	mn-CSEC - 1st n-bn-CSEC	0.069
	mn-CSEC - 2nd n-bn-CSEC	0.115
	1st n-bn-CSEC - 2nd n-bn-CSEC	0.010
NW(µm)	mn-CSEC - 1st n-bn-CSEC	0.007
	mn-CSEC - 2nd n-bn-CSEC	0.527
	1st n-bn-CSEC - 2nd n-bn-CSEC	0.009
NAI	mn-CSEC - 1st n-bn-CSEC	0.000
	mn-CSEC - 2nd n-bn-CSEC	0.000
	1st n-bn-CSEC - 2nd n-bn-CSEC	0.714
NPI	mn-CSEC - 1st n-bn-CSEC	0.012
	mn-CSEC - 2nd n-bn-CSEC	0.004
	1st n-bn-CSEC - 2nd n-bn-CSEC	0.724
NWI	mn-CSEC - 1st n-bn-CSEC	0.019
	mn-CSEC - 2nd n-bn-CSEC	0.141
	1st n-bn-CSEC - 2nd n-bn-CSEC	0.004

Abbreviations: mn-CSEC, mononucleated cervical-squamous-epithelial-cell; 1st n-bn-CSEC, first nucleus of binucleated cervical-squamous-epithelial-cell; 2nd n-bn-CSEC, second nucleus of binucleated cervical-squamous-epithelial-cell; p, significant level; CL, cell length (the longest cell diagonal that passes through the nucleus); CW, cell width (cell diagonal perpendicular to the diagonal of CL); CA, Cell area; CP, Cell perimeter; NA, nucleus area; NP, nucleus perimeter; NL, Nucleus length (the longest cell nucleus diagonal); NW, nucleus width (cell nucleus diagonal perpendicular to the diagonal of NL); NAI, nucleus area index (nucleus area:cell area)x100; NPI, nucleus perimeter index (nucleus perimeter:cell perimeter)x100; NLI, nucleus length index (nucleus length:cell length)x100; NWI, nucleus width index (nucleus width:cell width)x100; µm, milli-micron; μm^2, milli-micron square.

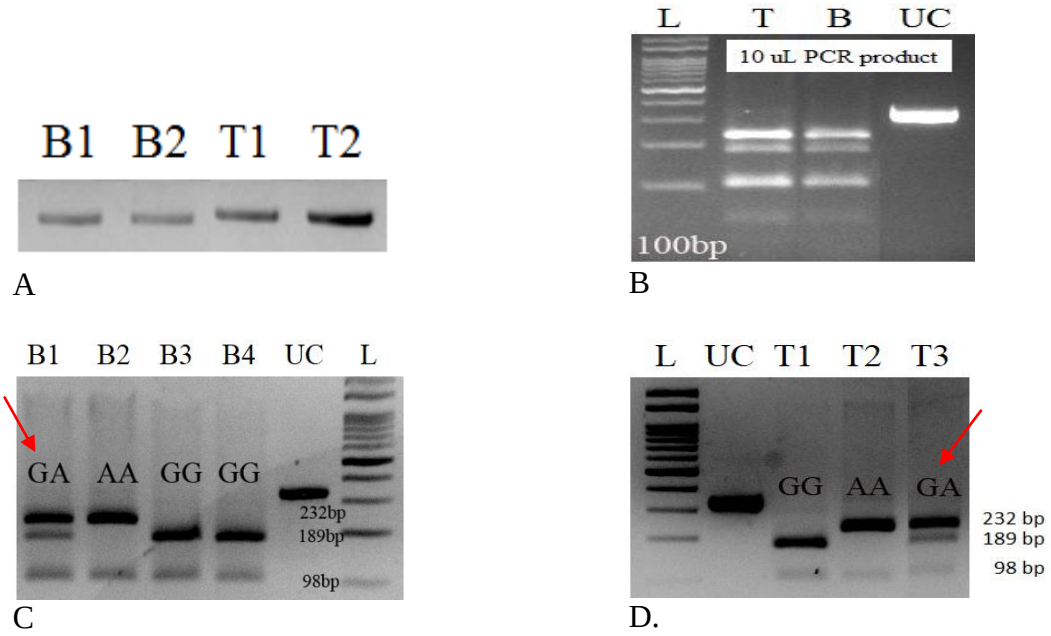
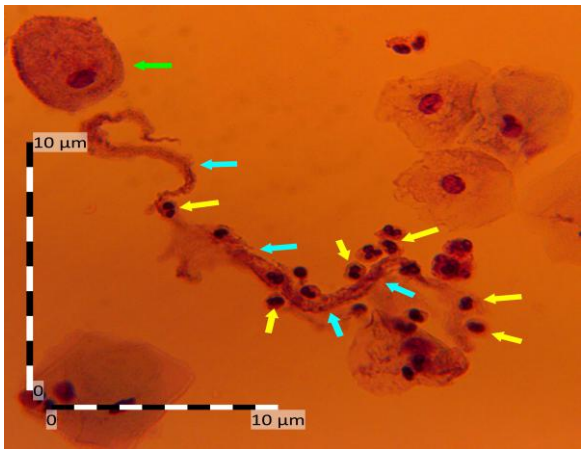


Figure 1. Detection of Fas-promoter-670 gene G/A polymorphism in blood and CSECs. (A) DNA extraction from blood and CSECs samples. (B). Optimization of restriction fragment length polymorphism from the ThinPrep Pap test of CSECs and blood samples with BstN1 enzyme. L, molecular weight marker; T, ThinPrep CSEC sample; B, blood sample; UC, running sample without BstN1 enzyme. (C) Genotyping of the Fas-promoter-670 gene from the blood sample. B1-B4, sample number; UC, running sample without BstN1 enzyme; L, molecular weight marker. The three possible genotypes were defined by three distinct patterns: AA(232, 98 bp), GA(232, 189, 98 bp), and GG (189, 98 bp). The subject in this case had the GA genotype of the Fas-promoter-670 gene (red arrow, B1 sample). (D) Genotyping of the Fas-promoter-670 gene from the ThinPrep CSEC sample. L, molecular weight marker; UC, running sample without BstN1 enzyme; T1-T3, sample number. The three possible genotypes were defined by three distinct patterns: AA(232, 98 bp), GA(232, 189, 98 bp), and GG (189, 98 bp). The subject in this case had the GA genotype of the Fas-promoter-670 gene (red arrow, T3 sample). CSEC, cervical squamous epithelial cell; PCR, polymerase chain reaction; bp, base pair.

A



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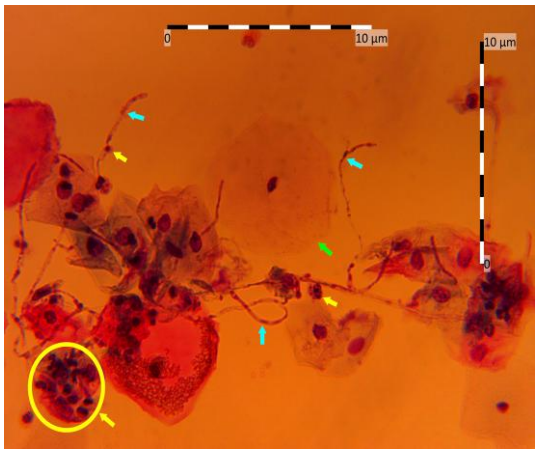
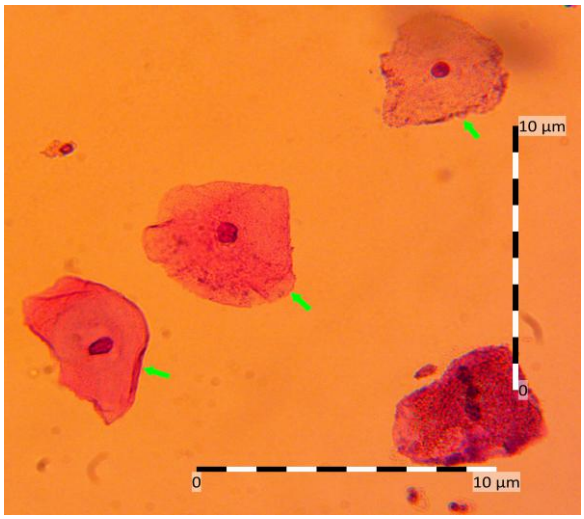


Figure 2. Appearance of CSEC and hyphae of *Candida sp.* in the subject with high risk HPV and *Candida sp* infection. (A) mn-CSEC (lime arrow) in the subject with high risk HPV and *Candida sp* infection in the early stage. Hyphae is indicated by the aqua arrow and the yellow arrow indicates the polymorphonuclear leukocyte. Magnification, 400x. (B) mn-CSEC (lime arrow) in the subject with high risk HPV and *Candida sp* infection in the advanced stage. Hyphae is indicated by the aqua arrow and the yellow arrow indicates a spore. Magnification 400x. mn-CSEC, mononucleated cervical squamous epithelial cell; CSEC, cervical squamous epithelial cell.

A



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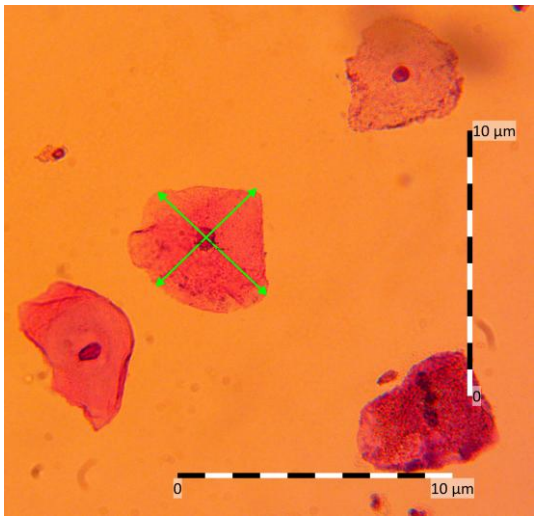

Figure 3. Appearance of mn-CSECs in the subject with high risk HPV and *Candida sp.* infection. (A) Appearance of mn-CSECs available for measurement (lime arrow). Magnification, 400x. (B) Cell length and cell width measurements result of mn-CSECs. Magnification 400x. mn-CSEC, mononucleated cervical squamous epithelial cell.

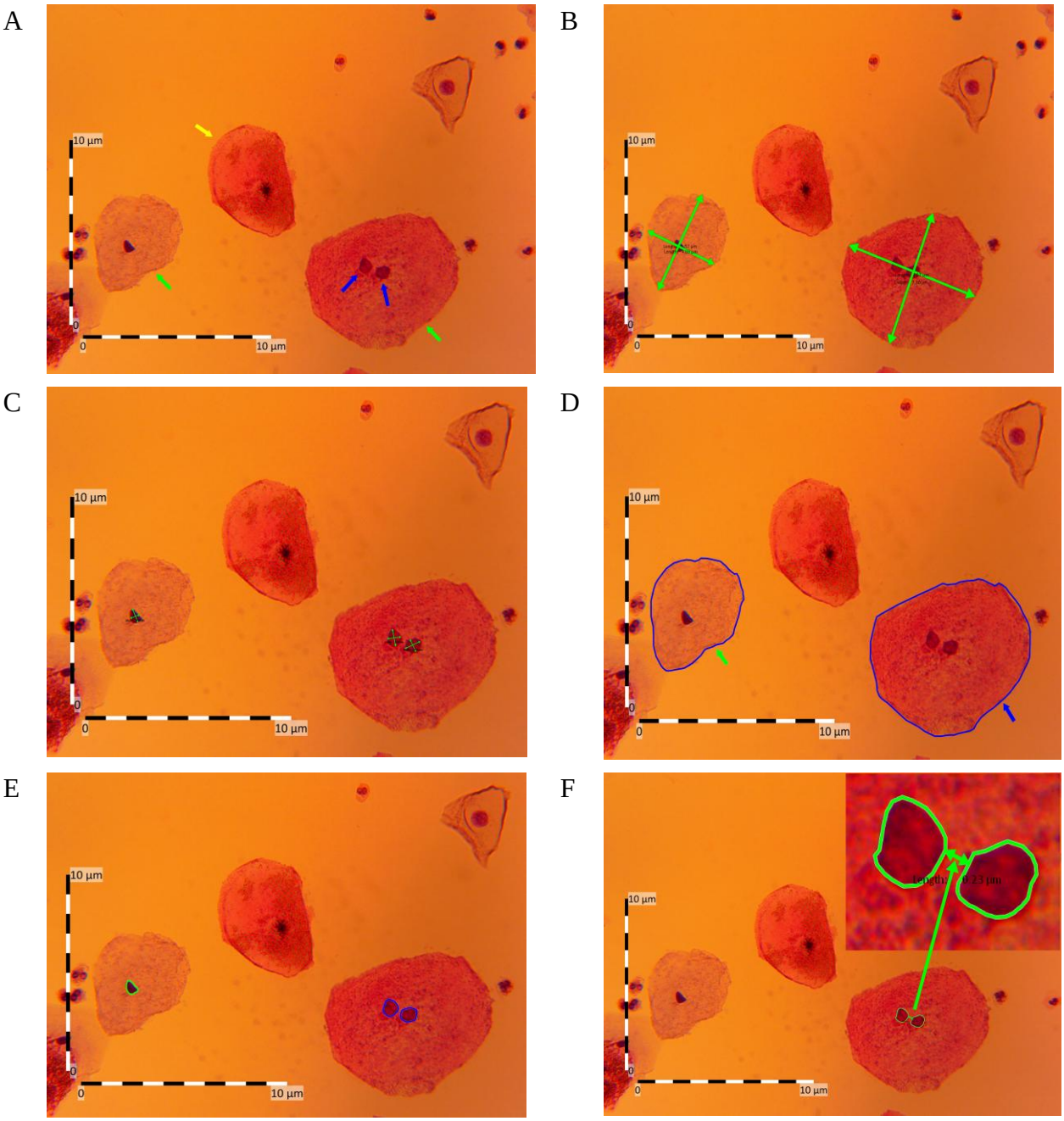

Figure 4. Comparison between mn-CSECs and bn-CSECs in the subject with high risk HPV and *Candida sp.* infection. (A) Appearance of mn-CSECs available for measurement (lime arrow) compared with mn-CSECs not available for measurement (yellow arrow) and bn-CSECs available for measurement (blue arrow). Magnification, 400x. (B) Comparison of cell length and cell width between mn-CSECs and bn-CSECs. Magnification 400x. (C) Comparison of nucleus length and nucleus width between mn-CSECs and bn-CSECs. Magnification, 400x. (D) Cell area and cell perimeter of mn-CSECs (lime arrow) and bn-CSECs (blue arrow). Magnification 400x. (E) Nucleus area and nucleus perimeter of mn-CSECs (lime polygon) and bn-CSECs (blue polygon). Magnification 400x. (F) Compression of bn-CSECs, demonstrated that between nucleus not pressing against each other. Magnification 400x. mn-CSEC, mononucleated cervical squamous epithelial cell; bn-CSEC, binucleated cervical squamous epithelial cell.

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The change of cell biometric and its nucleus on cervical-squamous-epithelial-cell with GA genotype of Fas-promoter-670 gene, high risk human papilloma virus and *Candida species* infection: A case report

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Abstract.

Cervical samples of patients with human papilloma virus (HPV) infections show positive results in HPV DNA testing. HPV infection can alter cervical squamous epithelial cells (CSECs) to be abnormal. Epigenetically, CSECs that change to be abnormal are affected by Fas-promoter-670 gene polymorphism. High risk HPV (hr-HPV) patients can be infected by other microbes, for example *Candida species (Candida sp.).* The purpose of the present study was to show CSEC biometrics based on epigenetics of the Fas-promoter-670 gene polymorphism in Indonesian women with hr-HPV and *Candida sp.* infection. Biometric quantification was performed based on the following analysis of CSECs. Indonesian hr-HPV women at the age of 28 years, with *Candida sp.* infection, underwent a Pap smear examination on April 21th, 2016 using the ThinPrep method and a blood test was also performed using the cubital vein (*vena cubiti*). Blood and ThinPrep samples were examined for the Fas-promoter-670 gene polymorphism. Epigenetically, the subjects had the GA genotype of the Fas-promoter-670 gene in the blood and ThinPrep samples. Patients with *Candida sp.* infection in the early stages were characterized by the appearance of polymorphonuclear leukocytes (PMN), whereas those in advanced stages presented without PMN on the hyphae. CSEC biometric measurements were performed quantitatively using mononuclear CSECs (mn-CSECs) and binucleated CSECs (bn-CSECs). Biometric measurements of the CSECs were performed quantitatively and assessed the length, width, area and perimeter of the cell and its nuclei. In the case investigated, cell length, cell width, nucleus area, nucleus perimeter and nucleus length index were significantly different between the mn-CSECs, the 1st nucleus of bn-CSECs and 2nd nucleus of bn-CSECs ($P<0.05$).

Key words

Epigeneticy, Fas promoter-670 gene, *Candida sp*., cervical-squamous-epithelial-cells, cell biometrics

Introduction

The ThinPrep Pap test can be used to identify precancerous lesions in patients so as to facilitate early detection for the management of further development of cervical cancer. ThinPrep Pap tests are currently the gold standard in liquid-based cytology testing, and their use is greatly improved compared with conventional Pap technology (1). It has been demonstrated that the ThinPrep Pap test has a high sensitivity and high specificity in screening for cervical cancer (2).

Precancerous cervical lesions are strongly associated with high risk human papilloma virus (hr-HPV) infection. It has been demonstrated that persistent infection with high risk types of HPV is the most significant factor for cervical cancer and precancerous lesion manifestation (3). A previous study has reported that incidence and mortality of cervical cancer and their relationship with the human development index in 185 countries in the world. Futhermore, screening methods include cytology (Papanicolaou test) and HPV testing, alone or in combination (4). However, HPV infection is not the only factor for the development of cervical cancer (5); genetic and environmental factors are also associated with the development of cervical cancer.

APO-1/Fas (CD95/TNFRSF6) receptor is a type I transmembrane glycoprotein that belongs to the tumor necrosis factor (TNF) or neuronal growth factor receptor superfamily (6). The human Fas-promoter-670 gene has been mapped, and is located at chromosome 10q24.1 (7) or 10.23 (8). This gene consists of 9 exons and 8 introns. Exon 1 comprises the 5'-untranslated region (UTR) and DNA sequence for the first 10 amino acids. Exons 2-5 encode the extracellular region, while exon 6 encodes the transmembrane region. Exons 7 and 8 encode the membrane proximal cytoplasmic 36 amino acids of the receptor. Exon 9 encodes the remaining 109 amino acids, including the 'death domain' and the 3'-UTR (6).

The Fas-promoter-670 gene plays a central role in the physiological regulation of programmed cell death (also termed apoptosis). Failure of this gene's regulation produces a death signal, which is reported in several types of cancer (9-10). It has been reported that a single nucleotide polymorphism at site 670 in the enhancer region of the Fas gene is associated with tumorigenesis (11). A study has demonstrated an association between Fas-promoter-670 gene polymorphism and cervical cancer. A statistically significant correlation was identified between the susceptibility for cervical cancer and the GA and combined GA+GG genotypes in a study that focused on an Indian population (12). An association between Fas-promoter-670 gene polymorphism and HPV infection has also been shown in cervical cancer in Japan (13). Another study in Asia has also demonstrated a positive association between Fas-promoter-670 gene polymorphism and cervical cancer (14). Additionally, a previous study has reported a case of a high risk Indonesian mother, whose Fas-promoter-670 gene in normal CSECs was mutated from AA to GA, whereas in the lymphocyte cells the genotype was AA (15).

A previous study has reported that the *Candida species (Candida sp.)* is of great concern due to its infection of human hosts, particularly patients with cancer (16). Invasive candidiasis is a serious infection that predominantly affects critically ill and immunocompromised patients. In general, *Candida albicans* is a species that is involved in

this infection (17). Furthermore, it has been stated that cancer patients have a greater risk for serious infections, such as *Candida sp.* Patients with candidemia have a serious problem due to high mortality, particularly if is caused by resistant *Candida spp*. (18).

Binucleation of CSECs in patients with *Candida sp.* infection has been reported. It has been stated that binucleation is a reactive cellular change in Pap smears due to *Candida* infection (19). A study result previously indicated that there is an effect of HPV infection on the number of nuclei in CSECs. Following HPV infection, the appearance of CSECs on the ThinPrep Pap slide demonstrate a different number of nuclei, exhibiting ≥1 nuclei. In the cells with >1 nucleus, if the nuclei pressed against each they were defined as positive compression, while the nuclei contacting but not pressing against each other were defined as negative compression (20). It has been reported that positive compression of binucleated cells may be present as a result of hr-HPV infection, which is caused due to inflammation in intraepithelial lesions or malignancy cases infected with *Candida sp.* (19). The present report evaluates the performance of CSEC biometrics and its nuclei with a GA genotype of the Fas-promoter-670 gene, and who has hr-HPV and *Candida sp.* infection.

Case report

A 28-year-old Indonesian women living with hr-HPV and *Candida sp.* infection on April 21th, 2016 underwent a Pap smear examination and a blood test. Before participating in this study, the participant provided informed consent. Ethical approval for the study was obtained from the *"Komisi Etik Riset Fakultas Kedokteran, Universitas Trisakti, Indonesia"* (approval no. 60/KER/FK/05/2013). Informed written consent was obtained from participants before data collection. The subject concerned was included in the community of Indonesian women living with hr-HPV based on evidence of laboratory examinations. In this case, the subject first has coitus at the age of 16 years, had free sex and had also never received HPV vaccination.

Blood samples (~10 µl) were taken from a cubital vein (*vena cubiti*) are inserted into an ethylene diamine tetra acetate (EDTA) tube. The Pap smear examination was performed according to the ThinPrep method to collect cytological material. Whole blood in the EDTA tube and cytological material in the ThinPrep solution were analyzed for Fas-promoter-670 gene polymorphisms by restriction fragment length polymorphism (RFLP) with *Bacillus stearothermophilus* N1 (BstN1) enzyme. DNA extraction from the blood and ThinPrep samples was performed using chloroform. In addition, the forward primer, 5'CTACCTAAGAGCTATCTACCGTTC3' and reverse primer, 5'GGCTGTCCATGTTGTGGCTGC3' were used. Epigenetically, the subject in this case had

the GA genotype of the Fas-promoter-670 gene both in the blood and ThinPrep samples (Fig. 1).

In addition to analyzing the Fas-promoter-670 gene polymorphism, cytological material in the ThinPrep solution also analyzed cytologically. ThinPrep 2000 was used to creates slides automatically based on liquid-based cervical cytology. Optilab Advance Plus and Image Raster 3 programs (PT MICONOS, Daerah Istimewa Yogyakarta, Indonesia; available at https://miconos.ac.id/new/support/download) were used by three observers. The presence of *Candida sp.* was based on the observation of hyphae under the microscope. *Candida sp.* infection can be divided into two stages, termed the early and advanced stages. *Candida sp.* infection in the early stage is characterized by the appearance of polymorphonuclear leukocytes (PMNs), whereas at the advanced stage no PMNs are observed on the hyphae of *Candida sp*. The spores of *Candida sp.* were observed qualitatively (Fig. 2). The biometric measurements of CSECs were performed quantitatively, focusing on the length, width, area and perimeter of the cell and its nuclei. Optilab plus and Image Raster 3 programs were used to analyze the biometric measurements by three observers. The measurements of the mononucleated-CSECs (mn-CSECs) (Fig. 3) and binucleated-CSECs (bn-CSECs) (Fig. 4) were as follows: Cell length (CL), cell width (CW), cell area (CA), cell perimeter (CP), nucleus length (NL), nucleus width (NW), nucleus area (NA), nucleus perimeter (NP), nucleus length index (NLI), nucleus width index (NWI), nucleus area index (NAI) and nucleus perimeter index (NPI). Based on the results of biometric measurements, CSECs in these cases were divided into three groups: i) mn-CSEC; 1^{st} nucleus of bn-CSEC (1^{st}-bn-CSEC); and iii) 2^{nd} nucleus of bn-CSEC (2^{nd}-bn-CSEC), which are presented in Table I. The CL, CW, NA, NP and NLI were not significantly different between the mn-CSECs, 1^{st}-bn-CSECs and 2^{nd}-bn-CSECs ($P>0.05$); however, CA, CP, NL, NW, NAI, NPI and NWI were significantly different ($P<0.05$). Multiple comparisons between the mn-CSECs, 1^{st}-bn-CSECs and 2^{nd}-bn-CSECs are presented in Table II. The CA and CP in mn-CSECs, 1^{st}-bn-CSECs and 2^{nd}-bn-CSECs were significantly different ($P<0.05$); however, 1^{st}-bn-CSECs was not significantly different compared with 2^{nd}-bn-CSECs ($P>0.05$). The NL and NW of mn-CSECs were significantly different compared with those of the 1^{st}-bn-CSECs, as well as for 1^{st}-bn-CSECs compared with 2^{nd}-bn-CSECs ($P<0.05$), while mn-CSECs were not significantly different compared with 2^{nd}-bn-CSECs ($P>0.05$). The NAI and NPI were significantly different between the mn-CSECs and 1^{st}-bn-CSECs or 2^{nd}-bn-CSECs ($P<0.05$); however, they were not significantly different for the 1^{st}-bn-CSECs compared with the 2^{nd}n-bn-CSECs ($P>0.05$). The NWI of mn-CSECs was significantly different compared with that of the 1^{st}-bn-CSECs, and the NWI of 1^{st}-bn-CSECs

was significantly different compared with that of the 2nd-bn-CSECs (P<0.05), while mn-CSECs did not significantly differ compared with 2nd-bn-CSECs in terms of NWI (P>0.05).

Discussion

DNA extraction from CSECs and leukocytes is useful for the genotyping of the Fas-promoter-670 gene, as well as for electrophoresis optimization. Genotyping of the Fas-promoter-670 gene in CSECs and leukocytes reveals that both have a GA genotype. To evaluate the exposure of HPV infection on the Fas-promoter-670 gene in CSECs, genotyping is necessary for both CSECs and leukocytes. The genotyping result of the Fas-promoter-670 gene in both CSECs and leucocytes of the present case demonstrated the same result, which suggests that the Fas-promoter-670 gene in CSECs does not mutate due to HPV infection. This is in contrast to a previous case, in which the Fas-promoter-670 gene in the CSECs had a GA genotype, whereas had an AA genotype in the leukocytes, therefore the Fas-promoter-670 gene in CSECs had mutated (15). This demonstrates that the Fas-promoter-670 gene in CSECs mutates due to the effect of HPV infection (local infection). We know that HPV infection is a local infection that is in the cervix.

The results of previous study indicated that persistent HPV infection is necessary for the development of cervical cancer. Furthermore, stated that genetic and epigenetic alterations in host cell genes are crucial for the progression of cervical precancerous lesions to invasive cancer (21). A recent study has reported that samples collected from patients with cervical cancer, tested positive for HPV DNA (22). Additionally, a study in Western Kenya also demonstrated associations between vaginal infections and potential high risk and hr-HPV genotypes. In detail, of the free sex workers analyzed, 33.3% had HIV and 57.7% harbored a potential hr-HPV and hr-HPV genotype (23). Based on numerous studies worldwide, the epidemiology of HPV infection and oncogenic properties of HPV type are due to different HPV genotypes. However, there are still many countries where population-based prevalence has not yet been identified. Furthermore, cervical cancer screening strategies are different between countries (24).

The results presented in the present case report are consistent with the results of recent research in Indonesia, which shows that HPV vaccination is not yet a priority for adolescents. This is due to a lack of education and the cost of HPV vaccination. Therefore a program is required to provide accurate information regarding HPV vaccination to the public, particularly teenagers (25).

This case is consistent with studies of the Japanese population, which demonstrated that a GG genotype is associated with an increased risk for the development of cervical cancer, with an odd ratio (OR) of 2.56 compared than the AA genotype. Furthermore, the G allele in the GA or GG genotype also increased the risk of cervical cancer, with an OR of 1.60 (26). Other studies investigating females in Northern India demonstrated that a GA genotype and a combination of GA and GG genotype significantly increased the risk of cervical cancer compared with an AA genotype (12). In addition, a study of Brazilian females <48 years old also reported that a GA genotype increases the risk of cervical cancer 5-fold compared with an AA genotype (27).

Research of a Greek population demonstrated that there is no significant association between EVER1/2 polymorphisms (rs2290907 and rs16970849) and cervical cancer. However, the study did provide additional data that also suggested no association between the FAS polymorphism (rs1800682) and the susceptibility to persistent precancerous lesions and cervical cancer. Current literature for EVER1/2 polymorphisms and cervical cancer is very limited worldwide therefore, prospective studies are needed to further clarify this point (28).

Candida sp., the most common *Candida albicans,* plays an important role in secondary infections of the vaginal and vulvar epithelium. *Candida sp.* is part of the normal flora in women and is often asymptomatic (29). In a study based in Brazil, which investigated 633 pregnant women, 158 specimens (24.1%) exhibited pathogenic infections, while 22.9% were infected with *Candida spp.* (30). A subsequent study in Brazil demonstrated that of the 263 patients analyzed, *Candida spp.* was isolated in 27%, and >60% of the isolates were identified as *Candida albicans.* In addition, *Candida non-albicans* was isolated at a rate of 8.6% in symptomatic patients and 14.3% in asymptomatic patients (31). Another study in Ethiopia reported a high prevalence rate of vulvovaginal candidiasis and *Candida non-albicans*. Therefore it is important to conduct continuous epidemiological surveys to measure changes in species distribution from *C. albicans* to *Candida non-albicans* (32). A study in West Kenya demonstrated that bacterial vaginosis was the most common infection (48.3%), followed by *Trichomonas vaginalis* (31.4%) and *Candida spp.* (19.9%). Significant associations between bacterial vaginosis and HPV 58, and between *Candida spp.* and HPV 16 and HPV 53 suggest the need for sexually transmitted disease management in a cervical cancer prevention program (23).

The appearance of PMN on hyphae of *Candida sp.* in the early stage indicate an infection and allergic reaction. By contrast, in the advanced stage of the infection the hyphae of *Candida sp.* do not present with PMN. It has also been reported that *Candida sp.* infection

produces a peptide toxin called candidalysin. The peptide toxin is produced by hyphae of *Candida albicans* and is characteristic of fungal pathogenesis. In addition, candidalysin is important for *C. albicans* mucosal infections. Candidalysin is known to activate epithelial cells to induce downstream innate immune responses that are associated with protection during vaginal infections. It has been reported that candidalysin plays an importance role in stimulating a strong pro-inflammatory response by neutrophil recruitment. Conversely, if candidalysin is not present, the inflammatory response decreases due to a lack of neutrophil recruitment (33).

Previous studies have shown that *Candida sp.* infects humans, particularly patients with cancer. The present study demonstrated that of the 68 blood samples, 5 (7.35%) were positive for the presence of *Candida spp,* 2 (40%) identified to be positive for *Candida albicans* and 3 (60%) were contained *Candida non-albicans* (16). The results of another study demonstrated that 8.7% of 150 samples were infected with *Candida sp.* (34).

A recent study reported that ThinPrep cervical cytology samples can be used in cervical cancer screening. There have also been reports of Indonesian mothers with hr-HPV and the Fas-promoter-670 gene in CSECs, which is mutated from AA to GA, and had normal CSEC characteristics (15). Based on the appearance of mn-CSEC and bn-CSEC, mn-CSEC characteristics in this case was normal, while bn-CSECs were abnormal. This is consistent with the fact that abnormality of bn-CSECs is due to cytokinesis failure. Futhermore, cytokinesis failure has potential of proliferation (35). Based on the results of cell biometric measurements (Tables I and II), there was a change in the size and shape of CSECs. The CA, CP, NL, NW, NAI, NPI, NWI measurements between mn-CSECs, 1^{st}-bn-CSECs and 2^{nd}-bn-CSECs were significantly different ($P<0.05$); however, CL, CW, NA, NP and NLI were not significantly different ($P>0.05$). The presents results and recent studies suggest that the growth rate of precancerous cells is significantly faster compared with normal cervical cells. However, the proliferation capacity of precancerous cells is similar to cervical cancer cells at the molecular level (36).

In the Pap smear method, the determination of CSEC characteristics can be used as a reference in the diagnosis of cervical cancer. A light microscope and Image Raster 3 are used to analyze CSEC morphology and assess measurements of the cell and its nuclei. Most hospitals in Indonesia have facilities, such as light microscope, the Image Raster 3 program is easy to obtain. This method may be simplest; however, cervical cancer can also be detected using a more sophisticated tool. The present study, which focused epigenetically on the Fas-promoter-670 gene and two contrasting pathogens HPV and *Candida sp.,* may hopefully

improve early screening methods of cervical cancer. Furthermore, the principles of CSEC measurements in this study can be used for the development of cervical cytopathology examinations based on 'biometric artificial intelligence'.

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Availability of data and materials

The datasets generated and analyzed in the present study are included in this published article.

Authors' contributions

Mauritius Lambertus Edy Parwanto, Raditya Wratsangka, Assangga Guyansyah and Reza Aditya Digambiro designed the study, collected the samples, carried out the ThinPrep, genetic and cell imaging analysis, participated in the collecting and interpretation of data, wrote the manuscript, and gave the final approval of the version to be published. David David, Hanslavina Arkeman, Kirana Anggraeni, Haryo Ganeca Widyatama, Hosea Jaya Edy, Yosua Jaya Edy and collected and analyzed the data, performed the literature review and wrote the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The authors declare that they have obtained written informed consent for the publication of the details relating to the patient in this report. All possible steps have been taken to safeguard the identity of the patient. This study has ethical clearance by the 'Komisi Etik Riset Fakultas Kedokteran, Universitas Trisakti, Indonesia'.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

Refereces

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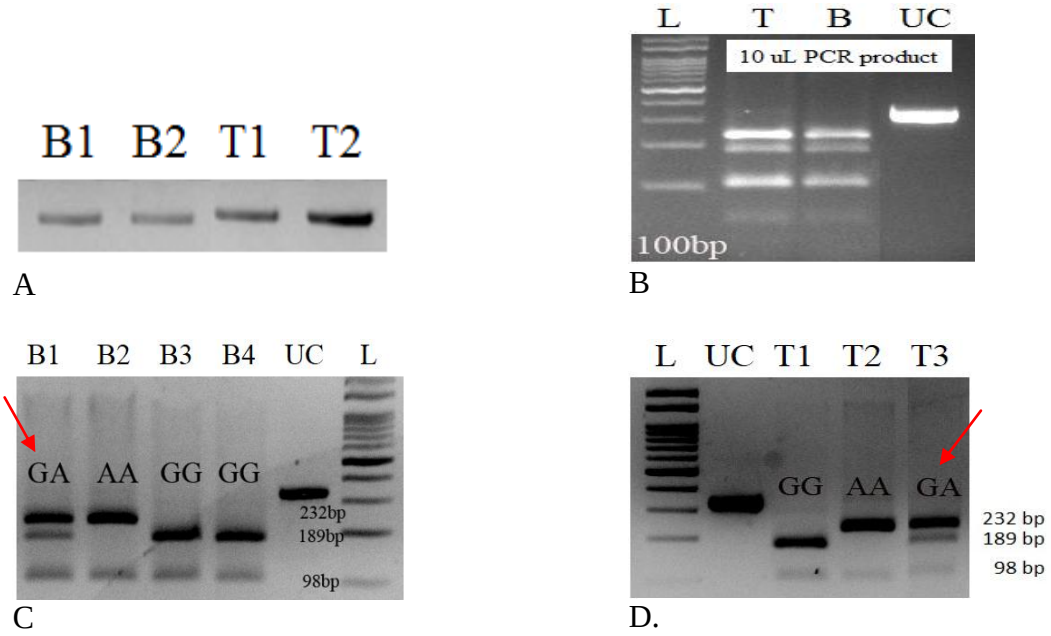
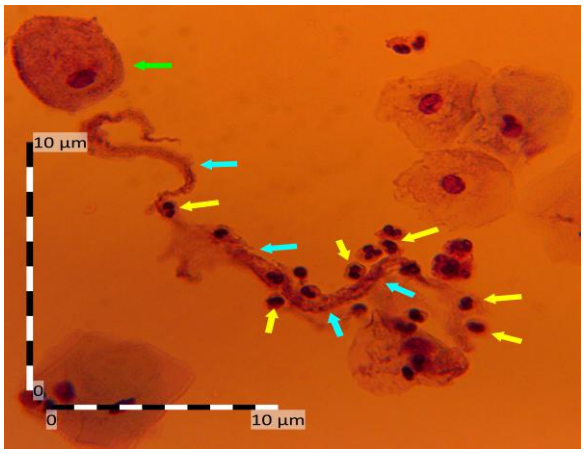


Figure 1. Detection of Fas-promoter-670 gene G/A polymorphism in blood and CSECs. (A) DNA extraction from blood and CSECs samples. (B). Optimization of restriction fragment length polymorphism from the ThinPrep Pap test of CSECs and blood samples with BstN1 enzyme. L, molecular weight marker; T, ThinPrep CSEC sample; B, blood sample; UC, running sample without BstN1 enzyme. (C) Genotyping of the Fas-promoter-670 gene from the blood sample. B1-B4, sample number; UC, running sample without BstN1 enzyme; L, molecular weight marker. The three possible genotypes were defined by three distinct patterns: AA(232, 98 bp), GA(232, 189, 98 bp), and GG (189, 98 bp). The subject in this case had the GA genotype of the Fas-promoter-670 gene (red arrow, B1 sample). (D) Genotyping of the Fas-promoter-670 gene from the ThinPrep CSEC sample. L, molecular weight marker; UC, running sample without BstN1 enzyme; T1-T3, sample number. The three possible genotypes were defined by three distinct patterns: AA(232, 98 bp), GA(232, 189, 98 bp), and GG (189, 98 bp). The subject in this case had the GA genotype of the Fas-promoter-670 gene (red arrow, T3 sample). CSEC, cervical squamous epithelial cell; PCR, polymerase chain reaction; bp, base pair.

A



B

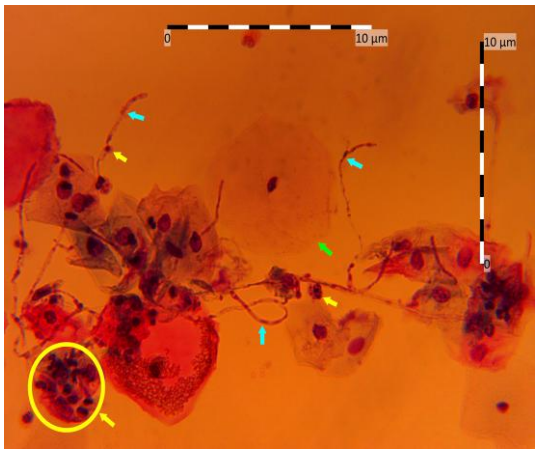
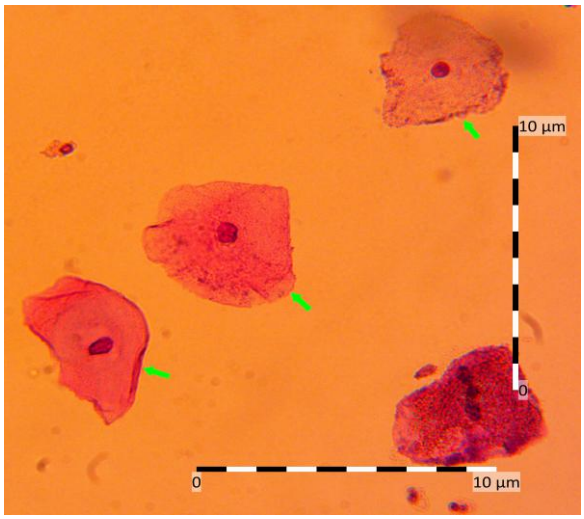


Figure 2. Appearance of CSEC and hyphae of *Candida sp.* in the subject with high risk HPV and *Candida sp* infection. (A) mn-CSEC (lime arrow) in the subject with high risk HPV and *Candida sp* infection in the early stage. Hyphae is indicated by the aqua arrow and the yellow arrow indicates the polymorphonuclear leukocyte. Magnification, 400x. (B) mn-CSEC (lime arrow) in the subject with high risk HPV and *Candida sp* infection in the advanced stage. Hyphae is indicated by the aqua arrow and the yellow arrow indicates a spore. Magnification 400x. mn-CSEC, mononucleated cervical squamous epithelial cell; CSEC, cervical squamous epithelial cell.

A



B

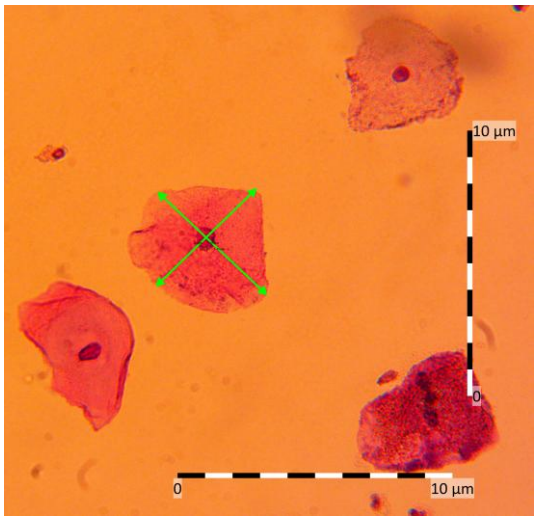


Figure 3. Appearance of mn-CSECs in the subject with high risk HPV and *Candida sp.* infection. (A) Appearance of mn-CSECs available for measurement (lime arrow). Magnification, 400x. (B) Cell length and cell width measurements result of mn-CSECs. Magnification 400x. mn-CSEC, mononucleated cervical squamous epithelial cell.

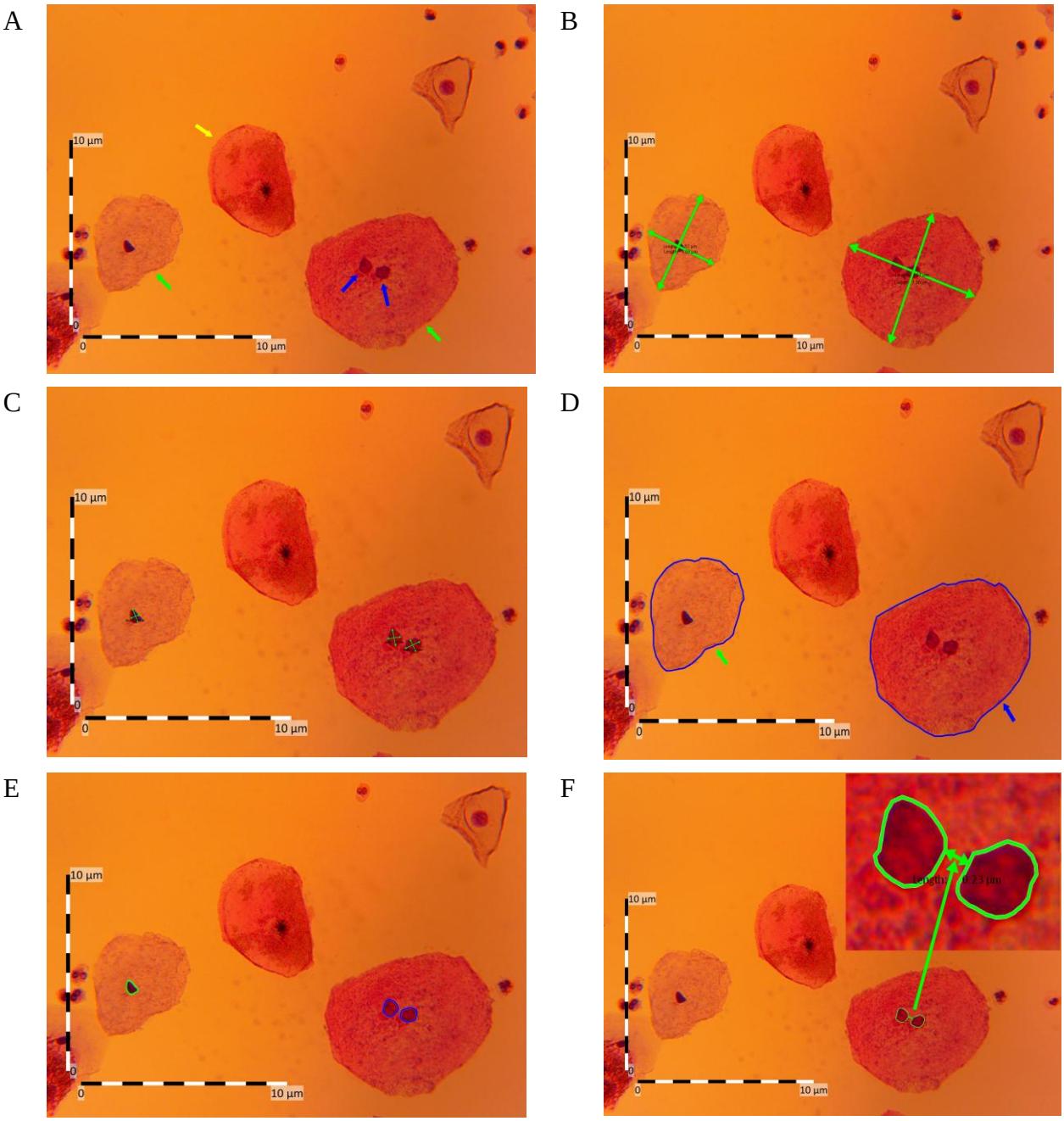


Figure 4. Comparison between mn-CSECs and bn-CSECs in the subject with high risk HPV and *Candida sp.* infection. (A) Appearance of mn-CSECs available for measurement (lime arrow) compared with mn-CSECs not available for measurement (yellow arrow) and bn-CSECs available for measurement (blue arrow). Magnification, 400x. (B) Comparison of cell length and cell width between mn-CSECs and bn-CSECs. Magnification 400x. (C) Comparison of nucleus length and nucleus width between mn-CSECs and bn-CSECs. Magnification, 400x. (D) Cell area and cell perimeter of mn-CSECs (lime arrow) and bn-CSECs (blue arrow). Magnification 400x. (E) Nucleus area and nucleus perimeter of mn-CSECs (lime polygon) and bn-CSECs (blue polygon). Magnification 400x. (F) Compression of bn-CSECs, demonstrated that between nucleus not pressing against each other. Magnification 400x. mn-CSEC, mononucleated cervical squamous epithelial cell; bn-CSEC, binucleated cervical squamous epithelial cell.

Table 1. Comparison of cell biometric and its nuclei between mn-CSEC and bn-CSEC in Indonesian women living with highrisk HPV and *Candida sp.* infection

Variable	mn-CSEC mean ± SD (n cell=27)	1st nucleus of bn-CSEC mean ± SD (n cell=6)	2nd nucleus of mn-CSEC mean ± SD (n cell=6)	P value
CL (µm)	5.44±0.31	5.72±0.61	5.73±0.26	0.900
CW (µm)	4.49±0.29	4.47±0.28	4.76±0.13	0.930
CA (μm^2)	20.45±1.23	25.45±2.78	25.61±0.71	0.000
CP (µm)	16.67±1.09	18.64±1.72	19.30±0.96	0.000
NA (μm^2)	0.47±0.05	0.45±0.04	0.45±0.03	0.403
NP (µm)	2.50±0.26	2.44±0.29	2.46±0.10	0.835
NL (µm)	0.83±0.08	0.76±0.09	0.89±0.05	0.034
NW(µm)	0.63±0.06	0.72±0.05	0.62±0.09	0.014
NAI	2.31±0.24	1.79±0.14	1.74±0.11	0.000
NPI	15.05±1.78	13.11±1.28	12.77±1.03	0.003
NLI	15.36±1.83	13.50±2.26	15.59±1.08	0.074
NWI	14.20±1.68	16.17±2.12	12.98±1.92	0.012

Abbreviations: mn-CSEC, mono nucleated cervical-squamous-epithelial-cell; bn-CSEC, binucleated cervical-squamous-epithelial-cell; 1th nucleus of bn-CSEC, first nucleus of binucleated-cervical squamous ephitelial cell; 2nd nucleus of bn-CSEC, second nucleus of binucleated cervical-squamous-epithelial-cell; SD, standard of deviation; n, sample size; p, significant level; CL, cell length (the longest cell diagonal that passes through the nucleus); CW, cell width (cell diagonal perpendicular to the diagonal of CL); CA, cell area; CP, cell perimeter; NA, nucleus area; NP, nucleus perimeter; NL, nucleus length (the longest cell nucleus diagonal); NW, nucleus width (cell nucleus diagonal perpendicular to the diagonal of NL); NAI, nucleus area index (nucleus area:cell area)x100; NPI, nucleus perimeter index (nucleus perimeter:cell perimeter)x100; NLI, nucleus length index (nucleus length:cell length)x100; NWI, nucleus width index (nucleus width:cell width)x100; µm, milli-micron; μm^2, milli-micron square.

Table II. Multiple comparison between mn-CSEC, 1st n-bn-CSEC and 2nd n-bn-CSEC in Indonesian women living with highrisk HPV and *Candida sp.* infection

Variable	Group	p value
CA (μm^2)	mn-CSEC - 1st n-bn-CSEC	0.000
	mn-CSEC - 2nd n-bn-CSEC	0.000
	1st n-bn-CSEC - 2nd n-bn-CSEC	0.848
CP (µm)	mn-CSEC - 1st n-bn-CSEC	0.001
	mn-CSEC - 2nd n-bn-CSEC	0.000
	1st n-bn-CSEC - 2nd n-bn-CSEC	0.336
NL(µm)	mn-CSEC - 1st n-bn-CSEC	0.069
	mn-CSEC - 2nd n-bn-CSEC	0.115
	1st n-bn-CSEC - 2nd n-bn-CSEC	0.010
NW(µm)	mn-CSEC - 1st n-bn-CSEC	0.007
	mn-CSEC - 2nd n-bn-CSEC	0.527
	1st n-bn-CSEC - 2nd n-bn-CSEC	0.009
NAI	mn-CSEC - 1st n-bn-CSEC	0.000
	mn-CSEC - 2nd n-bn-CSEC	0.000
	1st n-bn-CSEC - 2nd n-bn-CSEC	0.714
NPI	mn-CSEC - 1st n-bn-CSEC	0.012
	mn-CSEC - 2nd n-bn-CSEC	0.004
	1st n-bn-CSEC - 2nd n-bn-CSEC	0.724
NWI	mn-CSEC - 1st n-bn-CSEC	0.019
	mn-CSEC - 2nd n-bn-CSEC	0.141
	1st n-bn-CSEC - 2nd n-bn-CSEC	0.004

Abbreviations: mn-CSEC, mononucleated cervical-squamous-epithelial-cell; 1st n-bn-CSEC, first nucleus of binucleated cervical-squamous-epithelial-cell; 2nd n-bn-CSEC, second nucleus of binucleated cervical-squamous-epithelial-cell; p, significant level; CL, cell length (the longest cell diagonal that passes through the nucleus); CW, cell width (cell diagonal perpendicular to the diagonal of CL); CA, Cell area; CP, Cell perimeter; NA, nucleus area; NP, nucleus perimeter; NL, Nucleus length (the longest cell nucleus diagonal); NW, nucleus width (cell nucleus diagonal perpendicular to the diagonal of NL); NAI, nucleus area index (nucleus area:cell area)x100; NPI, nucleus perimeter index (nucleus perimeter:cell perimeter)x100; NLI, nucleus length index (nucleus length:cell length)x100; NWI, nucleus width index (nucleus width:cell width)x100; µm, milli-micron; μm^2, milli-micron square.

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Dear: Mauritius Lambertus Edy Parwanto[1*], Raditya Wratsangka[2], Assangga Guyansyah[2], Kirana Anggraeni[2], Reza Aditya Digambiro[3], David Tjahyadi[4], Hanslavina Arkeman[4], Haryo Ganeca Widyatama[4], Hosea Jaya Edy[5], Yosua Jaya Edy[6,7]
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I am very excited to accept your paper entitled:
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