EXPRESSION IN CERVICAL CELLS OF WOMEN IN INDONESIA, A PRELIMINARY STUDY

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Abstract—Cervical cancer is the major cause of death of women in Indonesia after breast cancer. It is believed that the integration of Human Papilloma Virus plays a critical role in the development of cervical cancer. A recent study discovered “hot spots” contained candidate genes that are linked to the integration of DNA HPV to the host’s genome. This preliminary study aims to investigate the expression of these genes in HPV-uninfected cervical cells. Most of the genes were expressed in HPV-uninfected cervical cells and their expression levels were significantly different from each other qualitatively and quantitatively, suggesting that these genes might have an important role in maintaining a normal function of cervical cells. Perturbation in the expression level of these genes might be linked to the developing of pathological conditions, such as cervical cancer.

Index Terms—Cervical cancer, DNA, HMGA2, HPV, Integration, KLF5, KLF12, SEMA3D.

1 INTRODUCTION
Cervical cancer is the second most cause of death after breast cancer among cancer-related death in women worldwide, including in developing countries. In 2013, the prevalence of cervical cancer in Indonesia was 0.8% higher than the prevalence of breast cancer (0.5%) [1]. World Health Organization predicts that there will be a significant increase in cervical cancer cases in Indonesia in 2030, up to 7 folds [2]. Cervical cancer is caused by the infection of Human Papilloma Virus (HPV), mostly 16 and 18 subtypes. HPV causes cancer in susceptible epithelial areas, such as the cervix. Cervix contains ectocervix lined by stratified epithelium, endocervix lined by columnar epithelium and transformation zone (TZ) [3]. Cervical cancer usually occurs at TZ where metaplastic development occurs from columnar simple epithelium to squamous epithelium. The infection of HPV in this area facilitates the deregulation of viral gene expression and neoplastic development. Most of the infections will be eradicated by the host’s immune system, however, some infections will be persisted over time leading to cancer [4]. Thus, other virus-related factors and the host’s immune system are compulsory to initiate an early sign of cancer [5]. HPV DNA integration to the host’s genome is believed to be a critical factor in the development of cervical cancer [6]. A recent bioinformatics study has identified “hot spot” for integrating HPV to host’s genome where some genes are located, such as Kruppel like factor 5 (KLF5), Kruppel like factor 12 (KLF12), High mobility group AT-hook 2 (HMGA2) and Semaphorin 3D (SEMA3D) [7]. Those genes have been linked to cell proliferation, invasion and apoptosis, which are the characteristic of cancer cells [8], [9], [10], [11]. However, little is known about the expression of these genes in cervical cells. This study aims to investigate the expression of those genes in the normal cervix of women in Indonesia.

2 METHODS

2.1 Ethical Approvals
This study was approved by the Faculty of Medicine Ethics Committee (ref 150/KER/FK/IX/2019) and methods were conducted per the guidelines and regulations of this approval.

2.2 Sample Collection
Cervical swab samples were collected from eight women without cervical cancer confirmed with the Papanicolaou test. Informed consent was obtained from all women as this study was approved by the Faculty of Medicine Ethics Committee (ref 150/KER/FK/IX/2019). All samples were transported to the laboratory in a cool box filled with dry ice. Samples were then kept in -20C freezer for further use.

2.3 DNA Extraction
DNA was extracted from each sample using Quick-DNA Miniprep Plus Kit (Zymo Research Corp, Irvine, CA, USA) according to the manufacturer’s protocol. In brief, the sample was mixed with biofluid and cell buffer and proteinase K. Sample was then vortexed 15 s and incubated at 55C for 10 mins. Genomic binding buffer was added and the mixture was transferred to a Zymo-Spin™ IIC-XLR Column in a collection tube then centrifuged at 12,000g for 1 min. For washing the sample, DNA pre- and wash buffers were added and the sample was centrifuged at 12,000 xg for 1 min. DNA elution buffer then added to elute the DNA. DNA purity was evaluated by a spectrophotometer. Only DNA samples with an A260/A280 ratio of 1.8-2.0 were included in this study.

2.4 Optimising primer pairs
To conduct a PCR and quantitative real-time PCR (qPCR), primers (Geneaid, Table 1) were designed against the published reference DNA sequences using Primer3 plus (Rozen and Skaltsky 2000) and Net primer (PREMIER Biosoft) software (primer pair 1) or taken from previous studies (primer pair 2). To test the primer pairs, the DNA was extracted from blood and cervical swab samples then diluted to 5
different concentrations (1:5, 1:10, 1:50, 1:100 and 1:200) to create a standard curve of cycle threshold (Ct) versus concentrations using the qPCR. Primer pairs that had a single sharp peak and achieved an amplification efficiency of 0.8 – 1 were used for further analysis.

2.5 Gene Expression

The expression of genes was determined using conventional PCR and qPCR. For the conventional PCR, DNA was amplified in duplicate at 95 °C for 30s then 60 °C for 30s and 72 °C for 1 min for 39 cycles using Labcycler 48 (SensoQuest GmbH). Amplification of DNA dilutions was prepared in 50μL reactions containing 2μL of 200ng DNA, 25μL DreamTaq Green PCR Master Mix (2X) (Thermoscience), 2μL each of forward and reserve primers (Geneaid, Table 1) for the target genes and 24.6μL of RNAse free water. Amplified DNA was then loaded to a gel. For qPCR, DNA was amplified in duplicate at 95 °C for 5s then 60 °C for 10s and 72 °C for 10s for 40 cycles using a Rotor Gene 3000 (Qiagen). Amplification of DNA dilutions was prepared in 20μL reactions contained 10μL 2x SensiFAST SYBR No-Rox Mix, 10uM each primer (Geneaid, Table 1) and 6.4μL RNase free water. Ct values were then calculated using the Rotor Gene 3000 software (Q series; Qiagen GmbH) at a threshold of 0.05 normalized fluorescence units. Gene expression was calculated by the mean of 2-∆Ct, where ∆Ct denotes the target gene Ct – glyceraldehyde-3-phosphate dehydrogenase (GAPDH) Ct. GAPDH was applied as a housekeeping gene due to its stable expression in HPV infected and uninfected cervical cell samples [12] and showed relatively constant expression in our samples.

2.6 Electrophoresis

Amplified DNA was loaded to an electrophoresis system in 0.9% agarose gels, in 1x TAE buffer. FloroSafe DNA Stain (1st Base) was added to visualize the DNA band. Fragment sizes were determined using UVI-DOC FireReader (UVitec, UK). Subsequently, ImageJ was used to analyze the intensity of DNA fragments.

2.7 Statistical Analyses

Statistical analyses were determined using Microsoft Office Excel 2010 and GraphPad Prism Version 6.00 (GraphPad Software Inc.). All data that were nor normally distributed were first log-transformed. The 2-ΔCt data for each cervical cell sample was compared using ANOVA with Tukey’s post-hoc test or student t-test. A value of P<0.05 was considered to be significant.

3 RESULTS

3.1 Demographic Characteristic of Women

On average, the age of subjects was 54 years old. Most of them were employed and had been married for almost 20 years. In general, all women had a regular menstrual cycle and were using contraception (Table 2). All samples did not show any abnormalities caused by HPV infection.

3.2 Primer Pairs

Two primer pairs of each gene were tested and optimized by generating an efficiency graph for each primer pair. The efficiency of KLF5 and HMGA2 primer pairs 1 were 0.82 and 0.76, respectively, whereas SEMA3D and KLF12 were 0.88 and 2, respectively. For primer pairs 2, KLF5 and HMGA2 had lower efficiency rates, which were 0.56 and 0.54, respectively. The efficiency of SEMA3D and KLF12 primer pair 2 was higher than they primer pair 1, which were 0.89 and 0.8, respectively. Thus, primer pair 1 of KLF5 and HMGA2, as well as primer pair 2 of SEMA3D and KLF12 were chosen to be used for further analysis.

3.3 The Expression of Genes

We analyzed qualitatively and quantitatively the expression of KLF5, HMGA2, SEMA3D, and KLF12. Amplified DNA was loaded and run into an agarose gel and subsequently, a picture was taken under UV lights. KLF5 and HMGA2 were qualitatively expressed in cervical cells of 8 women involved in this study (Fig. 1&2). However, SEMA3D and KLF12 were not detected in a loaded agarose gel.
For quantitative analysis, all genes were analyzed using qPCR. KLF5 was significantly upregulated in HPV uninfected cervical cells compared to HMGA2 and SEMA3D (Fig. 3), suggesting that KLF5 might have an important role in HPV-uninfected cervical cells. We did not observe the expression of KLF12 in all samples using the 2 primer pairs.

3.4 DNA Fragment Analysis
We analyzed the DNA fragment using ImageJ software. Using an integrated density tool in ImageJ software, the density of DNA fragments was measured and then compared with each other. Since we did not observe SEMA3D and KLF12 DNA fragments in the agarose gel, we only analyzed KLF5 and HMGA2. The density of KLF5 DNA fragments was significantly higher than HMGA2, suggesting that KLF5 might be involved more in the critical function of HPV uninfected cervical cells than HMGA2 (Fig. 4).

4 DISCUSSIONS
In this preliminary study we conducted gene expression analyses qualitatively and quantitatively in HPV-uninfected cervical cells of women in Indonesia, focusing on 4 genes of interest; KLF5, HMGA2, SEMA3D, and KLF12. We optimized each pair of primers using an efficiency graph and generated primer pair for each gene. We believe this is a critical study of the development of abnormalities in cervical cancer, including cervical cancer. Since every dysfunction of cervical cells has been linked to changes in gene expression and our genes of interest are believed to be involved in the early integration of HPV in the host's cervical cell, KLF5, HMGA2, SEMA3D, and KLF12 were examined. There are three zones of the epithelium in the cervix and one of them is called transformation zone, where columnar epithelium change into squamous cells [13].

<table>
<thead>
<tr>
<th>No.</th>
<th>Date of Birth</th>
<th>Employment Status</th>
<th>Marital Status</th>
<th>Length of Marriage (years)</th>
<th>Menstrual Cycle</th>
<th>Contraception type</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>29-Jun-65</td>
<td>Employed</td>
<td>Married</td>
<td>11</td>
<td>Irregular</td>
<td>IUD</td>
</tr>
<tr>
<td>2</td>
<td>29-Mei-65</td>
<td>Unemployed</td>
<td>Married</td>
<td>18</td>
<td>Irregular</td>
<td>-</td>
</tr>
<tr>
<td>3</td>
<td>27-Mar-58</td>
<td>Employed</td>
<td>Widowed</td>
<td>20</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>4</td>
<td>02-Dos-66</td>
<td>Employed</td>
<td>Married</td>
<td>21</td>
<td>Irregular</td>
<td>Permanent</td>
</tr>
<tr>
<td>5</td>
<td>16-Oct-70</td>
<td>Employed</td>
<td>Married</td>
<td>15</td>
<td>Regular</td>
<td>Permanent</td>
</tr>
<tr>
<td>6</td>
<td>27-Oct-66</td>
<td>Employed</td>
<td>Married</td>
<td>20</td>
<td>Regular</td>
<td>IUD</td>
</tr>
<tr>
<td>7</td>
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<td>Married</td>
<td>12</td>
<td>Regular</td>
<td>IUD</td>
</tr>
<tr>
<td>8</td>
<td>8-Oct-60</td>
<td>Employed</td>
<td>Married</td>
<td>24</td>
<td>Regular</td>
<td>IUD</td>
</tr>
</tbody>
</table>
This is the place where cancer cells develop due to metaplastic changes in this area [14]. It is believed that HPV infects these cells leading to the deregulation of gene expression. KLF5 has been linked to the development of cancer in various human cell lines, such as urine bladder [15], breast cancer [16], and colon-rectal cancer [17]. These studies have shown that KLF5 might have a critical function in proliferation, migration, apoptotic and invasion process of cancer cells, suggesting that changes in the expression level of KLF5 might disrupt the normal function of cervical cells leading to the development of the pathological condition, such as cancer. HMGA2 encodes the protein family of the non-histone chromosome and acts as a transcription factor. HMGA2 is located in chromosome 12 containing 5 exons [18]. Studies have suggested that the overexpression of HMGA2 protein has been linked to numbers of cancer, such as lung, ovarian, breast, pancreatic, prostate and colorectal cancers [19, 20]. HMGA2 is believed to be involved in cell proliferation, apoptosis and cell migration in cancer cell growth [21, 22]. Downregulation of HMGA2 has been shown to down-regulate the expression of Bcl-2 as well as up-regulate the expression of Caspase3 leading to a decrease of cell proliferation and an increase of apoptosis and vice versa [21]. SEMA3D encodes a semaphorin protein that belongs to the semaphorin III family. Semaphorins have been involved in axon guidance during the development of neurons [23]. It is believed that SEMA3D has an important function in the development of neuron, immune, cardiovascular, hepatic, gastrointestinal, as well as musculoskeletal systems [24]. SEMA3D is critical for the regulation of cell migration [25] and has been linked to colorectal cancer [26] and pancreatic ductal adenocarcinoma [27]. KLF12 encodes an activator protein-2 alpha (AP-2 alpha) which is a developmentally-regulated transcription factor [28]. KLF12 has been linked to several cancers, including lung cancer and gastric cancer. It is thought that KLF12 is involved in apoptosis and cell cycle. Deregulation of KLF12 might lead to an alteration in apoptosis and cell cycle regulation [11]. Overall, these genes have some unique features. Most of the genes were involved in cell proliferation. SEMA3D is involved in cell migration. These are the characteristics that are needed by cancer cells to develop and become metastases.

5 CONCLUSION

Most genes of interest associated with HPV-integration were expressed in human HPV-uninfected cervical cells. Their levels of expression were significantly different and the most upregulated gene was KLF5, suggesting its important role in maintaining the function of human HPV-uninfected cervical cells. Perturbations in the expression levels of these genes might be critical for the early development of cervical cancer.

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