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## ***Krüpple-Like Factor 5 Has a Strong Correlation with Reversionless 3-Like in Cervical Cancer Cells***

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### **Abstract**

*Cervical cancer is the fourth most frequent cancer in women caused by Human Papilloma Viruses (HPV) type 16 and 18 infections. A recent study suggested that HPV integration to the human genome can alter the expression of some genes, including KLF5. KLF5 plays a critical role in cell proliferation, an important process in cervical cancer pathogenesis. REV3L is believed to be involved in cell proliferation. This study aims to correlate KLF5 to REV3L in cervical cancer cells. This cross-sectional study was conducted in Trisakti Health Centre, Jakarta. Cervical tissues (6 normal and 4 cancerous tissues) were collected from 10 women. Normal tissues were obtained using the Pap smear technique, whereas cancer tissues were obtained using biopsy. All samples then transferred to a clinical laboratory for pathological testing and biomolecular laboratory for gene expression testing using qPCR. REV3L was upregulated in cervical cancer samples compared to normal ( $p < 0.05$ ) and it strongly correlated with KLF5 ( $R = 0.787$ ). There was no significant difference in the expression level of KLF5 between normal and cancer cells ( $p > 0.05$ ). There was a strong positive correlation between KLF5 and REV3L expression levels, suggesting that KLF5 and REV3L might affect each other in cell proliferation, which is a critical process in the development of cervical cancer. Taken together, KLF5 might be involved in regulating the expression of REV3L. These genes might have a critical role in the pathogenesis of cervical cancer cells.*

**Keywords:** *cervical cancer, cell proliferation, HPV, KLF5, REV3L*

### **1. Introduction**

Cervical cancer is the fourth most frequent cancer in women, reaching 570,000 new cases in 2018 worldwide [1]. Around 90% of women in developing countries are affected by this disease, leading to a high mortality rate. However, there is no effective treatment due to its complicated mechanism of development [1]. Most cervical cancer cases were provoked by the Human Papilloma Virus (HPV) infection type 16 and 18 [2]. A study has suggested that HPV integration with the human genome can affect some gene expression alterations, suggesting a critical starting point of cervical cancer pathogenesis [3]-[5]. A recent bioinformatics study has found several “hot-spots” genes for HPV to integrate, one of them is *Krüpple-Like Factor 5 (KLF5)* [6].

*Krüpple-Like Factor 5 (KLF5)* is a regulator gene for cellular physiological processes such as cell proliferation, angiogenesis, cell apoptosis, and cell migration [7], [8]. *KLF5* is an oncogenic gene, and its expression is related to pathogenesis and the development of cancer through cell mutation and proliferation [9]. *KLF5* is believed to be involved in cell proliferation [10]-[12]. One of the genes that is involved in cell proliferation is *Reversionless 3-Like (REV3L)*. *REV3L* encodes DNA Polymerase zeta protein, an enzyme that is responsible for correcting a lesion in a newly formed DNA strand due to the DNA replication process [13], [14]. This study correlates *KLF5* expression and cervical cancer cell proliferation through a cell proliferation-related gene, *Reversionless 3-Like (REV3L)*.

## 2. Methodology

### 2.1. Ethics Approvals

This study was approved by the Research Ethics Committee, Faculty of Medicine, Trisakti University (ref 150/KER/FK/IX/2019).

### 2.2. Sample Collection

All samples were obtained from 10 women (6 normal and 4 cervical cancer) in Trisakti Health Centre, Jakarta, Indonesia. After informed consent was explained and signed, samples were collected. All normal samples were taken using a Pap-Smear technique, whether cancer tissues were taken using biopsy. All samples then brought to the clinical laboratory for pathological testing and Biomolecular Laboratory in Faculty of Medicine, Trisakti University, Jakarta, Indonesia for further use.

### 2.3. DNA Extraction

Total DNA from the tissues was extracted with DNA Miniprep Plus Kit (Zymo Research Corp, Irvine, CA, USA) as per manufacturer's protocol. In brief, cell buffer and proteinase K were added to the tissue in a 1.5 mL microcentrifuge tube. The mixture was then vortexed for 15s and incubated at 55°C for 10 mins. Genomic binding buffer was then added and the mixture was transferred to a Zymo-Spin™ IIC-XLR Column in a collection tube. The column and collection tubes were then centrifuged at 12,000x g for 1 min. After discharging the liquid in the collection tube, DNA prewash buffer was then added and the sample was centrifuged at 12,000 xg for 1 min. DNA wash buffer was then added and the sample was centrifuged at 12,000 xg for 1 min twice. Finally, DNA elution buffer was added to elute the DNA. The DNA concentration and purity were confirmed using a Nanophotometer (NanoPhotometer P300, Implen GmbH, Munich, Germany) based on the 260λ and 280λ (wavelength) absorbance. Only samples that had a 260/280λ absorbance ratio 1.8-2.0 were used.

### 2.4. Primer Design

All primers used in this study (Table.1) were designed based on the reference sequence using Primer Premier 3 plus software [15]. To test for possible cross-reactivity with other genes, primers were aligned with the GeneBank database using the BLAST tool.

### 2.5. Optimising Primer

All primer-pairs were optimized to test the specificity and the sensitivity of each primer using extracted DNA from blood. For testing the combination of primers, the DNA was diluted into 5 different concentrations (1:5; 1:10; 1:50; 1:100; 1:200) to create an efficiency curve. Primer pairs that had a single sharp peak and achieved an amplification efficiency of 0.8-1 and R<sup>2</sup> value > 0.9 were used for further analysis.

**Table 1. *KLF5*, *REV3L* and *GAPDH* primer pairs**

Gene Name	Gene Symbol	Pair	Primers (5' → 3')	Size (bp)
Krüppel-Like Factor 5	<i>KLF5</i>	1	F: CCCTTGACATACACAATGC	136
			R: GGATGGAGGTGGGGTTAAAT	
		2	F: CCCTTGACATACACAATGC	206
			R: GTTAACTGGCAGGGTGGTG	
Reversionless 3-Like	<i>REV3L</i>	1	F: CGCGTCAGTTGGGACTTAAAG	98
			R: ACTATCGCCAACCTCAATGC	
		2	F: TGATGTCTTCAGCTGGTATCATGA	79
			R: (Not specified in image)	

Gene Name	Gene Symbol	Pair	Primers (5' → 3')	Size (bp)
		3	R: CCGCCCTTCAGGTTCACTT	150
			F: GCTCCAGTATGTGTACCATCTTGT	
			R: ATGGATATCTCGAAGTAACACGTC	
Glyceraldehyde-3-phosphate dehydrogenase	<i>GAPDH</i>		F: GTCTCCTCTTGACTTCAACAGCG	132
			R: ACCACCCTGTTGCTGTAGCCAA	

## 2.6. Gene Expression

DNA samples were amplified using the SensiFAST SYBR No-Rox Mix in a DNA samples were amplified using the SensiFAST SYBR No-Rox Mix in a Quantitative Real-time PCR was conducted using a Rotor-Gene 3000 series thermal cycler (Qiagen GmbH) in duplicate as per manufacturer's instruction. In brief, 40 cycles of DNA amplification at 95°C for 5s, 60°C for 10s and then 72°C for 10s was applied in analyzing the gene expression. Amplification of DNA dilutions was prepared in 20µL reactions containing 2µL of 1:20 of 200 ng DNA dilution, 10µL SensiFAST SYBR NO-Rox Mix, 0.8µL each of forward and reserve primers (Integrated DNA Technologies, Iowa, USA, Table 1) for the target genes and 6.4µL RNase-free water. Ct-values were calculated using the Rotor-Gene 3000 software (Q series; Qiagen GmbH) at a threshold of 0.05 normalised fluorescence units. Gene expression was then determined by the mean of  $2^{-\Delta Ct}$ . The delta Ct ( $\Delta Ct$ ) denotes the target gene Ct – GAPDH Ct. GAPDH was used as a housekeeping gene due to its stable expression in various tissues, including human cervical tissues [16]. Qualitative analysis was conducted on the electrophoresis system to confirm qPCR amplification results. Amplified DNA was run on a 0.9% agarose gel in 1X TAE buffer in 75V for 90 minutes. DNA fragments were stained using FloroSafe DNA stain and visualized using UVITEC (UVITECH Cambridge, Cambridge, UK).

## 2.7. Statistical Analysis

Statistical analysis was carried out using Prism 8 (GraphPad, La Jolla, CA, USA). A significant difference between the two groups (normal cervix and cervical cancer) was calculated using an independent t-test if the data were normally distributed. Otherwise, the Mann-Whitney U test was used. A value of  $p < 0.05$  was considered statistically significant. To determine the correlation between *KLF5* and *REV3L*, Pearson's R was used.

## 3. Results and Discussion

In this study, we conducted a gene expression analysis qualitatively and quantitatively in one of the HPV-integrated genes, *Krüppel-Like Factor 5*. We believe that perturbation of *KLF5* gene expression is crucial in the early development of cervical cancer [6].

### 3.1. Optimizing Primer Pairs

All primers were tested and optimized by generating an efficiency graph. The efficiency of each primer-pair of *KLF5* was 1.83 and 0.87 respectively. The efficiency of each *REV3L* primer pair was 0.97, 1.34, and 0.97 respectively. Only primer pairs that had an amplification efficiency of 0.8-1.0 were used for further analyzes, thus *KLF5* primer-pair 2 (Figure 1) and *REV3L* primer-pair 3 (Figure 2) were used for further analysis.

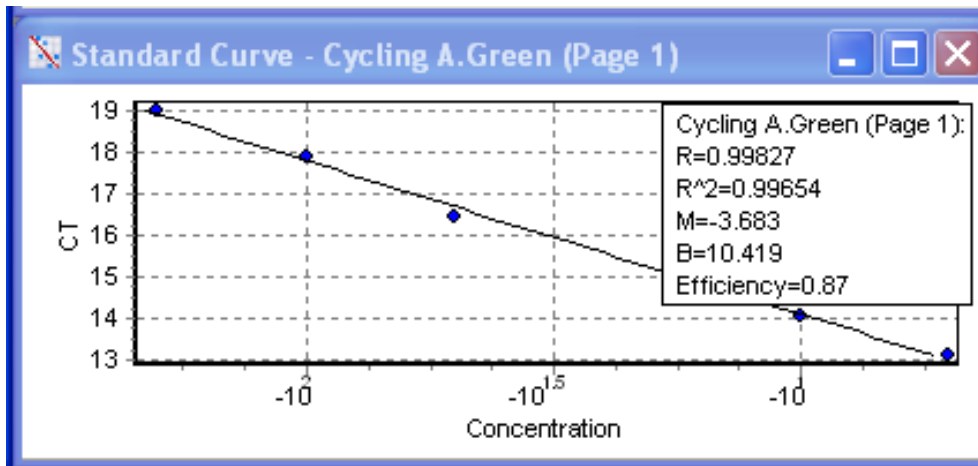


Figure 1. Efficiency Graph of *KLF5* primer pair 2

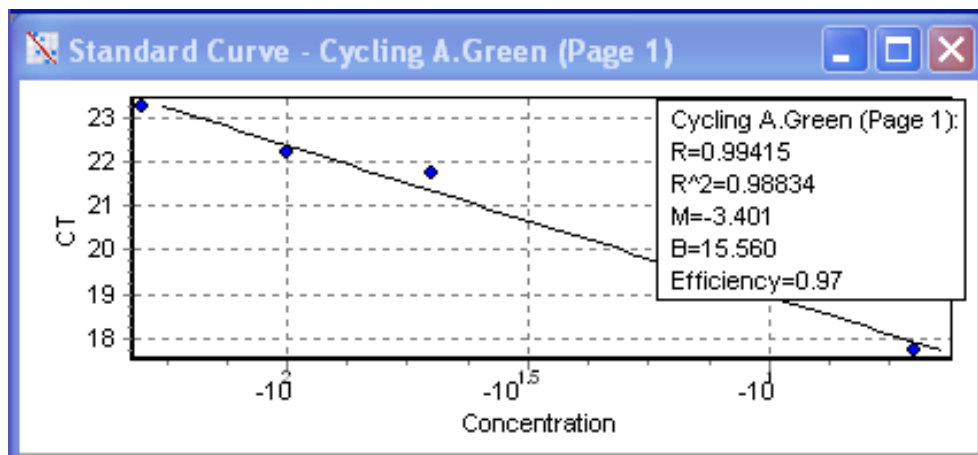
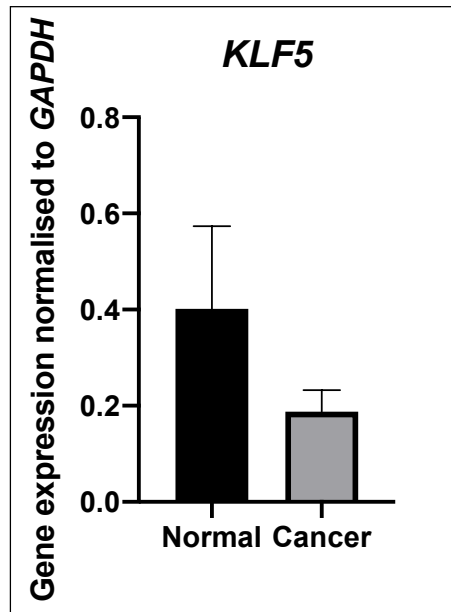


Figure 2. Efficiency Graph of *REV3L* primer pair 3

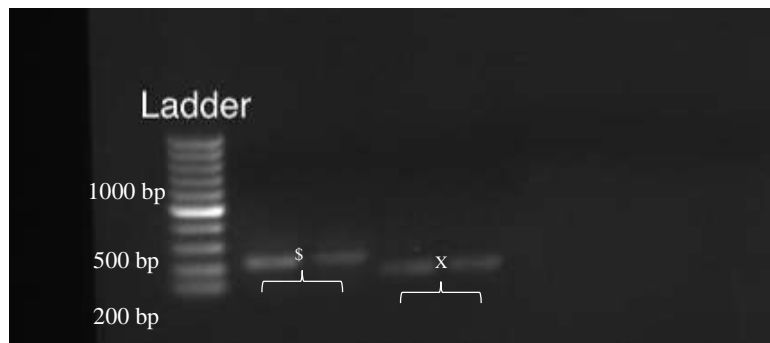
### 3.2. *KLF5* Expression on Normal and Cervical Cancer Cells

Quantitative analysis showed that *KLF5* expression was downregulated in cervical cancer cells (Figure 3), however, we did not find any significant differences between the two groups ( $p=0.3548$ ).

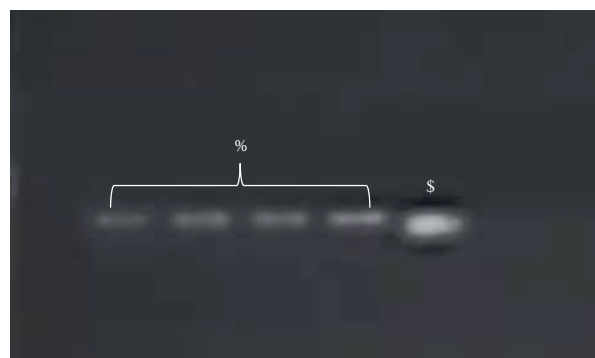


**Figure 3. Expression levels of *KLF5* in human normal (n=6) and cervical cancer cells (n=4). Data are presented as mean±s.e.m. T-test was used to analyze the data**

Qualitative analysis showed that *KLF5* was expressed in both normal (Figure 4 and 5) and cervical cancer cells (Figure 5), suggesting that *KLF5* might be involved in physiological cellular processes on both cells.



**Figure 4. The DNA Fragments of *KLF5* (marked with \$) and *REV3L* (marked with x) of normal cervical cells**



**Figure 5. The DNA Fragments of *KLF5* of cancerous (marked with %) and normal (marked with \$) cervical cells**

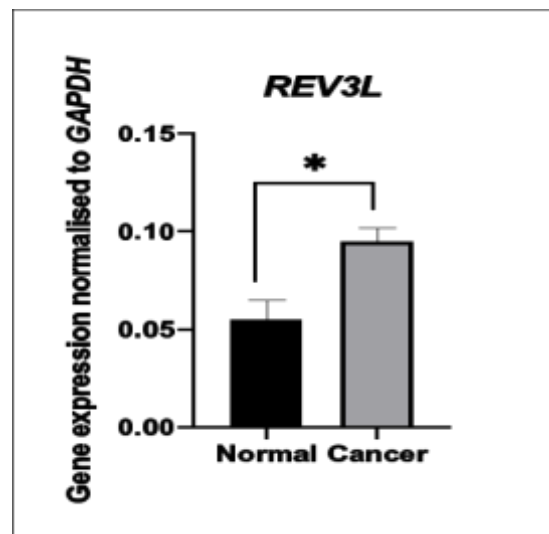
*KLF5* was expressed in both normal and cancerous cervical cells, indicating that it might be involved both in normal and pathological events in cervical cells. Although we did not find any significant difference between normal and cancer groups, *KLF5* was downregulated in cancer cells.

*KLF5* was highly expressed in various types of human tissue, such as skin, cervix, urinary bladder, adipose, tonsil, bone marrow, bronchus, and lung [7], [8]. *KLF5* has been linked to cell mutation in cancer, increasing cell proliferation and the development of cancer cells. An in-vitro study using urinary bladder cell lines, overexpression of *KLF5* increases the expression of CCNE1 protein, a protein that is involved in cell proliferation. On the other hand, the downregulation of *KLF5* decreases the expression of CCNE1 protein [17]. In the breast cancer cell line, *KLF5* induces cell proliferation, migration, and invasion by binding to Tumor Necrosis Factor, Alpha Induced Protein 2 (*TNFAIP2*) gene promoter. *TNFAIP2* is believed to be involved in angiogenesis, spermatogenesis, antiviral responses and apoptosis [18]. A mutation of *KLF5* enhances cell proliferation in colon-rectal cancer *in-vitro* [19]. *KLF5* has been shown to mediate cell proliferation via Cyclin D1 protein. Cyclin D1 protein is believed to make the G1 phase shorter in the cell cycle, thus induce the initiation of DNA replication [20]. Taken together, our findings suggest that HPV infection does not alter *KLF5* expression significantly in cancer cells, and other HPV-integrated genes may play a part in cervical cancer pathogenesis and development.

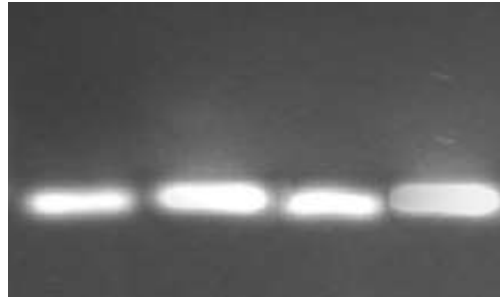
### 3.3. *REV3L* Expression on Normal and Cervical Cancer Cells

Quantitative analysis showed that *REV3L* expression was significantly upregulated in cervical cancer cells ( $p = 0.0165$ ) (Figure 6). Qualitative analysis showed that *REV3L* was expressed in both normal (Figure 2) and cervical cancer cells (Figure 7), suggesting that *REV3L* might be involved in physiological cellular processes on both cells.

*KLF5* expression may have an essential role in the cell proliferation process [17]. One of the genes that might ubiquitously play a role in cervical cell proliferation is *REV3L* [13]. *REV3L* expression might be various in every different type of human tissue. *REV3L* was upregulated in glioma cells [21], however, *REV3L* was downregulated in colon cancer [22], gastric, and lung cancer [23]. *REV3L* was expressed in both normal and cancerous cervical cells, indicating that *REV3L* might be involved in both normal and pathological events in cervical cells. *REV3L* was expressed in both normal and cervical cancer tissues, which indicate that *REV3L* plays an essential role in both normal and cervical cancer cell proliferation. *REV3L* was significantly upregulated in cervical cancer cells, which might indicate an enhancement of cell proliferation in cervical cancer tissue.



**Figure 6. Expression levels of *REV3L* in human normal (n=6) and cervical cancer (n=4) cells. Data are presented as mean±s.e.m. T-test was used to analyze the data. \*  $P<0.05$**

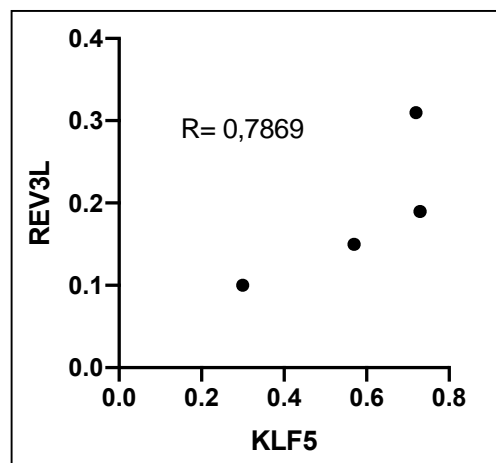


**Figure 7. Electrophoresis results of *REV3L* on cervical cancer cells**

DNA tends to be damaged in living cells. Although DNA repair processes are targeting the damaged DNA, some lesions persist and interfere with DNA replication. To overcome this problem, a process called “translesion DNA synthesis” uses an enzyme to supply the cell additional time to restore the damage before completing the DNA replication process. This enzyme is called DNA polymerase zeta and is encoded by *REV3L*. Altered expression of *REV3L* is believed to cause an error accumulation, enhancing the development of cancer [19].

### 3.5. Correlation between *KLF5* and *REV3L* on Cervical Cancer Cells

*KLF5* expression may have an essential role in the cell proliferation process [7]. One of the genes that might ubiquitously play a role in cervical cell proliferation is *REV3L* [8]. To test whether *KLF5* expression may have a vital role in cell proliferation, we conduct a correlation test between *KLF5* and *REV3L*. There was a strong correlation between *KLF5* expression and *REV3L* expression. ( $R=0.7869$ ) (Figure 8), suggesting *KLF5* has been linked to *REV3L* expression and cervical cancer cell proliferation.



**Figure 8. Scatter plots showing related gene expression levels of *KLF5* versus *REV3L* in cervical cancer cells (n=4). Data are presented as normalised gene expression to *GAPDH*. Pearson’s correlation coefficient ( $R$ ) test was used to analyze the data**

As expected, our results showed that the expression level of *KLF5* was strongly positively correlated with *REV3L*. The important role of *KLF5* in cell proliferation has been



extensively studied [7], [14], [18], [19]. *REV3L* encodes a DNA polymerase zeta, an enzyme that is involved in repairing the damaged DNA in DNA replication [8], [13]. Our study marks the importance of *KLF5* in the DNA replication process via its interaction with *REV3L*. Further study is needed to analyze the role and regulation of *KLF5* in the DNA proliferation of cervical cancer cells.

#### 4. Conclusion

*KLF5* and *REV3L* were expressed in normal and cancerous cervical cells, suggesting vital roles of those genes in physiological processes in both cell types. There was no significant difference in the expression of *KLF5* in cervical cancer cells compared to normal cells, suggesting that other HPV-integrated genes might play a role in cervical cancer pathogenesis. *REV3L* was significantly upregulated in a cervical cancer cell, suggesting that this gene might play a critical role in cervical cancer cell proliferation. We found that there was a strong positive correlation between *KLF5* expression and *REV3L* expression, suggesting *KLF5* might be linked to *REV3L* expression in cervical cancer cell proliferation.

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