Thesis progression

Thesis title: Investigation of circular RNA dysregulation mediated by human papillomavirus oncogenes and its functional role in cervical carcinogenesis

Thesis progression title: Validation of five downregulated circRNAs identified by RNA sequencing in normal cervical scrape samples and cervical cancer cell lines

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1. Background and rationale

Cervical cancer (CC) remains a major global health burden, with an estimated 662,301 new cases and 348,874 deaths worldwide in 2022 across both sexes and all age groups (J. Wu et al., 2025). In Thailand, CC ranked as the third most common cancer among women in 2023, with an estimated 9,158 new diagnoses and 4,705 deaths annually (ICO/IARC HPV Information Centre, 2023). Persistent infection with high-risk human papillomavirus (HR-HPV), particularly HPV16 and HPV18, is the primary etiological factor, with HPV16 accounting for ~55% of global CC cases (Graham, 2017). Typically, 90% of HPV infections can resolve spontaneously within two years due to the immune system response (Della Fera et al., 2021). However, persistent HR-HPV infection, together with cofactors such as long-term oral contraceptive use, smoking, immunosuppression, and multiple HPV infections, can drive the progression of precancerous lesions to invasive carcinoma, which generally takes over 10 years (Okunade, 2020). This long period provides a critical opportunity for prevention and therapeutic intervention before progression to cancer. Furthermore, complex interactions between viral oncoproteins and host cellular pathways, particularly those involving regulatory proteins and non-coding RNAs (ncRNA) also play a critical role in cervical carcinogenesis (Yadav et al., 2023). Despite extensive research on CC, substantial knowledge gaps remain regarding the prevalence and associated factors of cervical intraepithelial lesions, as well as the challenges involved in screening.

HPV is a double-stranded DNA virus without an envelope, typically spread through sexual contact, which infects basal epithelial cells and replicates within the nucleus (Mcmurray et al., 2000). Its genome is approximately 7–8 kilobase pairs in size and exhibits icosahedral capsid symmetry (Qian et al., 2018). The genome is organized into three major portions: a 1-

kb long control region (LCR) containing cis-elements essential for replication and transcription; a 4-kb early (E) region encoding nonstructural proteins E1, E2, E4, E5, E6, and E7; and a 3-kb late (L) region encoding the capsid proteins L1 and L2 (Basera et al., 2022; Münger et al., 2004). The main players in the multistep transformation process affecting the infected cervical cells are E6 and E7 proteins encoded by HR-HPVs act as viral oncoproteins (Della Fera et al., 2021). Mechanistically, E6 promotes degradation of p53, while E7 inhibits the retinoblastoma protein (pRb), disrupting apoptosis and cell cycle control and leading to uncontrolled cellular proliferation (Pal & Kundu, 2020). Moreover, E6/E7 expression from HR-HPV has been identified as a key factor that induces epigenetic changes, which have been shown to play a crucial role in CC development and progression (Durzynska et al., 2017). Therefore, understanding the distinct functions of E6 and E7 is essential in the context of CC.

Circular RNAs (circRNAs) are a class of noncoding RNAs generated from precursor mRNAs (pre-mRNAs) through a back-splicing process, in which a downstream 5′ donor site is joined to an upstream 3′ acceptor site (Qian et al., 2018). Unlike linear RNAs, circRNAs are resistant to exonuclease digestion due to their covalently closed loop structure (Lasda & Parker, 2014). They also play important roles in physiological processes, owing to their evolutionary conservation, abundance, cell- and tissue-specific expression, and remarkable stability (Misir et al., 2022; Okunade, 2020). Recent studies have demonstrated that circRNAs regulate tumor cell proliferation, survival, and metastasis by acting as either tumor suppressors or oncogenes (Huang et al., 2021). In addition, circRNAs are aberrantly expressed in various cancers, including CC, where they may serve as key regulators of cancer progression and patient survival (M. Wu et al., 2022). MicroRNAs (miRNAs), in contrast, are small noncoding RNAs that mediate RNA silencing and post-transcriptional regulation of gene expression (Smith et al., 2021). A well-known function of circRNAs is their ability to act as miRNA sponges, thereby regulating target gene expression by inhibiting miRNA activity (Shanmugapriya et al., 2018). CircRNA biogenesis is tightly regulated by trans-acting factors, with mechanisms that vary depending on the cellular and contextual environment (Li et al., 2018; Yang et al., 2022). Given their distinct expression patterns and functional roles in cancer progression, further research is needed to identify additional trans-acting factors involved in circRNA formation, as well as to clarify their functions in CC.

Currently, virus-derived circRNAs and the E7 oncoprotein have been shown to influence circRNA expression (Zheng et al., 2018). Abnormal expression of circRNAs is closely related to

the occurrence and development of CC (Luo et al., 2021). However, despite growing evidence highlighting their role, the precise functions of circRNAs in cancer progression and their regulatory association with the HPV16 E7 oncoprotein remain unclear. Understanding circRNA functions in HPV-related cervical carcinogenesis could provide valuable insights for developing prognostic biomarkers and therapeutic targets. Therefore, this study aims to identify and validate circRNAs associated with HPV16 E7 expression, investigate the functional roles of selected circRNAs in cell proliferation, migration, invasion, and apoptosis, and further elucidate the mechanisms by which their expression is regulated by trans-acting factors, as well as the processes through which HPV16 E7 influences these regulatory factors. The results of this study may provide the potential of circRNAs for guiding therapeutic interventions and improving clinical outcomes in CC.

2. Hypothesis and Objective

2.1 Hypothesis

HPV16 E7 alters circRNA expression by transcriptionally regulating trans-acting factors involved in back-splicing, thereby contributing to CC development.

2.2 Objectives

1. To identify circRNAs associated with HPV16 infection.

2. To determine the effects of the candidate circRNA on cell proliferation, migration, invasion, and apoptosis.

3. To identify of trans-acting factor that regulate the candidate circRNA.

4. To determine the regulation of trans-acting factors induced by HPV16 E7.

2.3 Conceptual framework

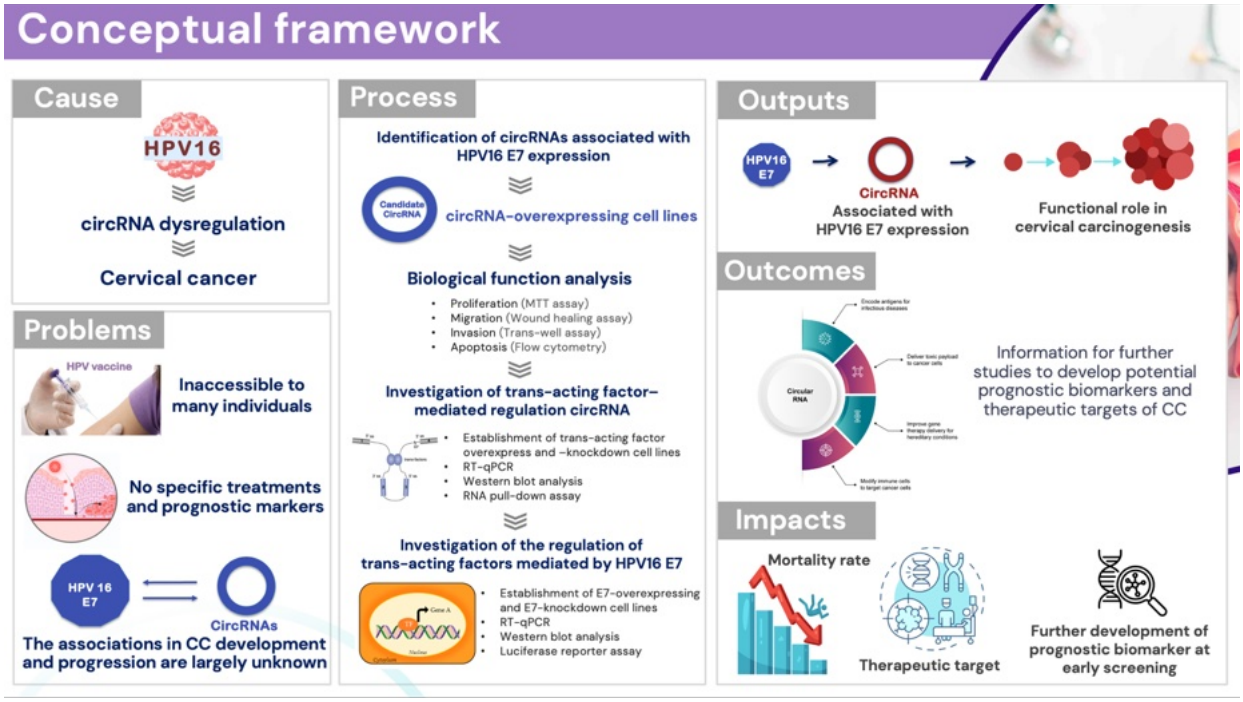

Figure 1 Conceptual framework

3. Study design

This study is divided into 4 parts.

Part I Identification of circRNAs associated with HPV16 expression.

Part II Investigation of candidate circRNA effects on cell proliferation, migration, invasion, and apoptosis.

Part III Investigation of trans-acting factor–mediated regulation of the candidate circRNA.

Part IV Investigation of the regulation of trans-acting factors mediated by HPV16 E7.

4. Materials and Methods

4.1 Clinical specimens

HPV-negative normal cervical scrape cells were left-over specimens from the previous project collected from the women undergoing cervical cancer screening at the Department of Obstetrics and Gynecology, Srinagarind Hospital, Khon Kaen University, Khon Kaen Province, Thailand (HE611591). All specimens were cytologically classified as no squamous intraepithelial lesion (noSIL; n = 10). Cervical scrape cells were preserved in 95% ethanol and stored at −80 °C until further processing.

4.2 Cervical cancer cell lines

Human CC cell lines, including SiHa (HPV16-positive), CaSki (HPV16-positive), HeLa (HPV18-positive), and C33A (HPV-negative), were used. All cell lines were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and antibiotics, including penicillin, streptomycin, gentamicin, and amphotericin B (fungizone). Cells were maintained in a humidified incubator at 37 °C with 5% CO_2.

4.3 Total RNA extraction

Cervical scrape cells in 95% ethanol or CC cell lines were pelletd by centrifugation at 300 ×g for 10 minutes at 4 °C. The supernatant was discarded, and the cell pellet was washed with sterile phosphate-buffered saline (PBS) using a volume equivalent to the removed supernatant. Total RNA was subsequently extracted using TRIzol™ reagent (Invitrogen) according to the manufacturer's instructions.

4.4 Divergent primer design

To specifically determine circRNA expression, divergent primers targeting the back-splice junction (BSJ) were designed to selectively amplify circRNAs without amplifying their corresponding linear transcripts. Briefly, CircBase IDs of the candidate circRNAs were used as input in the CircInteractome database to obtain the BSJ junction sequence templates. These templates represent the ligation of the 3′ downstream sequence to the 5′ upstream sequence, which is unique to circRNAs. Divergent primers spanning the BSJ were subsequently designed using Primer-BLAST. Primer specificity was further confirmed using circPrimer 2.0 to ensure exclusive detection of circRNAs. The primers used for amplification are listed in **Table 1.**

Table 1. Divergent designed for circRNAs detection.

CircRNAs	Primers (5′ → 3′)	Length (nt)	Product size (bp)
1. hsa_circ_0024604	Forward; AAGCAGAAAGTTGAGCGCAT	20	101
	Reverse; TGGTGACTCTCGGTTCAAAATAC	23	
2. hsa_circ_0001897	Forward; GCTGGCCTTGGGAGGTTATT	20	140

CircRNAs	Primers (5′ → 3′)	Length (nt)	Product size (bp)
	Reverse; GGCCCACTGTCATCCAAGAA	20	
3. hsa_circ_0027089	Forward; CAACATGGGTGGTGATGAGGA	21	109
	Reverse; TGAATCATCTTCCCAGTCTTTCCA	24	
4. hsa_circ_0071099	Forward; TGCATAGATGCTTCCTTACGTG	22	148
	Reverse; TCTGTCACAGCATCACCGAT	20	
5. hsa_circ_0001824	Forward; CCTGCTAAAGAAAAGTGACGGG	22	100
	Reverse; TGAAAGAAATGTGGCATGTGAGA	23	

4.3 Reverse transcription quantitative PCR (RT-qPCR)

Total RNA was reverse transcribed into complementary DNA (cDNA) using the PrimeScript™ RT Reagent Kit (Takara Bio Inc., Kusatsu, Japan) with random hexamer primers. SsoAdvanced™ Universal SYBR Green Supermix (Bio-Rad Inc., Hercules, CA) was used for the qPCR reaction. The expression of circRNA was determined by using the threshold cycle (Ct) method, and relative expression levels were calculated via the $2^{-\Delta CT}$ method. Amplification was conducted on the Applied Biosystems ABI/QuantStudio 6 system (Applied Biosystems, Waltham, USA). GAPDH was used as the internal reference for normalization.

5. Result

Validation of circRNA expression in cervical scrape cells and CC cell lines

Based on our prior *in silico* analysis, the top five downregulated circRNAs were identified by RNA sequencing data as being significantly decreased in HPV16-positive normal tissues, HPV16-positive HSIL tissues, and HPV16-positive CC tissues compared with HR-HPV–

negative normal cervical tissues. These circRNAs may be regulated by trans-acting factors through HPV16-mediated transcriptional regulation. Therefore, five downregulated circRNAs—hsa_circ_0024604, hsa_circ_0001897, hsa_circ_0027089, hsa_circ_0071099, and hsa_circ_0001824—were selected for further investigation of their expression profiles in cervical scrape cells and CC cell lines.

The expression levels of these circRNAs were examined by RT-qPCR in HPV-negative normal cervical cells and CC cell lines, as shown in **Figures 2A–2E**. The results demonstrated that hsa_circ_0024604 (Figures 2A) was significantly downregulated in CaSki, SiHa, HeLa, and C33A cells. In addition, hsa_circ_0001897 (**Figure 2B**) and hsa_circ_0027089 (**Figure 2C**) were significantly downregulated in CaSki, SiHa, and HeLa cells, but showed no statistically significant change in the HPV-negative C33A cells. In contrast, hsa_circ_0071099 (**Figures 2D**) did not exhibit a statistically significant difference in CaSki cells, whereas hsa_circ_0001824 (**Figure 2E**) was slightly upregulated in SiHa, HeLa, and C33A cells and was significantly upregulated in CaSki cells.

Based on the study objective of identifying circRNAs associated with HPV infection, hsa_circ_0001897 and hsa_circ_0027089 were selected for further analysis due to their consistent downregulation in HPV-positive CC cell lines.

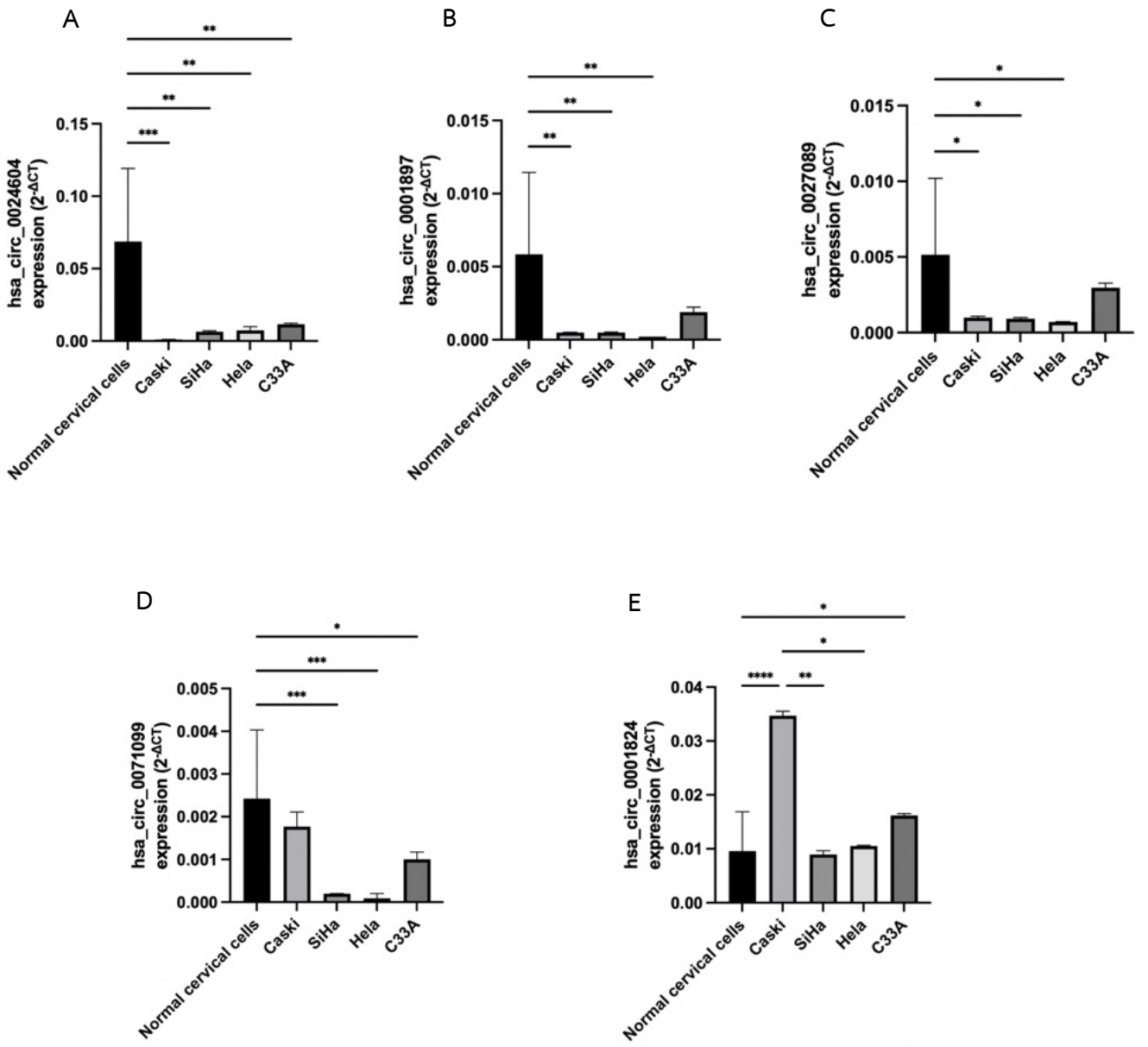

Figure 2. Relative expression levels of the top five circRNAs. The expression of (A) hsa_circ_0024604, (B) hsa_circ_0001897, (C) hsa_circ_0027089, (D) hsa_circ_0071099, and (E) hsa_circ_0001824 was determined in normal cervical cells and compared with CC cell lines (CaSki, SiHa, HeLa, and C33A) using RT-qPCR. Gene expression was normalized to GAPDH and calculated via the $2^{-\Delta CT}$ method. *:p<0.01, **:p < 0.001, ***:p < 0.0001.

6. Conclusion

Among the top five candidate circRNAs identified through *in silico* analysis, hsa_circ_0001897 and hsa_circ_0027089 were significantly downregulated in HPV-positive CC cell lines, but not in the HPV-negative C33A cells. These findings suggest that the expression of these circRNAs may be associated with HPV-related regulatory mechanisms, supporting their selection for further investigation.

7. Thesis plan

Activities	Timeline											
	2025		2026				2027				2028	
	Q3	Q4	Q1	Q2	Q3	Q4	Q1	Q2	Q3	Q4	Q1	Q2
1. Literature review and planning												
2. Qualifying Examination												
3. Proposal examination												
Part I Identification of circRNAs associated with HPV16 expression												
4. Assessment of HPV16-associated circRNA expression in HPV16 (+) CC cell lines and HPV16 (-) cervical scrape samples using RT-qPCR												
5. Confirmation of candidate circRNA sequences using Sanger sequencing												
6. Investigation of candidate circRNA localization using fluorescent *in situ* hybridization (FISH) in CC cell lines												
Part II Investigation of candidate circRNA effects on cell proliferation, migration, invasion, and apoptosis												
7. Construction of circRNA-overexpressing cell lines												
8. Investigation of circRNA-overexpressing cell lines compare to MOCK cells												
Part III Investigation of trans-acting factor–mediated regulation of the candidate circRNA												
9. Selection of potential circRNA trans-acting factors using *in silico* analysis												
10. Investigation of the expression levels of potential trans-acting factors in HPV16 (+) CC cell lines compare to HPV16 (-) CC cell lines using RT-qPCR and western blot analysis												
11. Construction of trans-acting factor–overexpressing cell lines and design of siRNAs for knockdown												
12. Determination of the effects of trans-acting factor overexpression or knockdown on circRNA expression levels using RT-qPCR												

Activities	Timeline											
	2025		2026				2027				2028	
	Q3	Q4	Q1	Q2	Q3	Q4	Q1	Q2	Q3	Q4	Q1	Q2
13. Assessment of the interaction between circRNA and trans-acting factors using an RNA pull-down assay												
Part IV Investigation of the regulation of trans-acting factors mediated by HPV16 E7												
14. Analysis of potential transcription factor regulating the trans-acting factor using the JASPAR database												
15. Analysis of transcription factor expression in HPV16 (+) and HPV16 (-) CC cell lines by RT-qPCR												
16. Establishment of E7-overexpressing and E7-knockdown cell lines												
17. Determination of transcription factor and trans-acting factor expression in E7-overexpressing and knockdown cell lines using RT-qPCR and western blot analysis												
18. Analysis of interactions between transcription factor and the promoter of the trans-acting factor using a luciferase reporter assay												
17. Manuscript preparation												
18. Thesis defense												

Finish

Ongoing

Furter work

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