THE EXPRESSION OF HPV16/18 E6 AND E7 ONCOPROTEINS IN CERVICAL LESIONS AND THE DEVELOPMENT OF APTAMER TARGETING HPV16 E6 ONCOPROTEIN.

DR NADIAH BT AHMAD SABRI

DISSERTATION SUBMITTED IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF PATHOLOGY

(ANATOMIC PATHOLOGY)

UNIVERSITI SAINS MALAYSIA

2022

ACKNOWLEDGEMENT

Praises to Allah that all knowledge belongs to Him.

Alhamdulillah, for the opportunity to be humbled in this journey of learning.

To those who I cherish.

Husband, Dr Abdul Rahman Nik Mazian.

Daughters, Iffah Layyinah, Aya Sofia and Jannah Aliyah.

And to those who I acknowledge and appreciate wholeheartedly.

Supervisor, Assoc. Prof Anani Aila Mat Zin.

Co supervisor, Dr Shazana Hilda Shamsuddin.

Medical lab technologist, Puan Ummi Atikah Ayub.

Thank you for the endless support and guidance.

May all the knowledge shared be an aamal jariah and benefited others.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATION

AC	: Adenocarcinoma
AIS	: Adenocarcinoma in situ
ASC	: Adenosquamous carcinoma
CIN	: Cervical intraepithelial neoplasia.
DNA	: Deoxyribonucleic acid
FFPE	: Formalin fixed paraffin embedded
HPV	: Human Papilloma Virus
HR-HPV	: High Risk type Human Papilloma Virus
HSIL	: High grade squamous intraepithelial lesion.
LSIL	: Low grade squamous intraepithelial lesion.
PCR	: Polymerase chain reaction
RNA	: Ribonucleic acid
SCC	: Squamous cell carcinoma
SELEX	: Systematic evolution of ligands by exponential enrichment

ABSTRAK

Latar Belakang: Virus papilloma manusia (HPV) berisiko tinggi terutamanya HPV16 dan HPV18 telah menjadi agen penyebab utama barah serviks dimana patogeniknya adalah disebabkan oleh ekspresi onkoprotein virus E6 dan E7 berlebihan. Kajian ini bertujuan untuk menilai ekspresi onkoprotein HPV dalam tisu terbenam parafin diawet formalin (FFPE) dalam gred tisu serviks yang berbeza menggunakan antibodi dan juga aptamer yang direka bentuk dalam siliko.

Kaedah: Sebanyak 102 tisu serviks FFPE berbeza gred telah dikumpul dari Hospital Universiti Sains Malaysia (HUSM) dan Hospital Raja Perempuan Zainab II (HRPZII) dari tahun 2014 hingga 2022. Ekspresi onkoprotein E6 dan E7 HPV16 dan HPV18 adalah dinilai menggunakan kaedah pewarnaan imunohistokimia. DNA-Aptamer baru yang menyasarkan HPV16-E6 onkoprotein direka bentuk dalam siliko dan dicirikan sebagai bioreseptor alternatif dalam aplikasi pewarnaan histokimia.

Keputusan: Perkaitan yang signifikan (p=0.001) ekspresi HPV18-E7 dengan penggredan histologi tisu serviks keseluruhan telah dicatatkan. Sementara itu, ekspresi HPV16-E7 dikaitkan dengan ketara (p=0.001) dengan penggredan histologi tisu serviks positif DNA HPV16. Aptahistokimia menghasilkan ciri pewarnaan khusus nuclear dengan pengurangan ketara pewarnaan tidak spesifik latar belakang berbanding immunohistokimia.

Kesimpulan: Ekspresi imunohistokimia onkoprotein E6 dan E7 HPV16 dan HPV18 telah terbukti sebagai alat diagnostik alternatif yang berkuasa dan boleh digunakan di makmal rendah sumber berbanding ujian molekul standard emas. Sementara itu, aplikasi aptamer dalam histokimia menunjukkan potensi menjanjikan serta spesifikasi yang lebih tinggi berbanding histokimia berasaskan antibodi.

Kata kunci: HPV16, HPV18, E6, E7, imunohistokimia, aptahistokimia.

ABSTRACT

Background: High risk human papilloma virus (HPV) particularly HPV16 and HPV18 had been the hallmark causative agent of cervical malignancy, in which overexpression of oncoprotein E6 and E7 induced the pathogenicity. The present study focused on evaluation HPV oncoproteins expression in formalin fixed paraffin embedded (FFPE) tissue in different grade of cervical lesions utilizing antibodies and in-silico designed DNA-Aptamer, as detection probe.

Methods: A total of 102 FFPE cervical tissues of different grades were collected from Hospital Universiti Sains Malaysia (HUSM) and Hospital Raja Perempuan Zainab II (HRPZII) from the year of 2014 until 2022. Immunohistochemical analysis of E6 and E7 oncoproteins of HPV16 and HPV18 were evaluated. Novel DNA-Aptamer targeting HPV16-E6 oncoprotein were in-silico designed and characterized as alternative bioreceptor using histochemical staining application.

Results: Increased expression of HPV18-E7 was seen with the progression of cervical lesions from the low-grade precursor to malignancy. Meanwhile, the HPV16-E7 expression was significantly associated with histological grade of HPV16-DNA confirmed positive cervical lesion ($p=0.001$). Aptahistochemistry using DNA-Aptamer targeting HPV16-E6 resulted in nuclear-specific staining in malignant cases with negligible non-specific background staining compared to the antibody-based immunohistochemistry.

Conclusion: Immunohistochemical expression of HPV oncoproteins is a powerful alternative diagnostic tool, applicable in low-resource laboratory setting compared to the gold standard molecular testing. Meanwhile the DNA-Aptamer showed promising

potential in the histochemical application with superior specificity than the immunohistochemical detection.

Keywords: HPV16, HPV18, E6, E7, immunohistochemistry, aptahistochemistry.

CHAPTER 1: INTRODUCTION

1.1 Epidemiology of cervical cancer.

Cervical cancer is one of the most prevalent cancers in women worldwide with significant public health issue. It primarily affects underdeveloped nations, where it can cause great emotional and financial distress. This condition is often known as "silent" cancer because most patients have no symptoms, however discovered by chance during a cervical biopsy for pre-invasive lesion (1).

With a projected 341,831 deaths worldwide from cervical cancer in 2020 alone, it is the fourth most frequent cancer in women after breast, colorectum & lung (2). In Malaysia, cervical cancer is the third most frequent cancer in women, and fifth-leading cause of cancer-related death with estimation of 991 cervical cancer deaths occur annually (3). National Cancer Registry 2012-2016 (4) reported highest incidence of cervical cancer at the age of 50-65 years. Between women aged 15 to 44 years, cervical cancer ranked second most common female cancer.

However, over the course of five years, the incidence rate dropped from 7.6 to 6.2 per 100,000 people. More than half of the cervical cancer cases (59%) are fortunately detected at an earlier stage (I and II) (4). This could be contributed by effective screening and early intervention programme that had been done since the 1969. In Kelantan, specifically, the age standardized incidence rate of cervical cancer is 4.7 per 100,000 populations. The slightly lower than the national incidence rate could be due to stricter religious practise or underreporting of cases when some of the people prefer seeking treatment from ‘traditional’ doctors rather than going to the hospital.

1.2 Overview of cervical cancer and it's precursor.

Cervix is located at the base of the uterus where squamous epithelium lines the ectocervix, and columnar epithelium lines the endocervical canal. The region of the squamocolumnar junction, or also called as transformation zone is where the metaplastic event occur and is the origin of nearly all cases of cervical carcinoma (5).

The progression spectrum of cervical carcinoma occurs from low grade squamous intra‑epithelial lesion (LSIL) then to high grade squamous intra‑epithelial lesion (HSIL) to invasive carcinoma and finally to metastatic cancer(6). Approximately 12% of LSIL cases will develop to HSIL, in which if the HSILs are left untreated, some of these lesions may develop into invasive carcinoma (7).

Previously, cervical intraepithelial lesions were categorised using the three-tiered classification, namely CIN 1, CIN 2 and CIN 3 (8). To improve diagnostic reproducibility and unify the terminology for HPV-related squamous lesions of all lower anogenital tract sites, a multidisciplinary consensus effort was launched in 2012, known as the Lower Anogenital Squamous Terminology (LAST) project. The adoption of a two-tiered nomenclature for HPV-related lesions which are low-grade squamous intraepithelial lesion (LSIL) and high-grade squamous intraepithelial lesion (HSIL) was one of the consensus guidelines that emerged as a result (8).

The Bethesda system utilized this dichotomous term for cytological interpretation, and later was adopted in histological reporting. Previous histologic classification of CIN 1 is equivalent to categorization of LSIL, wherelse CIN 2 and CIN 3 is now equivalent to categorization of HSIL (8).

LSIL appear as dysplasia in the lower third of the epithelium with presence of koilocytes which are cytopathic effect of HPV. Koilocytes are squamous epithelial cells exhibiting

large, coarse, hyperchromatic nuclei, with nuclear membrane irregularity known as "raisinoid" nuclei, as well as demarcated cytoplasmic halos. Meanwhile an abnormal parabasal-like cell proliferation with loss of polarity, overlapping nuclei, a high nuclear-to-cytoplasmic ratio, increase in mitoses and significant nuclear atypia, are all characteristics of HSILs, a type of precancerous lesion. CIN 2; where the dysplasia involving the lowest two thirds of the epithelium and CIN 3; in which the dysplasia extending to the upper third of the epithelium, are both included in HSIL (7).

Prior to 1960, squamous cell carcinoma (SCC) accounted for more than 90% of overall primary cervical malignancies. Squamous cell carcinoma can be further described according to histological pattern, namely non keratinizing SCC, keratinizing SCC and basaloid SCC. Nevertheless, the incidence of cervical SCC has gradually declined, in large part due to the efficient cytologic diagnosis and subsequent eradication of its precursors. Contrarily, there has been a significant rise in the age-adjusted incidence of cervical adenocarcinoma (7).

The current WHO Classification of Female Genital Tumours, 5th edition recommends the subdivision of cervical carcinoma into HPV-associated or HPV-independent, to be documented in pathology reports. This apply to the two major types of cervical carcinoma, which are SCC and adenocarcinoma. Distinguishing HPV association in cervical carcinoma require p16 immunostaining as a surrogate marker, or HPV molecular testing (9).

As of notes, there are also many other malignancies apart from SCC and adenocarcinoma that may arise from cervix, such as carcinosarcoma, adenosquamous carcinoma, mucoepidermoid carcinoma, adenosarcoma and neuroendocrine carcinoma.

1.3 Prevalence of HPV in cervical precursor lesions and cancer

A milestone paper by Walboomers et al. (10) concluded that worldwide human papillomavirus prevalence in cervical carcinomas was 99.7% and underestimation of HPV prevalence in cervical cancer is most likely due to the limitations of study methodologies. It was strikingly proven that almost all occurrences of cervical SCC and a significant part of endocervical adenocarcinoma are caused by HPV infection (11).

A collective data over 9 years from 4 major referral hospitals in Malaysia revealed HPV was detected in 92.5% of overall cervical cancer cases, specifically, 95.9% of squamous cell carcinomas and 84.3% of adenocarcinomas (12). Another local study found that 83.2% of women with cervical cancer and 0% of cancer-free women tested positive for HPV infection (13).

Human papillomavirus is a small non-enveloped circular double stranded DNA virus, measuring 50–55 nm in diameter from the family of Papillomaviridae (14). The viruses mostly affect epithelial cells in the skin or mucous membranes and commonly transmitted through sexual contact.

Regardless of age group, more than 90% of HPV infections typically resolved after 6 to 18 months. Such transitory infections will disappear in non-immunocompromised patients as a result of the host immune system (6). However, the development of CIN requires a chronic persistent infection. The HPV16 strain, for example, persists longer than other high-risk strains. There is a larger probability of HPV DNA integrating into the host genome in those cases of persistent infections.

Up to date, more than 100 types of HPV are identified, which can be generally divided to low risk and high-risk HPV. At least 14 are known to be oncogenic or high-risk HPV, found to be strongly linked to the development and spread of cervical cancer (15).

Worldwide, the most common high risk HPV types in cervical cancer were types 16 (57%), 18 (16%), 58 (5%), 33 (5%), 45 (5%), 31 (4%), 52 (3%), and 35 (2%) (16). This is concurrent with Malaysian prevalence data, which found out that in cervical squamous cell carcinoma, HPV 16 was the commonest (76.6%) genotype detected, followed by HPV 18 (35.0%). In contrast, HPV 18 (51.8%) was the commonest genotype detected in cervical adenocarcinoma followed by HPV 16 (48.2%). Overall, HPV 16 and HPV 18 genotypes were the commonest, detected in almost 70% of total cervical cancer cases. It is also revealed that multiple HPV infections were more prevalent (55.7%) than single HPV infections (36.8%) (12).

According to WHO, ideally p16 testing or HPV molecular typing is recommended for the diagnosis of HPV associated cervical cancer. HPV genotyping analysis is currently utilized in clinical practise as part of cervical cancer screening and prevention. When compared to cytology, using molecular HPV testing may offer the benefit of lowering the number of screening episodes from every 3 to every 5 years (17). Consequently, the financial burden of routine cytological screening tests is reduced. However, this test might not be accessible in healthcare facilities with limited resources. HPV molecular genotyping also is unable to determine if the patient has a temporary or chronic persistent HPV infection. The fact that the test couldn't specify if an infection was temporary or permanent, limits the accuracy of its predictions. Specific viral oncoproteins such as E6 and E7 may therefore be a more accurate diagnostic marker as they are consistently expressed in HPV-transformed cervical cells (12).

1.4 E6 and E7, the pivotal oncoproteins in cervical carcinogenesis.

The HPV viral genome has six early genes comprised of E1, E2, E4, E5, E6, and E7, and two late genes that are L1 and L2, encoding capsid proteins (14)

Infections started when the virus enter through an abrasion or exposed small wound within the cervical epithelium. Upon reaching the basement layer, the viral capsid protein, particularly HPV L1 and L2 internalized themselves into the mitotically active basal epithelial cells. This leads to production of circular episome which is situated extrachromosomal, harbouring the viral genome. During this early latent infection, only minimal copies of episome are maintained per nucleus, with very restricted ongoing cycles of replication (19).

HPV genome can either get integrated with the host genome or stay in an episomal form. In the absence of viral integration, the normal viral lifecycle produces morphologic changes characteristic of low-grade squamous intraepithelial lesion (LSIL). The infected basal cells will undergo maturation to superficial cells. There will be formation of koilocytotic cells (cells with an enlarged and irregular nucleus) which is associated with the presence and accumulation of viral E4 proteins. Koilocytosis contribute to viral dissemination in the upper layers of the epithelia due to keratinocyte fragility (19). Desquamation causes the viral virions to be released, infecting adjacent neighbouring cells.

To promote cervical cancer abnormalities, the virus must become integrated into the host genomic DNA and no longer stay in an episomal form. This event, which is essential for cancer progression, appears to be rare. Upon integration, the circular HPV genome is linearized, disrupting the E2 gene and resulting in its inactivation. E2 function was to

repress the viral E6 and E7 oncogenes promoter, but inactivation of E2 will lead to increased expression and stability of transcripts encoding the E6 and E7 proteins (19).

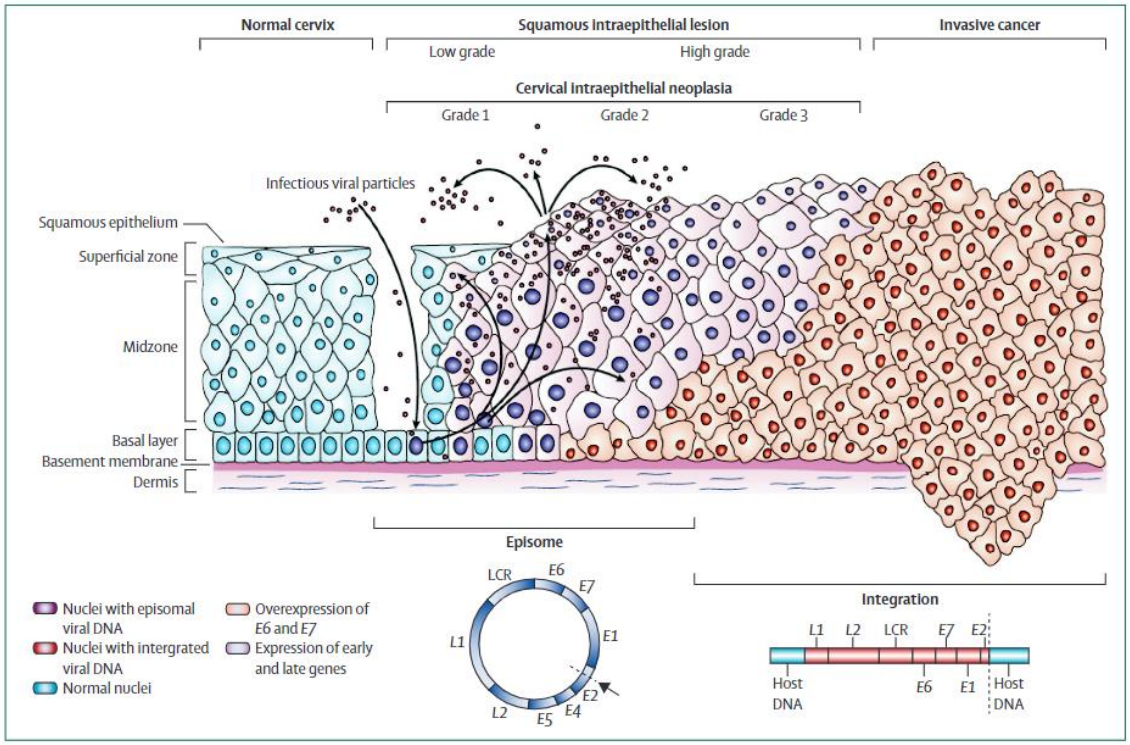


Image adapted from Crosbie et al. (6) depicting chronological event of HPV infection leading to cervical intraepithelial neoplasia and ultimately invasive carcinoma. Microwound and mild abrasions in the cervical epithelium are hypothesised to be the route through which the HPV enters and infected the basal cells. In the early infection phase, the viral DNA stay in episomal forms (purple nuclei). The expression of the E1 and E2 proteins is associated with early viral gene replication. As the cells differentiate and mature, they exhibit characteristic features of koilocytes with transcription of L1 and L2 as well as E4 within the superficial epithelial layers. Later, the L1 and L2 encapsidate the viral genomes, creating progeny virions in the nucleus. The shedding of these virions caused a new infectious life cycle. Persistent chronic infection with high-risk HPV increases the likability of viral DNA integration into the nucleus of host cells. In the untreated lesion, the integration of the HPV genome into the host chromosomes (red nuclei) will lead to disruption of E2, and consequent elevation of E6 and E7 oncogene expression, which are linked to the development of microinvasive and invasive malignancy.

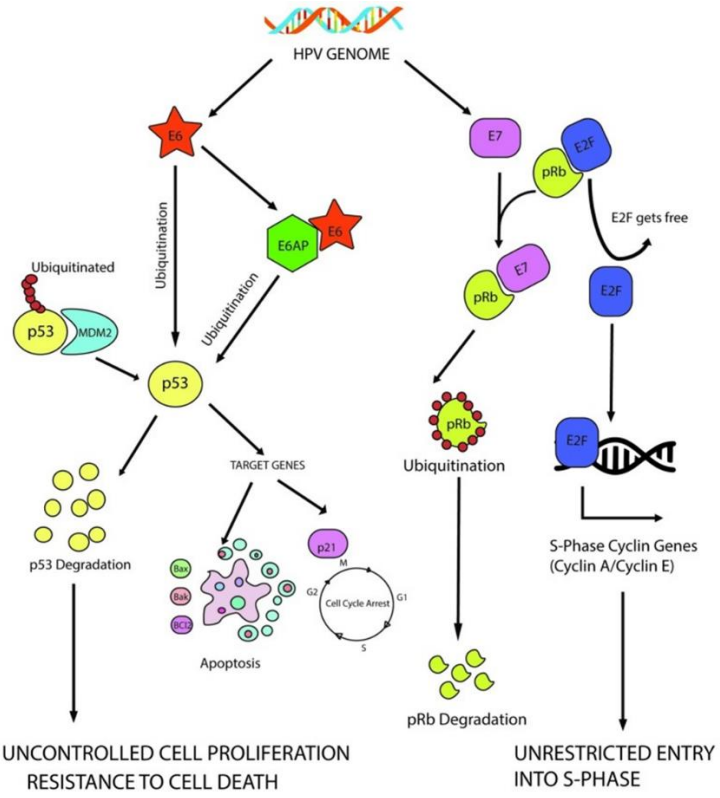
The 53 kD molecular weight protein, p53, is the most thoroughly studied tumour suppressor protein to date and is frequently referred to as the "guardian of the genome" since it controls a cell's course under stress. p53 acts as a transcription factor for either cell cycle arrest or apoptosis whenever a cell undergoes stress, in the form of oxidative damage or another kind. However, when p53 is inhibited by E6, a number of cellular changes can occur that cause a cell to become oncogenic, one of which is the development

of unchecked cell proliferation (6). It was discovered that E6 degrades p53 by ubiquitination with the aid of E6AP (E6-associated protein also known as UBE3A) (14).

A complex consist of trimeric E6/E6AP/p53 is formed when HPV E6 bind to E6AP at the region of LxxLL sequence within the conserved domain, which lead to the degradation of p53 (20). This will cause the cells to be able to bypass preventive checkpoints, resulting to uncontrolled cellular division. Therefore, interaction and disruption of p53 by E6 is an important step in maintaining constant cellular growth.

Likewise, unchecked cells proliferation is also contributed by E7-mediated inhibition of retinoblastoma protein (pRb) (6). Normally, pRb–E2F interaction will be the cell's checkpoint for transition through G1-S phase. In bounded condition of pRb protein with E2F transcription factor, no gene will be transcribed to allow cells to enter S phase. However, the presence of E7 which bind to pRb protein will cause disruption to this cell cycle. The E2F transcription factor will be free and released from pRb protein, causing transcription of cyclin E, cyclin A and p16, thus forcing premature S-phase entry of the cells. In time, there will be p16 upregulation and cyclin-dependent kinase inhibitor (tumor supressor) inactivation. Apart from causing pRb degradation, HPV E7 increase p16 expression by epigenetic derepression through KDM6B (H3K27- specific demethylase 6B) (14).

All in all, E6 and E7 co-expression in HPV infected cells establishes an optimal environment for sustained proliferative signalling. E7-mediated pRb disintegration will lead to abnormal cell growth. This could be sustained by p53, the "guardian of the genome". However, co-existence of E6 hinder the sustainment by p53, causing evasion of all anti-tumorigenic checkpoints to allow the cells through uncontrollable cell division (14).



Diagrammatic illustration adapted from Pal et al. (14) on biomechanism of E6 and E7 oncoproteins causing deregulation of tumour suppressor genes. Unrestricted cell proliferation and resistance to apoptosis by neoplastic cells is attained through E6 targeting p53 and E7 binding to pRb.

Evasion of apoptosis by E6 in HPV infected cancer cells can happen through both, p53-dependent and also p53-independent pathway. Direct interaction of E6 with BAK can lead to in vivo degradation of BAK. Apart from that, intrinsic apoptosis mediated by BAK can also be blocked by E6 via p53-E6AP interaction. Meanwhile, extrinsic mode of apoptosis mediated by TNF is also hindered through binding of PDZ domain of E6 to TNFR1 causing prevention of TRADD interaction with it. HPV infected cervical cancer cells also often have altered c-myc expression. C-myc is another oncogene which is deregulated by HPV E6 and E7 causing disruption to cyclins, Cdks and E2F transcription factors (14).

1.5 E6 and E7 biomolecular structure.

The crucial function of E6 and E7 viral oncoproteins is to promote oncogenesis in HPV infected cells. They can cause all the hallmarks of a cancer cell during the viral genome replication process, including unchecked cellular proliferation, angiogenesis, invasion, metastasis, unrestrained telomerase activity, evasion of apoptosis, and growth suppressor activity (14).

Among all known HPV oncogenes, E7 was the earliest oncogene to be discovered. It is a very small protein (17 kDa) composed of about 100 amino acids, with three conserved regions namely CR1, CR2, CR3. Part of CR1 and almost whole CR2 from the amino terminal have similar sequence and function with Adenovirus E1A and Simian Virus 40 (SV40) protein (14). The high-affinity binding of E7 to tumour suppressor Rb is mediated by LXCXE motif, which is strictly conserved region encoded in CR2 (21).

Meanwhile, E6 is slightly larger protein, of 18kDa size with 150–160 amino acids. The high-risk HPV E6 proteins possess a short sequence of amino acids called PDZ binding motif (PBM) at the extreme carboxy terminus which can bound to PDZ domain containing substrates. This PDZ binding motif (PBM) can be considered as potential oncogenic marker as it is not found in most of low-risk viruses. The oncogenicity of the protein is thought to be due to the structural arrangement of two zinc finger binding domains by four Cys-X-X-Cys motifs. The PDZ-binding motif found in carboxy terminal domain is the site of interaction with many proteins (14).

Of all protein interactions that these oncoprotein have, p53 degradation by E6 and pRb inhibition by E7 is the most significant in cervical cancer pathogenesis (14).

1.6 Expression of E6 and E7 oncoproteins through immunohistochemical staining.

Effective screening coverage with prompt high-quality follow up and treatment is deemed to have improve the overall achievement in cervical cancer prevention (22). In many countries, cytology-based screening has shown to significantly lower cervical cancer incidence rates. However, the relatively limited sensitivity seen with a single Pap smear typically leads in higher false-negative findings and the need for multiple Pap tests. Nevertheless, when both cytological and viral-typing tests are used in combination, the sensitivity of screening test heightened (23). Contrary to cytology, which necessitates subjective visual identification, HPV tests are biochemical and easily standardised. Therefore, improved techniques for better risk categorization of HPV-positive women are desperately needed as cervical cancer prevention programmes increasingly include HPV testing.

Currently, molecular assays have been the mainstream to detect high risk HPVs in cervical samples. The absence of high-risk HPV detection is indicative of a low incidence risk of cervical intraepithelial neoplasia grade 3 (CIN3) or cancer (24). Overall, molecular based HPV testing, has the advantage of high sensitivity with high negative predictive value(25). Negativity of high-risk HPV means longer protection of up to 5 years and lower risk of developing high grade cervical lesion and subsequent carcinoma (26). However, there is low specificity of the HPV assays to discriminate between transient and persistent HPV infections, which the latter have a higher risk for carcinoma transformation.

Most of validated methodology to identify high risk HPV is by PCR-based genotyping approach using known primers, primarily utilizing the highly conserved L1 gene. This biomarker for the detection of HPV is fairly sensitive (27). However, high-grade CIN is

developed as a result of persistent overexpression of E6 and E7 oncoproteins. The tumour suppressor proteins p53 and Rb are particularly inactivated by these early genes E6 and E7, which promote carcinogenesis. L1 expression may diminish through the process of viral integration, but E6 and E7 expression persist (28). Therefore, current HPV DNA tests when used alone, has lack of specificity for high grade cervical lesion and malignancy precursor identification.

It has been determined that the overexpression of HPV E6 and E7 mRNAs serves as a marker for the progression from a temporary infection to a sinister infection that eventually led to cellular transformation. For cervical screening, E6 or E7 mRNA quantification has a lot of potential, especially in young women whose HPV infection is frequent but whose transformation and conversion to CIN2+ are rather uncommon (29). Because the mRNA assay is conducted using a PCR or an in-situ hybridization format, it is unfortunate that this test will be ineffective in a resource-constrained laboratory without access to molecular testing facilities.

A good alternative for E6 and E7 viral mRNA biomarkers is their oncoprotein expression in the cervical lesions. Immunohistochemistry studies have the benefit of reduced costs and simpler operations, hence deem more suitable in basic laboratory studies with limited facilities and resources. In current practise, the immunohistochemical identification of p16 overexpression is employed clinically as a substitute biomarker of HPV-associated carcinoma. Because there is no direct molecular link between HPV DNA integration and p16 expression, it occasionally resulted in discrepancies between p16 IHC and HPV DNA test results (30). It was later discovered that immunohistochemistry study can demonstratively show the presence of E6 and E7 oncoproteins. A study concluded that the expression of HPV16 E7 protein and the severity of the cancer correlated concordantly (18).

One study demonstrated the potential of a new monoclonal anti-E7 HPV 16 antibody distinguishing low-grade CIN and high-grade CIN by nuclear staining within the basal, parabasal, and superficial layers (31). A study on CIN lesion and cervical cancer shows positive rate of HPV16 E7 protein immunohistochemical stain increases, as pathogenic stage increased (11). Other studies found that E7-HPV 16 and 18 expression was more prevalent when the histopathological grade was higher (32). The detection of E7 protein expression may effectively distinguish malignant transformations from transient HPV infections. Additionally, E7 protein identification technique may be used in either immunocytochemical or immunohistochemical analyses of cervical samples and provide more reliable results for improved efficiency in cervical cancer screening and early diagnosis (33).

These results demonstrate the potential of E6 and E7 of HPV 16 and 18 protein biomarkers as adjunct diagnostic tool and for use in screening procedures alongside cytological tests. Since the majority of pathology laboratories should have access to the immunohistochemical staining technique, this can be a good alternative test when molecular testing is not practical. It is also crucial to highlight that, despite the fact that HPV molecular testing has been available in Malaysia since 2019, only selected healthcare centres have access to it, with majority of others continue to use the cytological-based screening strategy (17).

1.7 The potential of Aptamer as alternative synthetic bioreceptor in cervical cancer detection.

Immunohistochemical staining method is one of the commonly used techniques for biomarkers detection. It utilises antibody as the gold standard probe, which is commercially available and clinically approved. However, antibodies do inherent several limitations. They are genetically and chemically difficult to modify due to their intricate architecture. Their huge molecular size limit tissue penetrations when incorporating them on biomaterials. Additionally, antibodies are expensive and labour-intensive during mass manufacture. They are also heat labile, may harbour batch to batch variation, cross-reactivity and have limited shelf life (34).

On the other hand, aptamer is an emerging oligonucleotide based bioreceptor. It is a synthetic probe with promising potential in clinical application. Diagnostics, biosensing, bioimaging, drug delivery and therapies, environmental monitoring, and analytics are just a few of the many uses for nucleic acid aptamers. It is currently making ways as the next generation affinity molecules for immunoassays and related diagnostic point-of-care (POC) tests (35).

RNA or DNA aptamers are small, approximately 15–100 nucleotides base, fold into three-dimensional (3D) structures with specific binding affinity to targets of interest. They can bind to target proteins in a conformation-dependent manner, almost similar to antibodies, and can be conjugated with additional function alteration properties (36).

Aptamers can be synthesis chemically in large quantities, without the need of animal as with conventional antibodies production protocol (37). Aptamers are the preferred molecule for various diagnostic procedures because of their non-immunogenic nature, sturdy, easier conjugation property, speedy and less expensive manufacturing (38).

Conventionally, a selection procedure known as SELEX (Systematic Evolution of Ligands by Exponential enrichment) is utilized to isolate the aptamers. The potential sequence of aptamer is selected after several rounds of amplification and ligands evolution or enrichment, which is a tedious, expensive, and time-consuming procedure (35).

In silico methods using bioinformatics tools and computational analysis could be an alternative for SELEX as it reduces the time and cost associated with development and production of aptamers. The in-silico bio-computational method generate a three-dimensional (3D) RNA or DNA structure from primary sequence followed by molecular docking simulations. The binding affinity of aptamers in relation to their target analytes and the stabilization of required conformations can be verified and modified using software (39).

Aptamers is advantageous over antibodies, and they have been demonstratively applicable as probes for histochemical staining of formalin-fixed along with paraffin-embedded (FFPE) tissues. Their smaller size relative to antibodies may enhanced tissue penetration and increase biomarkers detection specificity. In addition, the aptamer-based tissue staining has shorter reaction times and less restricted antigen retrieval conditions. Few studies had characterized usage of aptamer in histochemical staining, for example, oestrogen receptor (ER) (38), human epidermal growth factor receptor 2 (HER2) (40), and CD30 (41). Therefore application of aptamer as alternative probe for affinity-histochemical staining has a huge potential in disease diagnosis, specifically cervical cancer.

Several studies developing aptamers against HPV specifically HPV16 E7 and E6 had been published (20,21,37). However, application of these aptamer in histochemical

staining of cervical cancer and precursors in paraffin block tissue has not yet been established. One might anticipate that synthetic aptamer probes will develop into another potent tool and option for pathologists in addition to the already utilized antibodies, upon conduction of further validation studies on clinical specimens.

CHAPTER 2: OBJECTIVE

2.1 GENERAL OBJECTIVE:

To determine the potential of E6 and E7 oncoproteins of HPV 16 and 18 as a new screening and diagnostic protein biomarkers and investigate Aptamer targeting HPV as an alternative synthetic bioreceptor in cervical cancer and precursor detection.

2.2 SPECIFIC OBJECTIVES:

1. To determine the expression of E6 and E7 oncoproteins from HPV 16 and 18 using immunohistochemical staining in cervical lesion.
2. To evaluate the clinicopathological data and its association with HPV E6 and E7 oncoproteins expression in cervical lesions from Hospital Universiti Sains Malaysia and Hospital Raja Perempuan Zainab II.
3. To design and characterize Aptamer targeting oncoprotein E6 of HPV16 using in silico bio-computational model.
4. To describe the expression of Aptamer targeting oncoprotein E6 of HPV 16 using affinity-histochemical staining in cervical malignancy.

CHAPTER 3: MANUSCRIPT

3.1 TITLE PAGE

Evaluation of E6 and E7 HPV oncoproteins expression and the potential of Aptamer as alternative synthetic bioreceptor in cervical cancer detection.

Author:

Nadiah AHMAD SABRI [1,2]

[1]Department of Pathology, School of Medical Sciences, Health Campus, USM, 16150 Kubang Kerian, Kelantan, Malaysia

[2]Hospital USM, Health Campus, USM, 16150 Kubang Kerian, Kelantan, Malaysia

Corresponding author:

Nadiah Ahmad Sabri (MD UKM)

Department of Pathology, School of Medical Sciences, Health Campus, USM, 16150 Kubang Kerian, Kelantan, Malaysia

Tel: +609-7673000; Fax: +609-7653370; Email: nadiah.9489@gmail.com

3.2 ABSTRACT

Background: High risk human papilloma virus (HPV) particularly HPV16 and HPV18 had been the hallmark causative agent of cervical malignancy, in which overexpression of oncoprotein E6 and E7 induced the pathogenicity. The present study focused on evaluation HPV oncoproteins expression in formalin fixed paraffin embedded (FFPE) tissue in different grade of cervical lesions utilizing antibodies and in-silico designed DNA-Aptamer, as detection probe.

Methods: A total of 102 FFPE cervical tissues of different grades were collected from Hospital Universiti Sains Malaysia (HUSM) and Hospital Raja Perempuan Zainab II (HRPZII) from the year of 2014 until 2022. Immunohistochemical analysis of E6 and E7 oncoproteins of HPV16 and HPV18 were evaluated. Novel DNA-Aptamer targeting HPV16-E6 oncoprotein were in-silico designed and characterized as alternative bioreceptor using histochemical staining application.

Results: Increased expression of HPV18-E7 was seen with the progression of cervical lesions from the low-grade precursor to malignancy. Meanwhile, the HPV16-E7 expression was significantly associated with histological grade of HPV16-DNA confirmed positive cervical lesion ($p=0.001$). Aptahistochemistry using DNA-Aptamer targeting HPV16-E6 resulted in nuclear-specific staining in malignant cases with negligible non-specific background staining compared to the antibody-based immunohistochemistry.

Conclusion: Immunohistochemical expression of HPV oncoproteins is a powerful alternative diagnostic tool, applicable in low-resource laboratory setting compared to the gold standard molecular testing. Meanwhile the DNA-Aptamer showed promising

potential in the histochemical application with superior specificity than the immunohistochemical detection.

Keywords: HPV16, HPV18, E6, E7, immunohistochemistry, aptahistochemistry.

3.3 BACKGROUND

Worldwide, cervical carcinoma is the fourth most common cancer in women after breast, colorectum & lung (1). In Malaysia, it ranked third with highest incidence of cervical cancer at the age of 50-65 years (2). Over the years, the incidence rate of cervical carcinoma has dropped largely due to efficient screening programme and subsequent eradication of its precursors. Cervical cancer is preventable if the precursor lesion is diagnosed at the early stage, due to the relatively slow progression to cervical carcinoma. Vaccination against the established causative agent that is high-risk Human Papilloma Virus (HR-HPV) also offer protective measure from HPV related outcome. However, low compliance rate in the cervical screening and inefficient HPV vaccination programme may hampered the mission in eliminating this cancer as a public health problem.

Cervical cancer screening programme heavily rely on cytological smear test, also known as Papanicolau test, both locally and worldwide since 1960s. Advancement in the technique has generated a liquid-based cytological test. Although these tests are highly specific, it has low sensitivity in detecting cervical cancer precursors (3). Inter-observer variability is substantial due to reliance on the subjective interpretation of the cytology as well as potential error in sampling. These are the drawbacks when using the cytological tests as primary screening.

In contrast, the molecular HPV testing enhances the detection sensitivity, but it suffers from low specificity and low positive predictive value (3). Utilisation of molecular HPV testing reduce the frequency of screening from every 3 to every 5 years. Hence, reducing the cost burden of frequent cytological smear test on the healthcare system. However, it cannot differentiate between patient's having transient or persistent HPV infections, whereby persistent infection has higher risk for neoplastic progression. Unfortunately, the

test is also quite expensive and may not be available at the low-resource healthcare setting.

At present moment, the most prominent validated biomarkers for HPV detection are nucleic acid based including arrays of HPV DNA and mRNA transcriptional genes of E6 and E7 (4). Protein-based biomarkers are limited, mostly targeting LI major capsid protein of HPV 16, 18 and other high-risk HPV. These showing that more validated biomarkers are imperative to improve the efficacy in detection.

Increasing studies have shown that E6 and E7 oncoproteins of high-risk HPV play major roles in tumour progression notably in persistent HPV infection (5). To promote cervical cancer abnormalities, the virus must become integrated into the host genomic DNA. E7 contributes to oncogenesis by binding and interfering retinoblastoma (RB) protein, targeting them for degradation. Meanwhile, E6 inhibits p53, causing inactivation of apoptosis. E6 and E7 co-expression in HPV infected cells establishes an optimal environment for sustained proliferative signalling, causing evasion of all anti-tumorigenic checkpoints to allow the cells through uncontrollable cell division (5). Taking these into consideration, this study will be evaluating the potential use of E6 and E7 oncoproteins derived from the HPV 16 and 18 as diagnostic protein biomarkers for cervical cancer screening and detection.

Of note, majority of the protein-based assays are antibody-dependent techniques including immunostaining and enzyme-linked immunosorbent assay (ELISA). For decades, antibodies have been widely used as the research reagent in most of the bioassays because of the specificity and sensitivity binding towards antigen. However, they inherent several limitations including expensive, animal-dependant production, batch to batch

variability, cross-reactivity, unstable at high-temperature and difficult to genetically or chemically modified for bioconjugation (6).

These drawbacks have stimulated the invention of alternative binding reagents including aptamer. Aptamer is an oligonucleotide-based synthetic bioreceptor that shows promising potentials in the biomedical application notably in medical diagnostics (7,8). It is cheap and can be rapidly produce for mass production, consistent batch production, highly thermostable and easily modified for various bioanalytical application (6).

Several studies have reported the application of Aptamer in histochemical staining such as for detection of ERα in human breast cancer (9), expression of CD30 in lymphoma tissue (10) and lung tumour tissue characterization (11). In the limited resource laboratory setting without any molecular diagnostic facilities, aptamer-based histochemical assay might be useful as a complementary diagnostic tool with the cytological smear testing in cervical cancer detection.

In this study, we validated E6 and E7 oncoproteins expression of HPV 16 and 18 in different grade of cervical lesions. The association of E6 and E7 oncoproteins expression with clinicopathological data were also investigated. Additionally, the potential of Aptamer as alternative synthetic bioreceptor in cervical cancer detection was evaluated using bio-computational and aptahistochemical analyses. The ultimate outcome of this study is to provide valuable information in the diagnostic aspect, notably in terms of the possibility to use viral HPV oncoproteins in FFPE tissues as adjunct diagnostic tools with cytological testing during screening of cervical cancer in low-resource laboratory setting.

3.4 METHODS

Selection of samples.

This is a cross-sectional study conducted in HUSM and HRPZII involving cases of cervical lesions diagnosed from 1st June 2014 until 31st May 2022. Selected cervical tissues were previously diagnosed histologically based on the grading of cervical lesions which includes non-neoplastic cervical lesion (n=10), low grade squamous intraepithelial lesion (n=30), high grade squamous intraepithelial lesion (n=32), adenocarcinoma in situ (n=2), squamous cell carcinoma (n=14), adenocarcinoma (n=10) and adenosquamous carcinoma (n=4). A number of these cervical lesions had a confirmed HPV-DNA status using PCR method (Abbott *m*200*sp*). Demographic information and clinicopathological report were retrospectively retrieved from the computerized database. The specimens with missing or inadequate tissue for histochemical staining are excluded from data pool. The study protocol was approved by Ethical Committee University Sains Malaysia (USM/JEPeM/21040324) and Medical Research and Ethics Committee (MREC) of Health Ministry, Malaysia (NMRR- 21-742-59355).

Immunohistochemical staining.

Briefly, selected FFPE tissues were sections to 4 µm thickness. Slides were deparaffinized in xylene for 5 min and rehydrated in a descending ethanol gradient at room temperature. Heat-induced antigen retrieval was performed at 97˚C in Tris EDTA pH 9.0 for E7 staining, and at 121˚C in citrate buffer pH 6.0 for E6 staining, respectively. Slides were incubated overnight at 4˚C with primary antibodies Santa Cruz® HPV16 E7 sc-6981 (1:25), Santa Cruz® HPV18 E7 sc-365035 (1:25) and Bioss® HPV16-E6 + HPV18-E6 bs-1719R (1:100). This is followed by incubation with mouse LINKER (Dako) and horseradish peroxidase (HRP)-conjugated secondary antibody (Dako) for 20 minutes at