DETERMINATION OF PROGNOSTIC MARKERS FOR CERVICAL CANCER

by

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LIST OF ABBREVIATIONS

A	Adenine
ABC	Avidin-biotin-complex
APAAP	Alkaline phosphatase anti-alkaline phosphatase
Bcl-2	B- cell lymphoma protein 2
bFGF	Basic fibroblast growth factor
BVC	Blood vessel counting
CIN	Cervical intraepithelial neoplasia
CIS	Carcinoma in situ
COX-2	Cyclooxygenase-2
DAB	3,3’ diaminobenzidine tetrahydrochloride
DNA	Deoxyribonucleic acid
E-cadherin	E (epithelial)-cadherin
EDTA	Ethylenediaminetetraacetic acid
FVIIIRA	Factor VIII- related antigen
H&E	Haematoxylin and eosin
H_2O_2	Hydrogen peroxide
HIF	Hypoxia inducible factors
HPE	Histopathological examination
HPV	Human papilloma virus
HRP	Horseradish Peroxidase
hTERT	Human telomerase reverse transcriptase
hTR	Human telomerase RNA component

IGF-R	Insulin-like growth factor receptor
Ig	Immunoglobulin
IHC	Immunohistochemistry
LSAB	Labelled Streptavidin-Biotin complex
MVD	Microvessel density
mRNA	Messenger ribonucleic acid
N-cadherin	N (nerve)-cadherin
NSAIDS	Nonsteroidal anti-inflammatory drugs
NSCLC	Non-small-cell lung cancer
PAP	Peroxidase anti-peroxidase
PR	Progesterone receptor
SCC	Squamous cell carcinoma
SCID	Severe combined immunodeficiency disease
STS	Soft tissue sarcomas
TBS	Tris buffered saline
TP1	Telomerase-associated protein 1
VEGF	Vascular endothelial growth factor
vWF	von Willebrand factor

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PENENTUAN PETANDA BIOMOLEKUL DI DALAM PROGNOSIS KANSER SERVIKS

ABSTRAK

Faktor biomolekul dijangka dapat membaiki dan meningkatkan ramalan berkaitan diagnosis dan prognosis dalam kanser. Pemahaman tentang tapak jalan khusus biomolekul akan meningkatkan lagi keupayaan terapi dalam penyembuhan kanser serviks agar pengubahsuaian kaedah terapi dapat dibuat mengikut kesesuaian individu. Kajian telah menunjukkan pengiraan salur darah mikro (*MVD*), kadar pengekspresan protein *cyclooxygenase-2 (COX-2)*, *E-cadherin, human telomerase reverse transcriptase (hTERT) dan Insulin-like growth factor receptor (IGF-R)* mempunyai peranan penting dalam perkembangan sel kanser dan di dalam meramalkan prognosis pesakit kanser serviks. Pembahagian sel kanser adalah berlebihan dan tidak terkawal serta memerlukan hanya sedikit faktor pertumbuhan kerana sel kanser mempunyai pengekspresan *IGF-R* yang berlebihan. Walaubagaimanapun tumor tidak dapat membesar melebihi 1-10mm^3 sekiranya tidak terdapat sistem vaskular yang menyediakan salur darah mikro melalui proses angiogenesis. Tambahan pula peningkatan pengekspresan protein *COX-2* merencat proses apoptosis dan meningkatkan proses angiogenesis serta perebakan tumor. Tumor malignan mempunyai potensi metastasis yang tinggi kerana perlekatan antara sel-sel tumor adalah lemah disebabkan oleh pengekspresan E-cadherin yang rendah. Sel kanser juga tidak melalui proses replikasi senesen dalam setiap peringkat pembahagian sel kerana enzim *hTERT* yang berlebihan sentiasa mensintesis telomerase baru yang menghalang proses apoptosis dari berlaku. Kajian retrospektif ini menggunakan sampel serviks normal,

sampel neoplasia intraepithelial serviks (CIN) dan kanser serviks yang telah didiagnosis secara histologi dari tahun 1998 hingga 2003 di HUSM. Kaedah immunohistokimia telah digunakan ke atas semua sampel untuk pengiraan *MVD* dan menganggarkan peratusan pengekspresan *COX-2, E-cadherin, hTERT* dan *IGF-R*. Kajian ini mendapati bahawa terdapat peningkatan signifikan ($p<0.001$) dalam *MVD, COX-2, E-cadherin, hTERT* dan *IGF-R* pada peringkat kanser yang lebih tinggi. Walaubagaimanapun terdapat penurunan yang signifikan bagi pengekspresan *E-cadherin* ($p<0.001$) di dalam sampel kanser berbanding sampel *normal* dan *CIN*. Kami juga mendapati bahawa faktor *MVD* adalah paling berkaitan dengan beberapa faktor klinikal termasuk status metastasis nodus limfa ($p=0.005$), penglibatan parametrium ($p=0.003$), penglibatan ruang vaskular ($p=0.004$), status kemasukan stroma ($p=0.003$) dan status pembezaan sel kanser ($p=0.002$). Pengekspresan protein *COX-2* pula menunjukkan perkaitan yang signifikan dengan status metastasis nodus limfa ($p=0.006$), penglibatan parametrium ($p= 0.004$) dan status pembezaan sel ($p=0.001$). Pengekspresan proten *E-cadherin*, *hTERT* dan *IGF-R* pula didapati hanya berkaitan dengan status metastasis nodus limfa dan status pembezaan sel tumor ($p<0.05$). Kemandirian pesakit kanser serviks didapati berkaitan dengan MVD, status pembezaan sel tumor, status metastasis nodus limfa dan status kemasukan ruang stroma ($p<0.05$). Bagi kemandirian terhadap penyakit, kami mendapati kadar pengekspresan COX-2, status kemasukan stroma, penglibatan ruang vaskular dan kemasukan parametrial adalah faktor prognosis yang signifikan bagi pesakit kanser serviks. Kesimpulannya, pengiraan salur darah mikro (*MVD*), kadar pengekspresan protein *cyclooxygenase-2 (COX-2)*, *E-cadherin, human telomerase reverse transcriptase*

(hTERT) dan Insulin-like growth factor receptor (IGF-R) ini diekspreskan dalam kadar yang berbeza pada peringkat histologi yang berlainan dalam kanser serviks dan berpotensi sebagai petanda diagnosis dan prognosis dalam kanser.

DETERMINATION OF PROGNOSTIC MARKERS IN CERVICAL CANCER

ABSTRACT

Biomolecular factors could possibly improve the prediction of prognosis and diagnosis in cancer. Understanding specific molecular pathways could possibly shed some light in improving therapeutic modalities so that the treatment plan can be modified to suit individual cases. Various markers including factor VIII related antigen (MVD), Cyclooxygenase-2 (COX-2), E-cadherin, human telomerase reverse transcriptase (hTERT) and insulin like growth factor receptor (IGF-R) have been shown to play important roles in carcinogenesis and in predicting the prognosis of cervical cancer. Proliferation of tumor cells is uncontrolled, excessive and requires less growth factor since tumor cells have aberrant expression of IGF-R. However, tumor cells cannot grow more than 1-10mm^3 unless it is vascularised through angiogenesis by the formation of microvessels. Furthermore high expression of COX-2 suppress apoptosis, promote angiogenesis and tumor invasion. Malignant tumor cells have high metastatic potential because the cells are less adherent to each other as a result of decreased expression of E-cadherin. Cancer cells also do not undergo replicative senescence during each cell division because the hTERT enzyme synthesizes new telomerase and prevent apoptosis. This retrospective study was done among normal cervix, cervical intraepithelial neoplasia (CIN) and squamous cell carcinoma (SCC) of cervix which have been histopathologically diagnosed from year 1998 to 2003 in HUSM. All samples were subjected to immunohistochemistry approach to quantify the microvessel density

(MVD) and to estimate the percentage of the expression of COX-2, E-cadherin, hTERT and IGF-R. Our results revealed that there were significantly increased expression of MVD, COX-2, hTERT and IGF-R as the disease become more cancerous ($p<0.001$). However, decreased expression of E-cadherin were seen in SCC samples compared to normal and CIN samples ($p<0.001$). MVD expression has been shown to be the most related with all clinical and clinicopathological parameters including lymph node metastasis ($p=0.005$), parametrial involvement ($p=0.003$), vascular space involvement ($p=0.004$), deep stromal invasion ($p=0.003$) and tumor differentiation ($p=0.002$). While COX-2 protein expression showed a significant correlation with lymph node metastasis ($p=0.006$), parametrial involvement ($p=0.004$) and tumor differentiation status ($p= 0.001$). E-cadherin, hTERT and IGF-R protein expression showed significant correlation only with lymph node metastasis and tumor differentiation ($p<0.05$). For overall survival, our results showed that MVD, tumor differentiation, lymph node status and deep stromal invasion were significant prognostic markers ($p<0.05$). For disease free survival we found that COX-2 protein expression, deep stromal invasion, vascular space involvement and parametrial involvement was a good prognostic markers ($p<0.05$). It is concluded that factor VIII related antigen (MVD), Cyclooxygenase-2 (COX-2), E-cadherin, human telomerase reverse transcriptase (hTERT) and insulin like growth factor receptor (IGF-R) are expressed in different histological stages of cervical cancer and may have potential values to be used as diagnostic or prognostic marker in cervical cancer.

CHAPTER 1

INTRODUCTION

1.1 Anatomy and physiology of the cervix

Cervix is a Latin word meaning the neck, thus cervix is the neck of the uterus. Cervix serves as a canal between the uterus and vagina. The adult cervix is cylindrical in shape. Figure 1.1 show anatomy of the cervix. The portion that projects into the vagina is called the ectocervix which consisting of anterior and posterior lips, contains an external os roughly in the center. The cervix is 2.5 to 3.0cm in length and 2.5cm in diameter in nulliparous women. While in multiparous woman, the cervix is larger and has a slit like horizontal os and healed lacerations may be present (Yao *et al*., 2002). Normal cervix has a smooth, glistening mucosal surface while cervix with squamous cell carcinoma usually appeared as a fungating red to yellow mass (Figure 1.2 and 1.3).

The cervix has several important functions. It acts as barrier between the sterile endometrial cavity and the bacteria-laden vagina. The alkaline to neutral cervical mucus inhibits vaginal floras, which generally favour an acid environment. The function of the cervix is to allow flow of menstrual blood from the uterus into the vagina, and direct the sperms into the uterus during intercourse. The physical, biochemical and immunologic properties of the cervical mucus are important for sperm transport (Iwasaka *et al.*, 1998).

During pregnancy, the cervix becomes enlarged as a result of edema and increased vascularity in the stroma. The squamous mucosa is thickened, and endocervical epithelium often extends onto the ectocervix. The opening of the cervical canal is normally very narrow. However under the influence of the body hormones and the pressure from the fetal head, this opening widens to about 4 inches during labor, to allow the birth of a baby. The isthmus acts as a sphincter during childbirth (Yao *et al*., 2002).

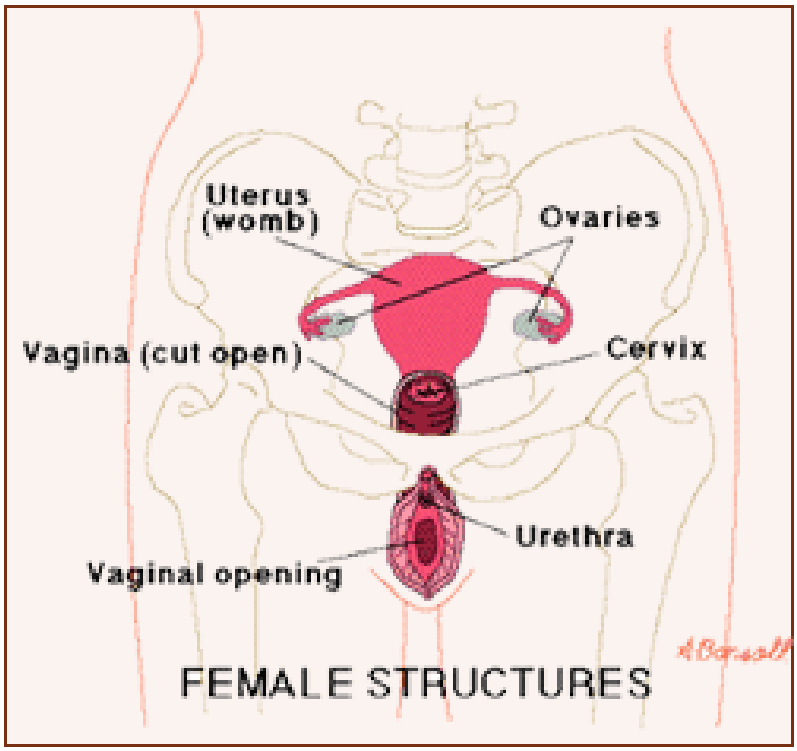

Figure 1.1 The anatomy of the cervix
(http://www.medindia.com/patients/patientinfo/images/cervix.gif)

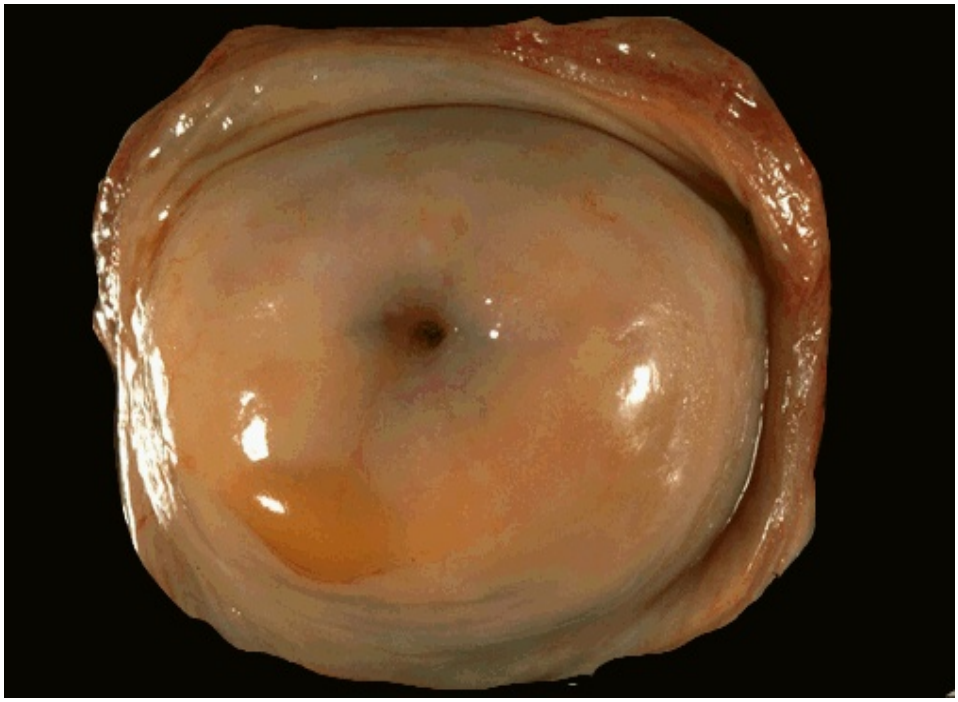

Figure 1.2 Normal cervix
(http:www.embryologist.com/images/femorgs.html)

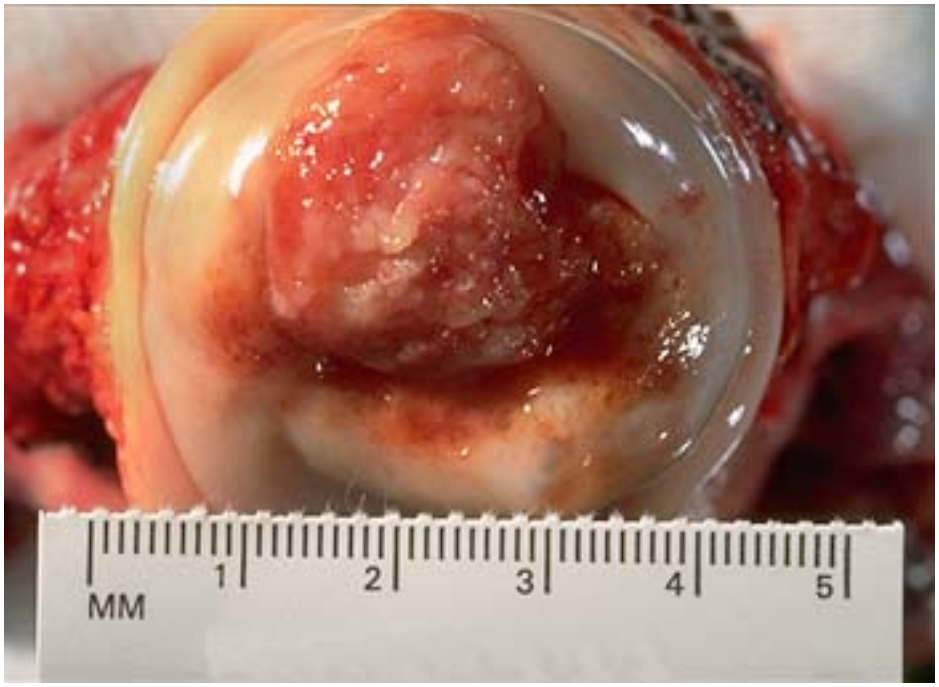

Figure 1.3 Cervical carcinoma
(http:www.gfmer/ch/selected_images_v2/detail_list.php)

1.2 Neoplasm

Neoplasm is a collection of diseases in which cell growth and division are unregulated. Classification of cancer is made according to the site of origin, histology and the extent of the disease. Without regulation, cells divide ceaselessly, piling up on top of each other to form tumors. When cells detach from a tumor and invade the surrounding tissues, the tumor is malignant, which is commonly termed as cancer. When the cells do not invade the surrounding tissues, the tumor is benign. Malignant tumor may spread through the bloodstream and lymphatic system to other parts of the body forming secondary tumors. This process is called metastasis. (Hopman *et al*., 2004).

Malignant neoplasms are characterized by a wide range of parenchyma cell differentiation, from well differentiated to completely undifferentiated. Malignant neoplasm that are composed of undifferentiated cells are said to be anaplastic (Woerner *et al*., 1995). Lack of differentiation or anaplasia is considered a hallmark of malignancy (Schneider *et al.*, 2002). Malignant neoplasm arises from a single cell which has undergone mutation. Mutations in genes give the cell increased growth advantages compared to others and allow them to escape normal controls on proliferation (Oliver *et al.*, 1998). The initial mutation will cause cell to divide to produce a genetically homogenous clone.

A tumor is said to be benign when its microscopic and gross characteristics are considered relatively innocent; implying that it will remain localized, it cannot spread to other sites, and it is generally amenable to local surgical removal and the patients usually survive (Schneider *et al*., 2002). Benign neoplasms are rarely life threatening, grow within a well defined capsule which limits their size and maintain the characteristics of the cell of origin and are thus usually well differentiated. Benign neoplasms are composed of well-differentiated cells that closely resemble their normal counterparts (Hopman *et al*., 2004).

Tumor cells show a number of features which differentiate them from normal cells. They are no longer as dependent on growth factors as normal cells either because they are capable of secreting their own growth factors to stimulate their own proliferation, a process termed autocrine stimulation or because growth factor receptors on the surface are altered in such a way that binding of growth factor is no longer necessary to stimulate proliferation (Schneider *et al*., 2002).

Tumor cells also differ than normal cells because they are anchorage independent while normal cells require contact with the surface in the extra cellular environment to be able to grow. Normal cells respond to the presence of other cells, and in culture will form a monolayer due to contact inhibition, whereas tumor cells lack this and often grow over or under each other (Wong *et al*., 2000). Normal cells stop proliferating once they reach a certain density but tumor cells

continue to proliferate. Study also found that tumor cells are less adhesive than normal cells (Macdonald *et al.,* 2000).

1.2.1 Cancer in Malaysia

A total of 21 464 cancer cases were diagnosed among Malaysian in Peninsular Malaysia in the year 2003, comprising 9 400 males and 12 064 females. The crude rate for males was 97.4 per 100 000 populations and 127.6 per 100 000 population for females. Cancer occurred at all ages. The median age at diagnosis for cancer in Malaysian males was 59 years and 53 years for Malaysian females. The five most frequent cancers in men were nasopharynx, leukaemia, lymphoma, lung, colon and rectum while cancer of the breast, cervix, ovary, uterus, thyroid gland and leukemia in woman (National Cancer Registry, 2003).

Cervical cancer is the second most common female cancer in the world after breast cancer. Cervical cancer is reported as being particularly common in less developed countries, including Southeast Asia. The incidence in Malaysia is 19.7 per 100,000 populations (National Cancer Registry, 2003). For the past twenty years or so the annual Reports of Ministry of Health recorded an average of 2 200 new cases per year (Ministry of Health Annual Reports 1980-2000). Cervical cancer incidence rate increased with age after 30 years. It has a peak

incidence rate at age 60-69 years, and declined thereafter (National Cancer Registry, 2003).

1.2.2 Cervical cancer

Cervical cancer develops in the lining of the cervix, the lower part of the uterus that enters the vagina. Cervix normally looks smooth, shining, and moist with thin film of mucus coating the surface, but it will look florid exophytic cauliflower-like growth when it become cancerous (Nor Hayati, 2003). Cervical cancer is thought to develop through a multistep process in which increasing severely premalignant dysplastic lesion called cervical intraepithelial neoplasia (CIN) I, II, III and carcinoma *in situ* progress to invasive carcinoma. Low grade CIN indicates a minimal change in the cells and high-grade CIN indicates a greater degree of abnormality (Iwasaka *et al.*, 1998).

Cervical cancer develops in the epithelia of the neck of the uterus from a single cell undergoing neoplastic transformation. Most of the time the process is initiated in a peculiar anatomical region known as transitional or transformation zone where the columnar epithelium of the inner part of the cervix, the endocervix merges with the nonkeratinizing squamous epithelium of the external part of the cervix, the ectocervix. It has been established the invasive cancer is the final stage of a continuous process historically described as a series of discrete stages (Woodman *et al*., 2001).

The association of HPV infection and cervical cancer is stronger than that between cigarette smoking and lung cancer. HPV infection is very common and it is the most common sexually acquired viral infection. There are more than 130 subtypes of HPV and about 70 subtypes infect human and 35 of them infect genital tract. In the presence of HPV, the benign metaplastic process is diverted into malignant transformation of CIN and then, in an unknown proportion of women, progressing to invasive squamous cell carcinoma (Bornstein *et al.*, 1995).

Treatment of locally advanced cervical cancer consists of radiotherapy and chemotherapy. However, results are better with this combination than with radiotherapy alone. The treatment still results in substantial morbidity (Maduro *et al*, 2003). Long term toxic effects of chemoradiotherapy in cervical cancer are still unclear because of the short follow-up of these patients. Further improvement in survival through intensifications of the standard treatment is limited by intrinsic and acquired tumor resistence, and short term and long term side effects. Tumor resistance is commonly caused by a loss of the tumor cells ability to enter apoptosis (Iwasaka *et al.*, 1998).

Understanding of specific molecular pathways leading to increased cell death could widen the therapeutic window. Better knowledge of the molecular mechanisms that underlie the apoptosis process should enable modulation of

this apoptotic pathway and might improve the treatment results. Combination of cell death by necrosis, which is commonly induced by radiotherapy, with cell death through apoptosis, might lead to higher overall tumor cell kill (Lyng *et al.,* 2000).

1.2.3 Human papillomavirus (HPV)

Human papilloma viruses are small, double stranded DNA viruses with a circular genome of about 7 900 nucleotide base pairs. Cervical infection by specific genital HPV types is a precursor event in the development of cervical cancer (Zeng *et al*., 2002). Genital HPV types are divided into groups based on the frequency of association with malignant tumors. Viral subtypes with high risk oncogenic potential include HPVs 16, 18, 45 and 56 (Munoz *et al*., 2003). Eleven subtypes are of intermediate risk for the development of neoplasia (HPVs 31, 33, 35, 39, 51, 52, 55, 58, 59, 66 and 68) and eight subtypes are of low risk (HPVs 6, 11, 26, 42, 44, 54, 70 and 73) (Joshi *et al*., 2005).

It was shown that more than 90% of cervical cancers contain HPV DNA so HPV infection can be viewed as a necessary cause of cervical cancer (Joshi *et al*., 2005). According to a proposed scheme for the natural history of HPV infection and cervical neoplasia, transient genital HPV infection commonly develop in women after the initiation of sexual intercourse. The prevalence of

genital HPV infection is highest in sexually active women in their teens and twenties and decreased markedly after age 30 years (Kumar *et al*., 2003a).

Infection of HPVs causes dysplastic lesions in the cervical epithelium that in many cases are self limiting, demonstrating effective host immune response to the virally infected cells (Joshi *et al*., 2005). However in some cases, the immune system fails to clear the infection which may become chronic and eventually lead to growth of malignant cells and the development of invasive cancer. It is evident that the majority of high risk HPV infections do not progress malignancy (Bosch *et al*., 2002). Other risk factor for malignant change are needed such as high viral load, p53 polymorphisms, immunosuppression, tobacco usage, multiple sexual partner and possibly synergistic effect of other concurrent viral infections such as herpes simplex (Kumar *et al*., 2003a).

The mechanism by which some viral infection remain low risk and some are high risk for developing high grade CIN or invasive cancer are different. In HPV infected lesions, the low risk viral DNA remains structurally as episomes. In the invasive cancers, the viral genome is usually found integrated into chromatin material of the host nucleus. In brief, much of current research shows that HPV interacts with host proteins to deregulate the cell cycle (Munoz *et al.*, 2003).

In the normal cell, an antioncogene such as retinoblastoma gene acts as a functional brake on cell division. E7 and related transforming proteins

competitively bind with retinoblastoma gene and inactivating it (Sherbet & Patil, 2003). Similarly a suppressor gene, p53 acts normally to suppress growth of tumor cells. The binding of the viral E6 oncoprotein in high risk HPV types with host cell protein, p53 promotes degradation of p53, which is functionally equivalent to mutational inactivation. Thus inhibition of cell growth is again lost, which represents an endogenous progression factor that important in the carcinogenic process (Furumoto & Irahara, 2002).

1.3 Immunohistochemistry in cancer research

Immunohistochemistry is a method for localizing specific antigens in tissues based on antigen-antibody recognization and it seeks to exploit the specificity provided by the binding of an antibody with its antigen at a light microscopic level. It has a long history, extending more than half a century from 1940. However, only since the early 1990s has the method found general applications in surgical pathology (Talbot *et al*., 2004).

Immunohistochemical technique is a specialized technique used to identify specific substances in tissue using derived antibodies and detection systems that allow visual identification. The identification of specific compounds enables the physician to determine more accurately the histogenesis of particular lesions and possibly provides prognostic information about them as well. Antibody molecules cannot be seen with the light microscope or even with the electron microscope

unless they are labeled or flagged by some methods that permit their visualization. Essentially, detection systems attach certain labels or flags to primary or secondary antibodies in order to visualize the target antibody-antigen localization in the tissue sections (Lee *et al*., 2003).

Immunohistochemistry staining is based on affinity between antigens and antibodies, which can be demonstrated by immunoflourescence technique or immunoenzyme technique (Farley *et al*., 2000). Antibodies are raised against antigenic determinants present on the compound in tissue and are detected by the use on the antibodies that are designed to recognize the immunoglobulin from the species exposed to the original compound. These detection antibodies (anti-antibodies from other species) are tagged with some reporter molecule such as fluorescein or enzyme that can catalyze a further reaction towards a visible product (Stabenow *et al*., 2005).

Immunoflourescence techniques involve two major types of labelling systems, either direct or indirect methods. The direct methods give a direct visualization of antigen or antibody by treatment of sample solution containing flourochrome labelled antibody. In the indirect method, samples are treated with unlabeled specific antibody for a particular antigen and followed by treatment with flourochrome labelled antibody specific for the first antibody (Leong *et al*., 2002).

In the immunoenzyme technique, for both direct and indirect methods, enzyme can be used as an alternative to fluorochrome to detect the antigens or antibody. Variation of immunoenzyme staining includes enzyme labelled antibody direct or indirectly and unlabeled antibody enzyme procedure or known as three layer techniques such as PAP (Peroxidase anti-peroxidase), APAAP (Alkaline phosphatase anti-alkaline phosphatase), and the Avidin-biotin-complex (ABC) method (Vasdev & Nayak., 2004).

The Avidin-biotin complex (ABC) or other methods of detection system is used on specimen of paraffin embedded tissue. The specific antibody to the compound of interest is allowed to combine with the antigen and is detected with an anti-antibody produced in another species that recognizes the first antibody as an antigen. This secondary antibody has biotin molecules attached to it, enabling further detection with the protein avidin (Vieira *et al*., 2004).

Biotin is a vitamin which is relatively small and its attachment to the chains of the immunoglobulin does not interfere with its ability to recognize the primary antibody. Avidin has a high affinity for the biotin molecule and the combination is virtually irreversible. Each avidin molecules has four combination sites for biotin, and each biotin molecule has two combination sites for avidin (Dabbs., 2002). The horseradish peroxidase (HRP) molecule is used as reporter molecule. This enzyme is bound by biotin and incorporated in an avidin-biotin complex in such a way that, when it is brought in proximity to the secondary biotinylated anti-

antibody, the complex binds through a remaining bind-binding site on one of the avidin molecules (Stabenow *et al*., 2005).

This essentially provides the enzyme at the site of the original primary antibody-antigen interaction. The enzyme then, acting on hydrogen peroxide, promotes the transfer of electrons from a chromogenic compound that precipitates as an insoluble pigment. The chromogen in use is 3,3'-Diaminobenzidine tetrahydrochloride (DAB). This molecule is essentially colorless in solution at dilute concentrations and precipitate dark brown during the oxidation. The precipitate is insoluble in alcohol, allowing the section to be counterstained with haematoxylin, dehydrated with ethanol, cleared with xylene and coverslipped (Nakano *et al*., 1990).

1.4 Prognostic markers in cervical cancer

Prognosis can be defined as an expert prediction of outcome which is based on an accurate diagnosis, knowledge of the natural history of the disease, the disease's response to treatment and the progression of the disease in the patient in question (Kenneth, 1995). Knowledge of prognostic factors could help in tailoring treatment so that overtreatmet can be avoided in low risk groups and adjuvant treatment only be given to patients with a high risk of relapse. Factors that are often been used as prognostic factors includes pelvic lymph node

metastasis, tumor diameter, deep stromal invasion and close resection margins (Kruse *et al*., 2004).

In cervical cancer, clinical stage is the principal prognostic factor that has been used. Other factors that are frequently identified as prognostic for diminished survival include increasing tumor size and number of involved lymph nodes, adenocarcinoma histology versus squamous cell cancers, and lymph-vascular space invasion (Lee *et al*., 2002a). Among the biological features that may have a predictive role in cancer of the cervix are haemoglobin level, tumor oxygenation status, squamous cell carcinoma antigen, HPV serotype, tumor vascularity, proliferative index and p53 mutation (Lyng *et al.,* 2000). Until now no adequate parameters are available in predicting the survival of the cervical cancer patients. Other more accurate prognostic parameters will allow not only improved understanding of the biologic behavior of the tumor but also could help define a subgroup of patients at risk for recurrence, which make it possible for individualized treatment (Woodman *et al*., 2001).

Biomolecular factors could possibly improve the prediction of prognosis and diagnosis in cancer. Because of their role in tumorigenesis lead to development of new treatment strategies. Among the potential prognostic markers being studied in the present include angiogenesis, cyclooxygenase-2, E-cadherin, human telomerase reverse transcriptase (hTERT) for telomerase activation and insulin like growth factor receptor (IGF-R).

We embarked on this study in order to determine the immunohistochemical expression of the above biological markers in different histological diagnosis of cervices samples. The hypotheses of this study are:

a) The number of microvessel density (MVD) and the percentage of expression of COX-2, hTERT, IGF-R will be directly proportional to the grade of the tumor and inversely proportional to the prognosis.

b) E-cadherin protein expression is inversely proportional to the grade of the tumor and the prognosis.

c) There is an association between the MVD, COX-2, E-cadherin, hTERT and IGF-R protein expression with clinical and clinicopathological factors of cervical cancer.

1.5 Research Objective

The general objective of this study was to determine the prognostic markers in cervical cancer.

The specific objectives are:

a) To quantify the microvessel density (MVD) and to estimate the percentage of the expression of insulin like growth factor receptor (IGF-R), cyclooxygenase-2 (COX-2), E-cadherin and human telomerase reverse transcriptase (hTERT) in normal cervix, cervix with CIN, cervix with HPV infection and cervix with SCC.

b) To determine whether the MVD, the percentage of the expression of IGF-R, COX-2, E-cadherin and hTERT can predict the risk of recurrence in CIN patients.

c) To investigate the association between MVD, IGF-R, COX-2, E-cadherin and hTERT with tumor differentiation, deep stromal invasion, parametrial involvement, vascular space involvement and lymph node metastasis in patients with SCC.

d) To investigate the association between MVD, IGF-R, COX-2, E-cadherin, hTERT, tumor differentiation, deep stromal invasion, parametrial invasion, vascular space involvement and lymph node metastasis in SCC's patients with prognosis of the patients.

CHAPTER 2

ANGIOGENESIS

2.1 Introduction: Angiogenesis in cervical cancer

Angiogenesis is the development of new blood vessels from preexisting capillaries. It is essential in tissue development, reproduction and wound healing (Branca *et al*., 2006c). However unregulated angiogenesis seem to play a critical role in several diseases including diabetes, arthritis and neoplasia. Angiogenesis has also been associated with the prognosis of neoplasm. Studies found that tumor with high microvessel density (MVD) seem to have a worse prognosis than those with low microvessel density.

Angiogenesis is essential for the development, growth and advancement of solid tumors (Abulafia & Sherer, 2000). Angiogenesis is induced by hypoxia with angiogenic transcription factor hypoxia inducible factors (HIF) (Fujita *et al*., 2006). Tumor angiogenesis is a complex multistep process and arises from an imbalance between positive and negative angiogenic stimuli. Several factors induce angiogenesis, but most important is basic fibroblast growth factor (bFGF) and vascular endothelial growth factor (VEGF). Although multiple cell types produce the angiogenic factors, the receptors are largely restricted to endothelial cell. Besides causing proliferations, they induce endothelial cells to secrete proteinase. This is to degrade the basement membrane, promote endothelial cell

migration and direct vascular tube formation from the expanding endothelial cell population (Fujimoto *et al*., 2006).

Neovascularization has a dual effect on tumor growths by perfusion supplies nutrients and oxygen to tumor. The newly formed endothelial cells stimulate the growth of adjacent tumor cells by secreting polypeptides, such as insulin like growth factors and interleukin (Lee *et al*., 2002b). Studies have found that tumor growth, after reaching the size of about 1-2mm^3 is strictly dependent on angiogenesis (Folkman & Shing, 1992). Angiogenesis is required not only for continued tumor growth but also for metastasis. Without access to the vasculature, the tumor cells cannot metastasize (Kumar *et al.*, 2003b).

Tumor angiogenesis is mainly evaluated on the basis of microvessel density (MVD). This can be measured using various endothelial markers such as CD31, CD34, ulex europaeus lectin I, von Willebrand factor (vWF) or usually known as factor VIII related antigen (FVIIIRA) (Dickinson *et al*., 1994). Factor VIII related antigen is a multimeric glycoprotein and was used to identify endothelial or megakaryocytic lineage of neoplasms. It has domain function to bind to platelet glycoprotein, collagen and heparin. Staining for factor VIII related antigen has been widely used to measure angiogenesis. Factor VIII related antigen remains a sensitive marker of benign blood vessels and has been used for the study of angiogenesis in various neoplasms such as breast cancer and cervical cancer (Weidner *et al*., 1991).

In cervical cancer, the measurement of the MVD has been found to provide prognostic information. Study by Tjalma *et al.* (1998) showed that there are correlation between tumor angiogenesis, expressed as microvessel density within the tumor stroma and pelvic lymph node metastasis in patients with early cervical cancer. They found that, the mean value of microvessel density in metastasized tumors (254 vessels per square millimeter of stroma) is significantly higher than in tumor with negative pelvic lymph nodes (144 vessels per square millimeter of stroma). Statistical multivariate analysis also found a significant correlation between MVD and disease free survival (Tjalma *et al*.,1998).

In numerous invasive cancers, including those originating in the uterine cervix, increased vessel density is related to increased tumor growth, risk of metastasis, or decreased survival (Weidner, 1993; Dickinson *et al.*, 1994; Tjalma *et al.*, 1998). Other studies showed that patient with high tumor microvessel density have an increased risk to die from recurrent disease, as apposed to patient with a low tumor MVD, irrespective to pelvic node metastasis (Tjalma *et al.,* 2001). Microvessel counts were also demonstrated to be increased in CIN III compared to CIN I by Weidner (1995).

Among the advantages of angiogenesis research are ready accesses of drugs that target endothelium. Drug resistance is less likely to arise because the target is the proliferating endothelial cell rather than tumor cells with high

mutation rates and the capacity of the targeted drugs to block the tumor cells to spread via blood vessels and causing metastasis (Folkman, 1992).

2.2 Objectives of the study

The general objective of this study was to quantify the microvessel density (MVD) in normal cervix, cervix with CIN, cervix with HPV infection and cervix with SCC. The specific objectives are:

a) To determine the MVD in normal cervix, CIN, and SCC.

b) To determine the MVD in cervix with HPV infection in patients with CIN and SCC.

c) To investigate the relationship of MVD with the risk of recurrence in CIN patients.

d) To investigate the relations between MVD with tumor differentiation, deep stromal invasion, parametrial involvement, vascular space involvement and lymph node metastasis in patients with SCC and their association with the prognosis of the patients.

The hypothesis we wish to test is that the number of microvessels is directly proportional to the grade of the tumor and inversely proportional to the prognosis.

2.3 Materials and methods

2.3.1 Study design

This is a retrospective study. The cases were tissue from patients either with CIN or SCC of cervix histopathologically diagnosed from 1998 to 2003 (6 years duration). Inclusion criteria for this study were cases with paraffin blocks and fulfill all of this criteria; which area of interest still exist, follow-up of patients were available for at least one year, patients undergo treatment for cancer and only patients with SCC included in this study.

Exclusion criteria for this study were cases where the paraffin blocks were not available, non-squamous cell carcinoma and cases which lack follow-up for one year or till their death which ever comes earlier. Patients with cancer of the cervix but did not undergo any treatment were also excluded from the study. The histopathology slides of those cases which fulfill these criteria were screened by pathologist (Dr. M. Madhavan) and the representative paraffin blocks were selected. From each chosen block, sections for haematoxylin and eosin (H&E) and immunohistochemistry were cut. Sections were stained with H&E to make sure that the area of interest still exists in the block.

Then the immunohistochemistry sections were stained with anti FVIII related antigen antibody for determination of MVD. Clinical data were collected from patient's medical record at the Hospital Universiti Sains Malaysia (HUSM) Registry Department. These include age of the patient, stage of the cancer, mode of treatment given, disease progression and the survival of the patients. Histological tumor cell types were assigned according to World Health Organization (WHO) classification. HPV status of the patients was determined by histology changes seen in the section by pathologist.

For SCC cases, staging was reviewed based on FIGO (International Federation of Gynecology and Obstetrics) staging system. Local tumor characteristic such as lymph node status, vascular space involvement, deep stromal invasion and parametrial involvement were determined from pathology report. Regular follow-up consisted of physical examination, colposcopy and smears of the vaginal vault or cervix. If indicated, a biopsy specimen was taken. The appearance of dysplastic cells at the time of biopsy of the cervix or in the vaginal vault in the patient had undergone hysterectomy was considered as recurrent disease. The age at diagnosis, histology diagnosis, primary treatment and appearance of recurrence were determined from the report. Microvessel density (MVD) was correlated with the clinical parameters and the prognosis of the patients using SPSS statistical software (version 12.0.1 USA). The study design was summarized in figure 2.1.

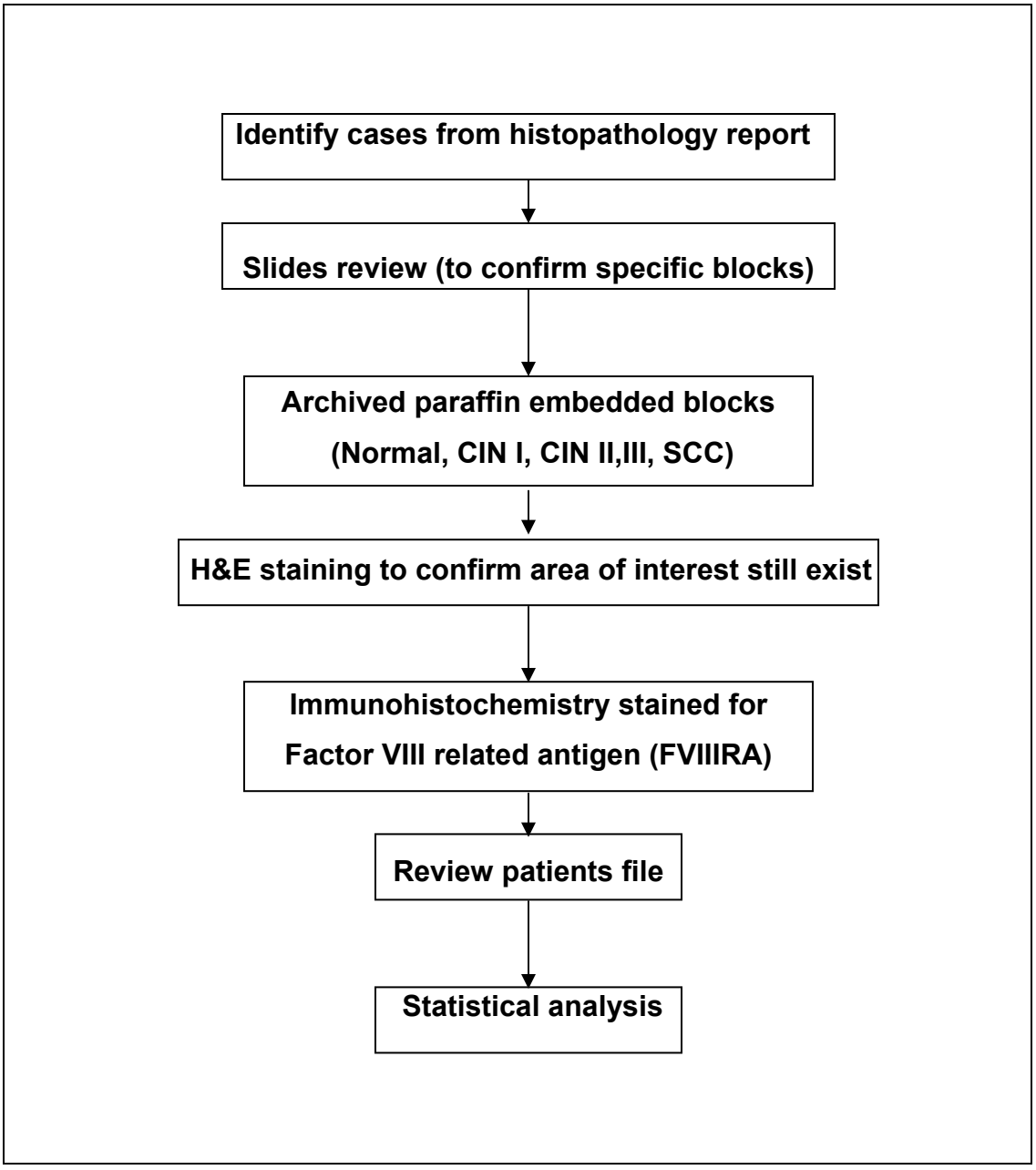

Figure 2.1 Flow chart of the study design