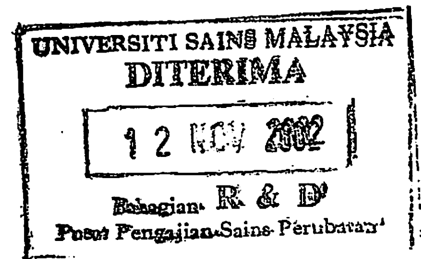
RESEARCH REPORT



Detection By In Situ Hybridization Of Specific High Risk HPV In Archival Tissue Of Invasive Cervical Carcinoma And Cervical Intraepithelial Neoplasia Using Specific Biotynylated HPV DNA Probe In Conjunction With P53 And Rb Gene Protein Expression

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1. INTRODUCTION

Cancer of the cervix is the second cause of cancer deaths among females in Malaysia after breast cancer. Penang, a state which has an up to date cancer registry records an annual incidence of 16 cases per 100,000 females. For the past twenty years or so Annual Reports of Ministry of Health recorded an average of 2200 new cases per year (Ministry of Health Annual Reports 1980-2000). This number is higher than the speculated 16 cases per 100,000 females in this country where the population of females is about 10.5 million

Cancer of the cervix is a preventive disease as the precursor cancer cells can be easily detected provided the women do regular screening tests called Pap Smear. The etiology is Human Papilloma Virus (HPV) in most cases and although there are ways to prevent transmission of the organisms, this is not often possible as the infection in the males are mainly asymptomatic.

Like other cancers, cancer of the cervix is graded according to its cellular differentiation. Several prognostic features recorded in the literature include the specific aggressive HPV strains, the angiogenetic factors, the small cell type and the presence of oncogene expressions at molecular level. In Malaysia, study on cancer of the cervix has been patchy and incomprehensive. With this in mind, we embarked on this study entitled " **Detection by in situ hybridization of specific high risk HPV in archival tissue of invasive cervical carcinoma and cervical intraepithelial neoplasia using specific biotynylated HPV DNA probe in conjunction with P53 and Rb gene protein expression**". This study comprises 2 parts; Identification of tumour suppressor genes, p53 and Rb gene in cervical lesions and identifying HPV subtypes in cervical cancer using in situ hybridization.

The identification of HPV by in situ hybridization did not materialize due to technical difficulty. We were not able to get any lesions positive although the H/E showed good koilocytic atypia. We are reporting here the findings of the first part of the research.

Clinicopathology of cervical cancers

Cervical Intraepithelial Lesion or CIN is a non invasive cervical lesion which demonstrates defective squamous cell maturation and differentiation. Depending on the thickness of this

defective maturation and differentiation, they are called CIN 1 when only a third of the cervical epithelium is involved. CIN 11 changes is when two third of the epithelium is involved and CIN 111 when the whole thickness of the epithelium is involved. This is the nomenclature widely used by histopathologists worldwide. Bethesda classification, is the classification employed by cytologists and cytopathologists for cytological diagnosis. The comparison between these classifications is illustrated below (Table 1).

Table 1: Terminologies for cervical cancer precursor lesions

WHO	Bethesda System
Mild Dysplasia (CIN I)	Low Grade Squamous Intraepithelial Lesion (LSIL)
Moderate Dysplasia (CIN II)	High Grade Squamous Intraepithelial Lesion (HSIL)
Severe Dysplasia (CIN III)	

WHO: World Health Organisation

There is an orderly program of maturation and differentiation throughout the epithelial thickness both at the morphological and at the molecular level (Stoler 1997). In an HPV infected cell, this orderly maturation does not occur. The infected cells are unable to divide. The viral DNA Early Region is detected within the suprabasal zone, and as the cell differentiates and approaches maturation there will be an induction in the synthesis of all viral genes and viral DNA. The production of virions will accumulate in the cells just beneath the uppermost epithelial surface. Such lesions are recognized as LSIL (Low Grade Intraepithelial Lesions) or mild dysplasia.

The HPV has many Early Regions and these are coded as E1 to E7. The nuclear enlargement and hyperchromasia that is recognized histologically as atypia, is the result of E6/E7 HPV oncoprotein-mediated activation of host DNA synthesis. The appearance of koilocytes is strongly associated with the presence of HPV E4 cytokeratin binding protein in an infected cell. Unlike the LSIL, the HSIL (High Grade Intraepithelial Lesion)does not attain a coordinate link between differentiation and viral early gene expression. The actual mechanism is unknown. Speculated mechanisms include viral integration or mutation in HPV E2, such that E2 - controlled regulation of E6/E7 expression is lost. This lead to loss of squamous cell differentiation and maturation morphologically seen as dysplasia.

These dysplastic cells are genetically unstable. They are at a greater risk of acquiring additional genetic errors...In an appropriate environmental milieu and under the influence of external mutagens, these cells can progress to malignant transformation

Cervical Carcinoma In General

There are estimated 40,000 cases of new invasive cervical cancers annually and nearly 1 million preinvasive (dysplastic/CIN/LSIL/HSIL) lesions .The World Health Organization (WHO) in collaboration with International Society of Gynaecological Pathologists classify cancer of the cervix as in table 2 below

Table 2. Modified World health Organization histological classification of epithelial tumours of the uterine cervix

Squamous cell carcinoma
- Microinvasive squamous cell carcinoma
- Invasive squamous cell carcinoma
- Verrucous carcinoma
- Warty (condylomatous) carcinoma
- Papillary squamous cell (transitional) carcinoma
- Lymphoepithelioma-like carcinoma

Adenocarcinoma
- Mucinous adenocarcinoma
  - Endocervical type
  - Intestinal type
  - Signet-ring type
- Endometrioid adenocarcinoma
  - Endometrioid adenocarcinoma with squamous metaplasia
- Clear cell adenocarcinoma
- Minimal deviation adenocarcinoma
  - Endocervical type (adenoma malignum)
  - Endometrioid type
- Serous adenocarcinoma

Mesonephric carcinoma

Well differentiated villoglandular adenocarcinoma

Other epithelial tumours

Adenosquamous carcinoma

Glassy cell carcinoma

Mucoepidermoid carcinoma

Adenoid cystic carcinoma

Adenoidbasal carcinoma

Carcinoid-like tumour

Small cell carcinoma

Undifferentiated carcinoma

The peak incidence of invasive cervical cancer occurs at age 40 – 45 years and 10 years younger for pre-invasive lesions (Crum, 1999). This cancer is highly associated with onset of sexual activity and HPV infection, acquired through sexual intercourse. (Crum,1999). HPV as the causative agent of more than 95% of cancer of the cervix is undisputed. There are numerous studies supporting this deduction.

Ferrera *et al.* (1999) employed general primer mediated PCR followed by PCR based sequencing technique to detect HPV DNA. They confirmed that HPV DNA was detected in 87% of all preinvasive and 95% of invasive cancers. In the control group of women, only 39% were positive for HPV DNA. They showed that HPV 16 ranked highest in pre-invasive, invasive cancers and in control group. Walboomers *et al.* (1999) utilized PCR-based test, deduced that, HPV prevalence in cervical carcinomas is close to 99.7%. Thus, it can be inferred that the cause of cervical cancer is infection by HPV.

Prevention of cervical cancer focuses on educating women on the strong causal link of HPV infection and the development of cervical cancer. One of the way is encouraging them to modify their sexual behaviour (Sidawy, 1997). Cervical carcinoma can, in theory, be prevented and treated by HPV vaccine therapy. HPV vaccine is still at the research phase. Therapeutic vaccines would eliminate HPV infection through cell mediated mechanisms, whereas prophylactic vaccines would act by inducing antivirion antibodies that prevent the infection. The prospect of effective vaccines is particularly appealing in countries in which massive cervical screening is not possible due to lack of funds.Three (3) HPV 18 E6 derived peptides (ELTEVFEFA,

KTVLELTEV and KLPDLCTEL) have been identified. The E7 derived peptide (TLQDIVLHL) was found to be highly immunogenic and could react against purified human CD8+ T cells (Rudolf et all, 2001).

Immunocompromised states such as AIDS promote HPV infection. As HIV infection rates rises, the increase in cervical cancer rates is expected. Frequent and stringent screening protocols and incorporation of adjunct screening modalities eg: tumour suppressor marker immunostaining, can aid in early and effective detection of cervical lesions in these immunocompromised population. Dorucci *et al.* (2001), examined 483 Italian women (median follow up of 7 years) between 1981 - 1995 and observed an increasing trend in the incidence of invasive cervical cancers in tandem with the rise of HIV related diseases. In Nigeria, where the incidence of HIV infection is high, the HPV and CIN prevalence is also high (Thomas 2001)

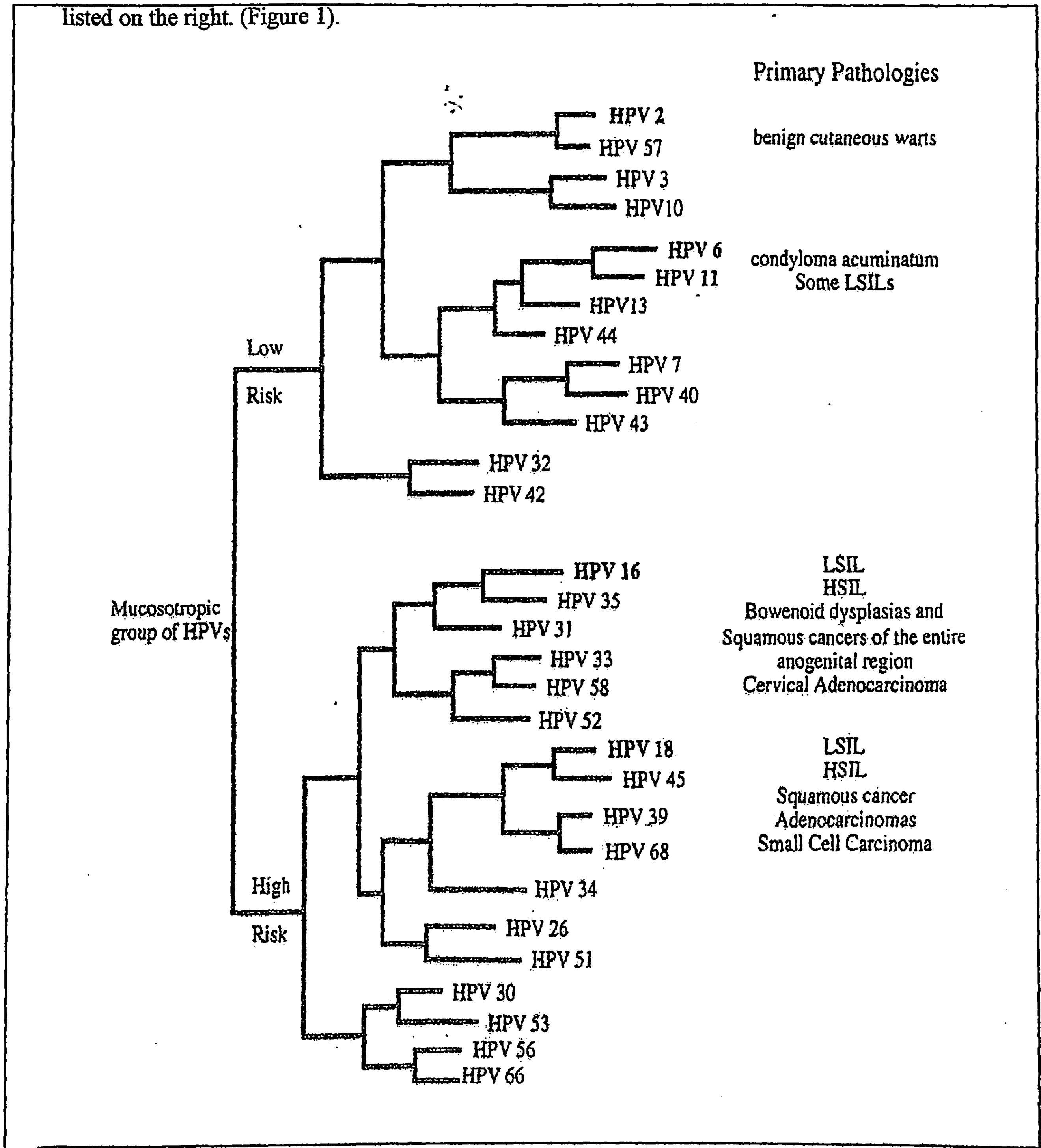
The Biology Of Human Papillomavirus

The Papilloma viruses are members of the Papovavirus family. All viruses of the family have in common small, double stranded DNA that replicate in the nucleus and have icosahedral protein capsules that form non - enveloped virions. Papilloma viruses have 55 nm, diameter capsids with 8,000 base pairs. The double stranded DNA genome has Early and Late Regions. There are seven or eight Early and two Late genetic open reading frames (ORFs), that encode all the gene products (Stoler, 1997).

What is a Papillomavirus

To date, there are more than 100 HPV types. A new HPV type should have less than 90% sequence homology in selected genomic regions compared to any of the known HPV types (Figure 1). Broadly speaking, there are cutaneotropic and mucosotropic groups of HPVs. The cutaneotropic HPV viruses cause warts on the upper parts of the body. The mucostropic viruses cause the genital lesions. The mucosotropic group, is classified into low risk and high risk HPVs. The low risk viruses are almost never found in invasive cancers. The high risk on the contrary, are those most often found in invasive cancers (Stoler, 1997).

Figure 1. Phylogenetic tree constructed for the mucosotropic group of HPVs based on the alignment of 384 nucleotides in the HPV E6 genes. The most common related pathologies are listed on the right. (Figure 1).



Functions of Viral Oncoproteins And Their Interactions With Cellular Proteins

HPV genomes codes for at least six different Early (E) and two Late (L) proteins (Zur Hausen 2000). Table 3 summarizes the functions of the different open reading frames of Human Papilloma virus (Arends *et al,* 1998 and Corden *et al,* 1999). High risk HPVs code for at least 3 proteins for growth stimulating and transforming proteins. These are E5, E6 and E7 (Zur Hausen, 2000).

Table 3 : Regulation of different open reading frames of Human Papillomavirus

Open Reading Frames (ORF)	Regulation and functions
E1	Negative regulators of replication
E2	Main viral transcription regulator
E3	Nonspecified
E4	Produces late cytoplasmic protein
E5	Early expansion protein of an infected cell clone
E6	1. Cause chromosomal instability of an infected host 2. Cause mutational consequences of a HPV positive host cell 3. Eliminate p53 gene
E7	1. Bind to Rb gene and Rb related pocket proteins 2. Enhance phosphorylation and degradation via ubiquination. 3. Regulate proliferation of cells infected by high risk HPV
E8	Nonspecified
L1	Regulator of major capsid protein
L2	Regulator of major capsid protein

The Mechanism of Cervical Carcinogenesis

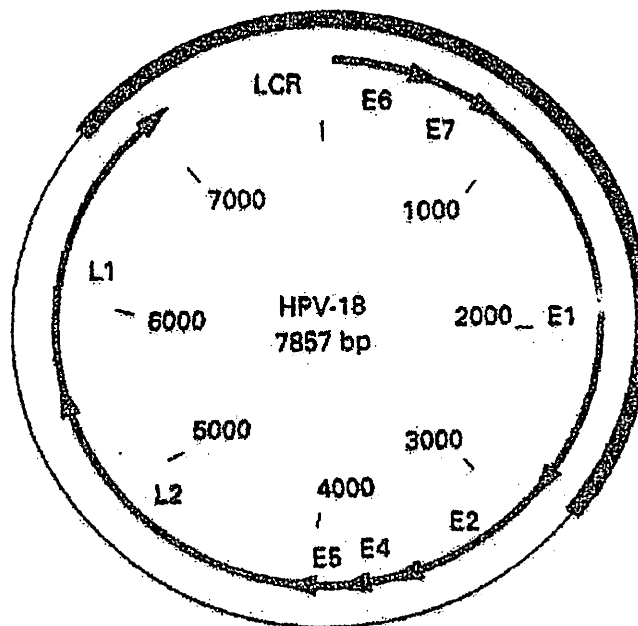
HPV infection plays a major role in cervical carcinogenesis. High risk HPV is consistently found in over 90% of cervical cancers and they possess transforming viral oncogenes (E6 and E7). The main mechanism of action is reduction of tumour suppressor genes p53 and the Retinoblastoma

(Rb) gene of the host cells. This is done by the E6 and E7 oncoproteins of the high risk HPV subtypes 16 and 18. The low risk HPV such as HPV6b or 11 do not exhibit such action (Arends *et al,* 1998).

Degradation of tumour suppressor p53 is mediated by E6 or E6-AP (E6 Associated Protein) ligase The close interaction between E6/E6-AP is potentially important in the recent observation of E6-AP mediated ubiquination and degradation of the src family tyrosine kinase Blk. The presence of E6 paradoxically blocks this degradation and thereby stabilizes the respective kinase and stimulates mitotic activity with consequent neoplastic growth.

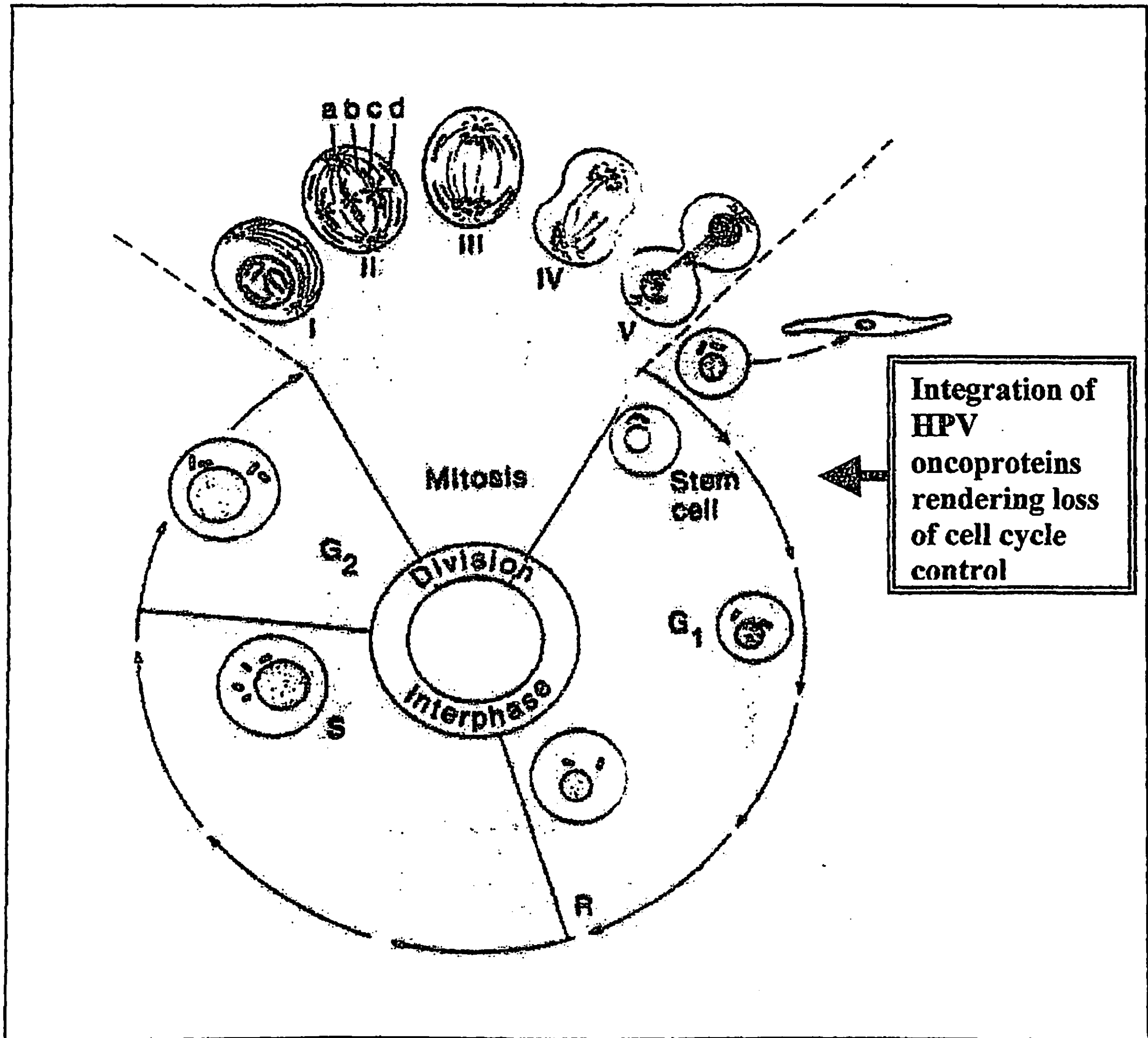
Similar to the functions of E6, E7 also binds to Rb - related pocket proteins. This binding results in phosphorylation of Rb proteins and subsequently enhances degradation by ubiquination. Following this molecular event, there will be release of transcription factors of the E2F family, which activates the cell's proliferative activity. Excessive uncontrolled cellular proliferation will result in neoplastic transformation (Zur Hansen, 2000). Integration of viral E6 and E7 oncoproteins are the key factor in cervical carcinogenesis (Figure 2)

Figure 2 : Diagrammatic representation of the region of the subgenomic fragments of high risk HPV created by restriction endonuclease digestion. The shaded region of the genome is that believed to be present after viral DNA integration.



Loss of p53 and Rb gene functions via rapid degradation by HPV E6 and E7 oncoproteins respectively, leads to loss of cell cycle growth regulation particularly within the transit phase, Go/G1 to S (Bahr 1997,.Arends *et al.* 1998) (Figure 3).

Figure 3: Integration of HPV in cell cycle

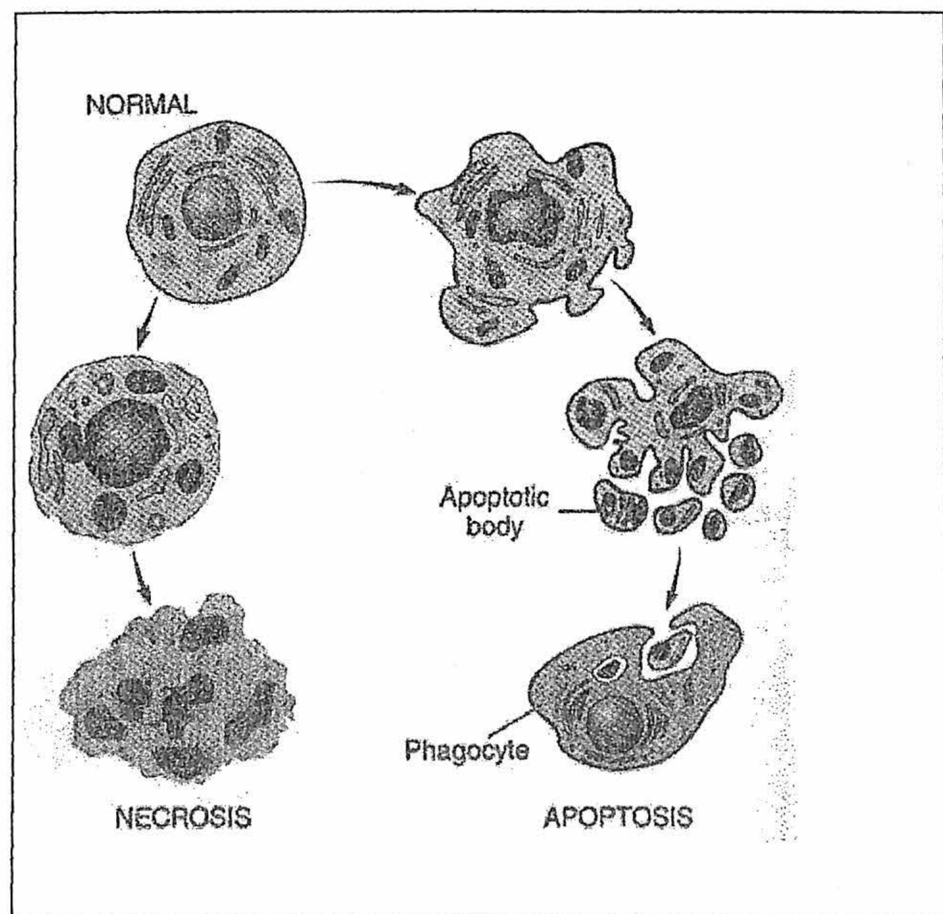


The inner cycle (refer figure 3) divides the life of the cell into periods of interphase and cell division. At the end of G_1 , one of the daughter cell, may continue to point R, the restriction point, ready to begin the DNA synthesis necessary for subsequent cell division. This is also a trigger point where a protein kinase has associated with a specific cyclin to initiate the DNA synthesis. In the S phase DNA is primarily synthesized. The product of this synthesis is sort out

at G2. Here again a cyclin dependant protein kinase(Cdk), with a specific cyclin, triggers the M phase. The mitotic events comprises of prophase, metaphase, anaphase and lastly, telophase,

Tumour suppressor gene, p53 also acts as a " guardian of the genome " and is involved in responding to DNA damage by inducing either growth arrest or cell death by apoptosis (Figure 4). Thus, HPV mediated loss of p53 function decreases the cancer cell's susceptibility to apoptosis. This promotes cellular survival. These cells have, abnormal and unstable genetic make up thus may transform to malignant cells (Arends *et al,* 1998).

Figure 4: Schematic representation of apoptosis (Cotran *et al,* 1999)



The sequential ultrastructural changes seen in coagulation necrosis (*left*) and apoptosis (*right*). In apoptosis, the initial changes consists of nuclear chromatin condensation and fragmentation, followed by cytoplasmic budding and phagocytosis of the extruded apoptotic bodies.

There is a preferential integration site within both the host and viral genomes (Corden *et al.* 1999). The site of HPV 18 DNA integration in a cell line derived from an HPV 18 DNA positive cervical carcinoma has been localized to chromosomes 8q24 and 2p24. The chromosomes positions 8q24-qter and 2p23-24 coincide with the location of c-myc and N-myc, respectively. The c-myc oncogene induces uncontrolled cellular proliferation and inactivates p53 gene thus reducing apoptosis (Soengas *et al,* 1999).

p53 tumour suppressor gene

P53 is a tumour suppressor gene. Abnormalities of p53 have been found to be the commonest molecular abnormality occurring in a wide range of human cancers. The loss of genetic information at the 17p locus where p53 gene is located is common in all neoplasia. Forty (40%) of the malignant tumours show detectable p53 abnormality. The most frequent is malignant melanoma (88%), testicular tumour (87%) and colorectal carcinoma (58%). Lower in the list are leukaemias (13%) and thyroid tumours (5%) (Dowell & Hall, 1994). The relationship of DNA and p53 is as follows :

a) DNA viruses including HPV, produces proteins that bind to and functionally inactivate p53 protein.
b) Wild type p53 possesses tumour suppressor activity, while the mutant protein does not
c) Wild type p53 can induce cell cycle arrest, a property lost by mutant protein
d) Wild type p53 protein can bind DNA in a sequence specific manner, whereas mutant p53 protein cannot, indicating that p53 is probably a transcriptional regulator
e) Abnormalities of p53 are common in neoplasia
f) Mutation of p53 gene is associated with conformational changes and altered protein stability
g) P53 has a role in apoptosis, particularly after genotoxic insults

Detection of p53 aberrations in normal cells could indicate likely transformation of the cell into neoplasia and its detection in neoplastic cells could predict \the aggressiveness of the cancer

Detection of p53

p53 abnormalities can be detected at chromosomal, gene and at protein level. For this study we utilized immunohistochemical method to detect p53 gene mutation at the protein level. The abnormal p53 protein or the mutant p53 is more stable than the wild type form (Rabah *et al,* 2000). The degraded p53 is a functional equivalence of a mutated p53 (Stoler, 1997). This result

in abnormal protein accumulating to levels where it can be detected by immunohistochemistry. This technique is fairly rapid and economical to be used in screening for p53 alterations. It can be performed on both cytological and histological material either paraffinized or fresh frozen tissues (Dwell & Hall, 1994).

HPV negative cervical carcinoma

A small percentage of cervical carcinoma is HPV negative. HPV negative cervical cell lines reveals mutation in TP53 gene. The mutation frequency range from 0% to 50% among HPV negative cervical cancer cell lines compared to HPV positive samples which showed only about 14% mutation frequency (Helland *et al* 1998). The site of mutation is mapped at codon 181, where a C → T transversion occurs leading to an Arginine to Cystein amino acid substitution. This knowledge gives the insight that genetic therapy could be developed targeting the specific mutation site.

The level of p53 in cells are regulated by its interaction with Mdm-2 protein (Prives & Hall, 1999). Mdm2 is one of the cellular proteins and mdm2 gene is one of the target genes of p53. A negative feedback loop exists in which the basal level of p53 activates the basal level of downstream target genes including mdm2. Mdm2 protein binds p53, inactivating the ability of p53 to function as a transcription factor. This is done by blocking access to the basal transcription apparatus and also targeting p53 for ubiquitin – mediated proteolytic degradation. Any insult will alter this signaling pathways resulting in alteration of the level and state of p53, perturbing the negative feedback loop (Figure 6). Disruption of p53 and mdm-2 interaction in vivo by a peptide leads to increased quantities of stabilized p53 (Figure 7)

Figure6: The relationship of p53 and Mdm protein

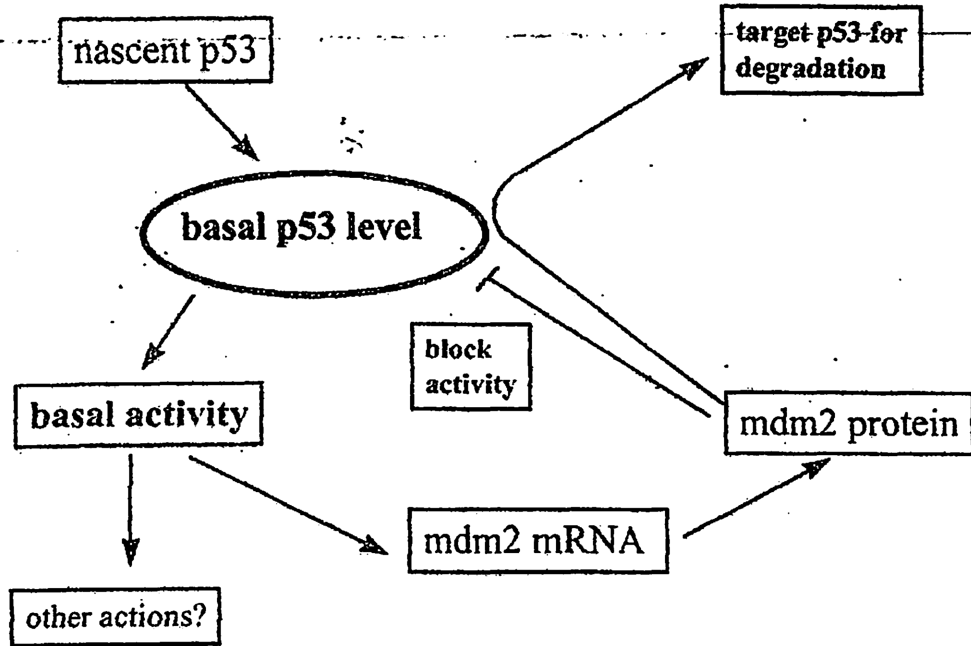
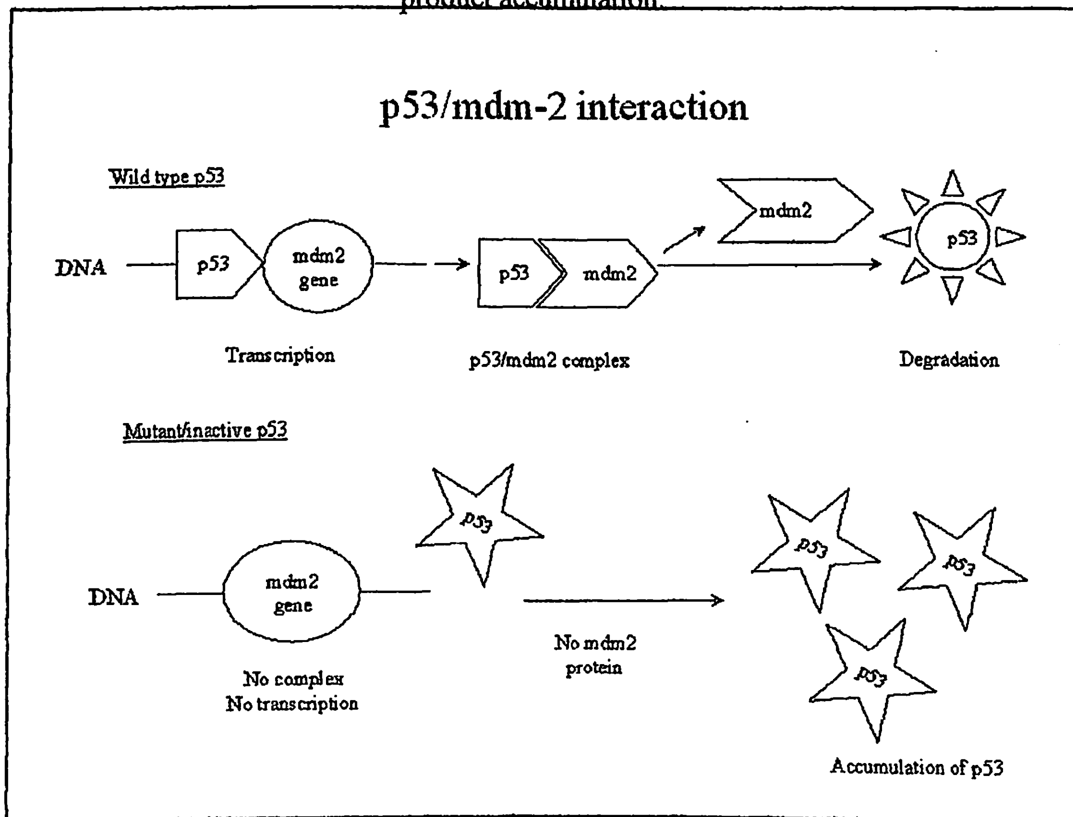


Figure 7 : Schematic presentation of p53 and mdm-2 interaction and the resultant product accumulation.



Therefore, not only viral oncoprotein can inactivate p53 other cellular proteins such as Mdm-2, are implicated in p53 inactivation. This finding is important in understanding HPV negative cervical carcinogenesis.

p53 index could be used as prognostic value by segregating cases into 2 groups of tumours with ' high level positivity' where greater than 20% of tumour cells are positive and low level where less than 20% of tumour cells are positive This classification is based on widespread over-expression of p53 in many aggressive cancers. This strongly correlates with molecular abnormality of the p53 gene. Contrastingly, a tumour with low percentage of positivity does not correlate with the presence of mutation. p53 protein product expression carries an important pathobiological significance as an independent predictor of adverse prognosis [Dowell & Hall (1994)].

The Rb (Retinoblastoma) gene

The Rb gene is located near chromosome 13q14.It is necessary for proper differentiation of the retinoblasts. The importance of the Rb gene, however goes far beyond retinoblastoma. There is good evidence that Rb gene produces an inhibition control on cell proliferation. DNA tumour virus transforming proteins such as HPV E7 oncoprotein, can bind and inactivate Rb gene via ubiquination and proteolysis (Wynford-Thomas, 1991). Rb gene is found to be inactivated in a variety of other cancers, including those of breasts, bladders, osteosarcoma and small cell undifferentiated carcinoma of the lung (Chandrasoma (1998). Rb gene is either completely absent or has significant deletions in tumours of epithelial origin such as squamous cell carcinoma of the head and neck (Stoler 1997)

Immunohistochemistry as opposed to other molecular methods

Immunohistochemistry is the method of choice in this study This technique detects the stabilized protein resulting from loss-of-function of p53 (Dowell & Hall, 1994). Detection of p53 by molecular methods yields lower frequencies of p53 abnormality as compared to immunostaining particularly those concerning malignant tumours of the brain, cervix and sarcoma.

The counting system is based on the scoring system suggested by Dowell & Hall (1994). For p53 to be of prognostic value the deduction of positivity index should be divided into a high level positivity [more than 20 % of the cells are positively stained] and low level

positivity corresponding to less than 20 % of the cells are positive stained by immunochemistry. Likewise this counting method is applied to Rb gene protein

We chose the indirect method for both p53 and Rb protein immunohistochemical staining. Robinson (1982) for the advantages below

a) It has the highest sensitivity as compared to the other methods

b) The quantity of the primary antibody used is small, thus more cost-effective

c) The antibody is easily available

d) The technique is rapid and cost effective

e) It can be performed on both histological and cytological (frozen and paraffin) material.

Compared to other molecular techniques, immunohistochemistry has superior pick up rate. This is because of its ability to detect stabilized protein even if a loss-of-function mutation has occurred outside the hot-spot region as described by Dowell & Hall (1994). In addition, altered p53 protein by alternative mechanisms such as viral oncoprotein induced degradation can also be detected by immunohistochemical method. The accuracy of any immunohistochemical analysis, depends on the following factors;

a) The sensitivity of the testing procedure

b) The detection system used

c) The optimum antibody dilution achieved

Common to all immunohistochemistry methods certain technical aspects can influence the final results including;

a) Over fixation causing antigenic loss

b) Lack of good tissue adhesion to slides causing reduced areas of tissue examined

c) The efficiency of the antigenic retrieval system

2. OBJECTIVES

General Objectives

a) To perform a laboratory based research on p53 and Rb gene protein products in cervical lesions seen at Hospital Univeristi Sains Malaysia [HUSM], Kubang Kerian between 1995 to early 1999.

b) To speculate the clinical significance of p53 and Rb gene protein products in cervical lesions.

Specific Objectives

a) To detect the presence of tumour suppressor p53 in cervical lesions using indirect immunohistochemistry method

b) To detect Rb protein products in cervical lesions using indirect immunohistochemistry method.

c) To compare and correlate statistically the presence or the absence of the tumour suppressor p53 and Rb protein index among different groups of cervical lesions studied.

3. MATERIAL AND METHODS

Materials

This is a retrospective study based on paraffinized archival tissue blocks of cervical biopsies and hysterectomy specimens. The demographic data and histopathology examination (HPE) reports were obtained from the registry book at the Department of Pathology, Hospital University Science Malaysia.

Inclusion Criteria

1) All cervical biopsies between 1995 to early 1999.
2) All conventional hysterectomy and Whertheim's hysterectomy specimens between 1995 to early 1999.

Exclusion criteria

Cases excluded from this study were those

a) in which the paraffin bocks were not available
b) in which the paraffin tissue blocks did not contain sufficient tissue left for 3 serial sections

Case groupings

Based on the HPE reports the cases were divided into three groups; the control group, the preinvasive group and overt carcinoma. The selection process was defined as follows:

a) **The Control group**

All normal, inflammatory and benign lesions such as benign endocervical polyp

b) **The preinvasive group**

All cervical lesions with HPV infection and Cervical Intraepithelial (CIN) lesions. These cases were further stratified into LSIL and HSIL according to the Bethesda System. The LSIL groups contains all HPV infected and CIN 1 cases. The HSIL component encompasses the CIN 11 and CIN 111 cases.

c) **The invasive group**

All malignant cervical epithelial lesions. They are further divided into;

(i) **Squamous cell carcinoma.** This is further divided into Keratinising Squamous Cell Carcinoma [SCCK] and Non Keratinising Squamous Cell Carcinoma [SCCNK]

(ii) **Adenocarcinoma** and other malignant lesions such as Neuroendocrine carcinoma

The Method

a) Preparation of tisssue sections

Three (3) serial sections 3-5 micron thick were done for each case. For each case one section was submitted for Hematoxylin & Eosin (H/E) staining. One section for p53 staining using immunohistochemistry and the remaining section for Rb protein using immunohistochemistry. The Hematoxylin & Eosin stained sections were examined under light microscopy for histological confirmation of the diagnosis recorded in the Pathology Registry book. The sections for immunohistochemical staining were mounted on Poly-L- Lysin coated slides

Immunohistochemical staining reagents

5 types of immunohistochemical reagents were used in this study

a. Primary antibody for p53 immunohistochemical staining
   Monoclonal Mouse Anti-Human p53 Protein (DAKO) Code no. M 7001,
   Lot 056
   Purified monoclonal antibody of mouse antiserum
   Mouse Ig concentration: 400 mg / L
   Isotype:Ig G 2b, kappa.
   Total protein concentration: 13.7 g/L

b. Secondary antibody for p53 immunohistochemical staining
   Biotinylated Rabbit Anti mouse Immunoglobulins (DAKO) Code No: E 354, Lot 113
   Biotinylated, affinity isolated rabbit anti Mouse immunoglobulins
   Solvent: 0.01 M phosphate buffer, 0.15 M NaCl, 15 mM NaN_3, pH 7.2
   Concentration of Specific Antibodies: 1.2 g / L
   Immunogen : Immunoglobulins, mainly Ig G, isolated from mouse serum.

c. Primary antibody for Rb gene protein immunohistochemical staining
   Epitope Specific Polyclonal Antibody to Retinoblastoma Protein
   Ab. No: 346P
   Epitope Designation QKM
   Source and Format: Rabbit Polyclonal Ig G, purified and diluted in PBS, pH 7.6
   Immunogen: A synthetic peptide from the C – terminus coupled to KLH human retinoblastoma gene product.
   Specification: Antigen retrieval Glyca Solution is recommended. The suggested positive control tissue is colon carcinoma.

d. Secondary antibody for Rb gene protein immunohistochemical staining

Biotinylated Goat Anti Rabbit Immunoglobulins (DAKO) Code No: E 432

Lot 014

Presentation: Biotinylated, affinity isolated goat anti rabbit immunoglobulin

Solvent: 0.01 M phosphate buffer, 0.15 M Na Cl, 15 mM NaN3, pH 7.2

Concentration of specific antibodies:1.0 g / L

Immunogen:Immunoglobulins, mainly Ig G, isolated from rabbit serum.

e. StreptABComplex / HRP

DAKO K 377

The package consists of;

1 ml of reagent A (Streptovidin) in 0.01 M phosphate buffer, 0.15 M Na Cl, 15 mM NaN3, pH 7.2

1 ml of reagent B (Biotinylated Horseradish Peroxidase) in 0.01 M phosphate buffer , 0.15 M NaCl, 15 mM NaN3, pH 7.2

1 mixing / storage bottle for the StreptABComplex

30 minutes before use, prepare the StrepAB Complex as follows;

5 ml of 0.05 M Tris / HCl, pH 7.6 was placed in the StrepABComplex mixing bottle

1 drop (45 UL) of reagent A was added

1 drop of reagent B was then added

The bottle was mixed well and left for 30 minutes before used.

Other staining chemicals used

a. Xylene (98.5 % ; Fisher chemical)
b. Ethanol (99 %)
c. 30 % Hydrogen peroxide (M = 34.01 g / mol, 1L = 1.11 kg; Merck)
d. Haematoxylin (Sigma)
e. Ammonia (BDH)
f. Tris (hydroxymethyl) ammonia (Sigma)
g. NaCl (99.9% ; BDH)
h. Sodium Azide (Sigma)
i. Diaminobenzidine Tetrahydrochloride (DAB) (79 % ; Sigma)
j. Bovine serum albumin (Sigma)

Types of solutions were prepared for staining: (Appendix C)

a. 0.05 M Tris Buffered Saline pH 7.6

b. TBS antibodies diluent

c. DAB substrate solution

d. PBS solution

Methods of p53 immunohistochemical staining

1. The sections were dried in the oven at 60 degree Celcius for 30 minutes.
2. Then the sections were deparaffinized with xylene and the hydrated sections to water.
3. The sections were put into 3% hydrogen peroxide in methanol for 15 minutes in order to remove the endogenous peroxidase from the tissue.
4. For antigen retrieval the slides were placed in a jar filled with Citrate buffer solution and heated in microwave oven for 5-10minutes at high power (750W). The slides were cooled with the solution to room temperature for about 20 minutes.
5. Sections were gently rinsed with distilled water and Tris Buffered Saline (TBS) for 2 minutes.
6. 2 – 3 drops of primary antibody (Monoclonal Mouse Anti Human p53 protein; DAKO) was applied to the sections in a dilution of 1:50 and incubated overnight.
7. Sections were rinsed with TBS.
8. 2 – 3 drops of secondary antibody Biotinylated Rabbit Anti – Mouse Immunoglobulins (DAKO) was applied to the sections in a dilution of 1:100 and sections were incubated for 30 minutes.
9. Sections were rinsed with TBS.
10. 2 – 3 drops of StreptAB Complex/HRP was applied to the sections in a dilution of 1: 100 for 30 minutes.
11. After that sections were rinsed with TBS.
12. 4 – 6 drops of HRP substrate chromogen (Diaminobenzidine) was applied to the sections and incubated for 5 minutes.
13. Sections were rinsed with tap water.
14. Sections were then counterstained with Haris Haematoxylin for 1 dip.
15. Then the sections were submitted to bluing process with ammonia, rehydrated, cleared and mounted.

All of the antibodies were diluted in TBS antibodies diluent.

All sections were incubated in the room temperature.

Methods of Rb gene protein immunohistochemical staining

1. The sections were dried in the oven at 60 degree Celsius for 30 minutes.
2. Then the sections were deparaffinized with xylene and hydrated sections to water.
3. The sections were put into 3% hydrogen peroxide in methanol for 15 minutes in order to remove the endogenous peroxidase from the tissue.
4. For antigen retrieval the slides were placed in a slide bath containing 400ml of working strength of Antigen Retrieval Glyca Solution. The slides are placed in microwave and turn to a rapid boil at high power of 700W to 1000W for aduration of 3 to 7 minutes. Then change to half the initial power and repeat the procedure and stop upon boiling and maintained it for 20 seconds. Repeat the same cycle for 3 to 5 times. Cool the slides with the Glyca solution to room temperature for about 20 minutes duration.
5. Sections were gently rinsed with distilled water and Phosphate Buffered Saline (PBS) for 15 minutes.
6. 2 – 3 drops of primary antibody (Epitope Specific Polyclonal Rabbit Antihuman Antibody to Retinoblastoma Protein BioGenex) was applied to the sections in a dilution of 1:50 and incubated for 30 minutes.
8. Sections were rinsed with PBS followed by subsequent wash with TBS.
9. 2 – 3 drops of secondary antibody Biotinylated Goat Anti Rabbit Immunoglobulins (DAKO No. E432) was applied to the sections in a dilution of 1:100 and sections were incubated for 30 minutes.
10. Sections were rinsed with TBS.
11. 2 – 3 drops of StreptABComplex/HRP was applied to the sections in a dilution of 1:100 for 30 minutes.
12. After that sections were rinsed with TBS.
13. 4 – 6 drops of HRP substrate chromogen (Diaminobenzidine) was applied to the sections and incubated for 5 minutes.
14. Sections were rinsed with tap water.
15. Sections were then counterstained with Haris Haematoxylin for 1 dip.
16. Then the sections were subjected to bluing process with ammonia, rehydrated, cleared and mounted.

All of the antibodies were diluted in TBS antibodies diluents.

All sections were incubated in the room temperature.

Types of solutions prepared for staining

1. 0.05 M Tris Buffered Saline pH 7.6
   a. Tris stock 0.05 M pH 7.6:
      Tris - 60.55 gm
      Distilled water - 980 ml
      1N HCL was added to give pH 7.6 and the mixture was made up to 1 Liter with distilled water.
   b. NaCl stock:
      NaCl (99.5 %) - 87.66 gm
      Distilled water - 1 Liter
   c. Both solutions were mixed. Equal parts of a and b solutions (100 ml each) was mixed and made up to 1 Liter with distilled water.
2. TBS antibodies Diluent
   TBS pH 7.6 - 100 ml
   Sodium azide - 0.1 gm
   Bovine Serum Albumin - 1.0 gm
   Above ingredients were mixed well
3. DAB substrate solution
   a. DAB - 6.0 mg
   b. 0.05 M TBS pH 7.6 - 10 ml
   c. 3% hydrogen peroxide - 0.1 ml
   d. DAB was dissolved in TBS and then was filtered. This product was added just before used.
4. PBS substrate solution
   a. Dissolve 1 tablet in 200 ml of water to obtain; 0.01 M Phosphate Buffer, 0.0027 M Potassium Chloride and 0.137 M Sodium Chloride
   b. Now the solution is at pH 7.4 at 25 degree Celcius
   c. Store solution at 0 – 5 degree Celcius
   d. Discard if turbidity develops

The microscopy and the scoring method

The immunostained sections were examined under light microscope. Tumour suppressor protein immuno-positivity was interpreted as number of cases expressing low or high positivity. The deduction of p53 and Rb index were based on nuclear and intracytoplasmic positive immunostaining respectively. The counting is standardized and the evaluation is based on an average of 10 high power fields, using a X 40 objective and a X10 ocular lens of a Nikon Y2 microscope. This is a semi-quantitative analysis, which is inter-observer dependent. The intensity of staining was not included as criteria in the evaluation.

The counting system is based on the scoring system suggested by Dowell & Hall (1994). If the case showed more than 20 % of cells positively stained, they are scored as 'high positivity' and those with less than 20 % of the cells positively stained, they are scored 'low' positivity.

Statistical Analysis

The data was statistically analyzed using SPSS univariate chi-square test. Chi-square test was employed to compare whether there was any difference in proportion (Dawson-Saunders & Trapp, 1994) between low and high positivity in he immnuno staining for p53 and Rb protein between the different groups of cervical lesions studied.

4. RESULTS

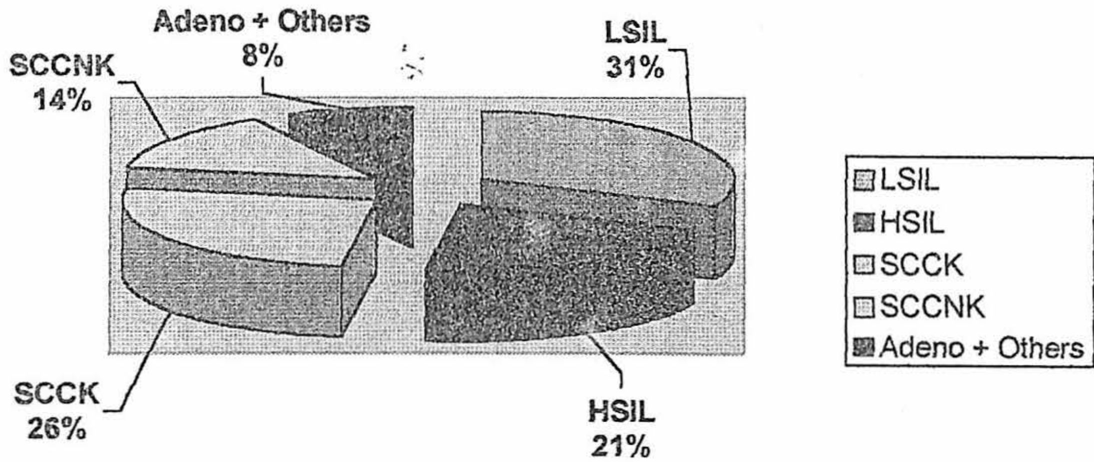
A total of 152 cases were included in this study. Of these 68 (44%) were preinvasive lesions [CIN changes], 62 (40.8%) were cancerous. Of the cancerous lesions, squamous cell carcinoma were 52 (82.8%) and adenocarcinoma makes up the rest. The majority of the squamous cell carcinoma is of the keratinising variant (65.3%). The proportion of the cases is as in table 4 and pie chart in figure 8 below.

Table 4 : The proportion of the cases in this study

Case Group	Designation of Case Group	Number of cases
Control	1	22
LSIL	2	41
HSIL	3	27
SCC K	4	34
SCC NK	5	18
ADENOCARCINOMA	6	10

Key: LSIL=Low grade intraepithelial lesions, HSIL= High grade intraepithelial lesions, SCC K=Keratinising squamous cell carcinoma, SCC NK= NonKeratinising Squamous Cell Carcinoma

Figure 8 : Pie chart exhibiting percentages among different cervical lesions in this study



Pie chart exhibiting percentages among different cervical lesions in this study.

Table 5 : Racial distribution according to case groups studied in %.

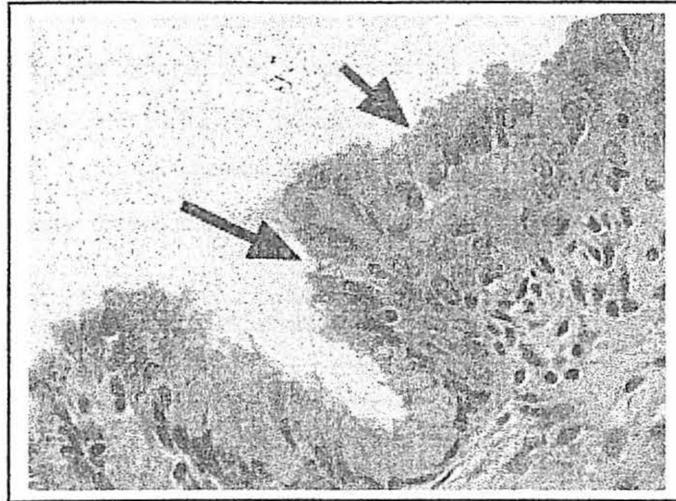
Race	Control	LSIL	HSIL	Squamous Carcinoma (SCC & SCCNK)	Adenocarcinoma & Others
Malay	18 (82%)	20 (49%)	16 (59%)	47 (90%)	5 (50%)
Chinese	2 (9%)	21 (51%)	11 (41%)	3 (6%)	5 (50%)
Siamese & Indian	2 (9%)			2 (4%)	
Total	22 (100%)	41 (100%)	27 (100%)	52 (100%)	10 (100%)

Table 6: The Dermography Of The Patients

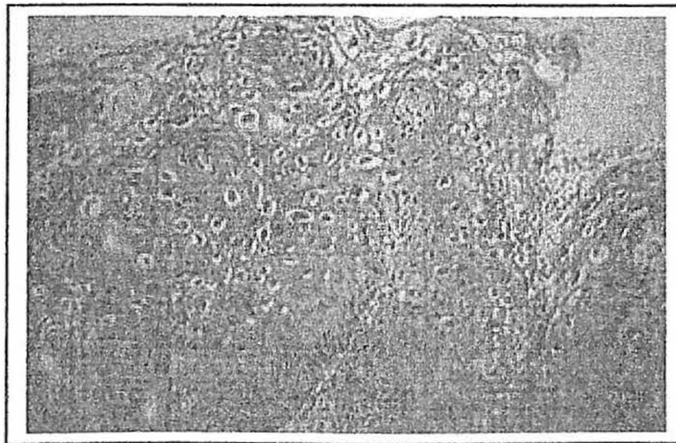
Age/years	Control	LSIL	HSIL	SCC [SCCK and SCCNK]	Adenocarcinoma
< 30	0	1	0	0	0
31-39	6	6	6	0	3
40-49	8	17	7	21	3
50-59	4	12	7	14	2
60-69	4	4	7	15	2
70-79	0	0	0	2	0
>80	0	1	0	0	0
Total	22	41	27	52	10

The Histology of various precursor and invasive cervical cancer lesions

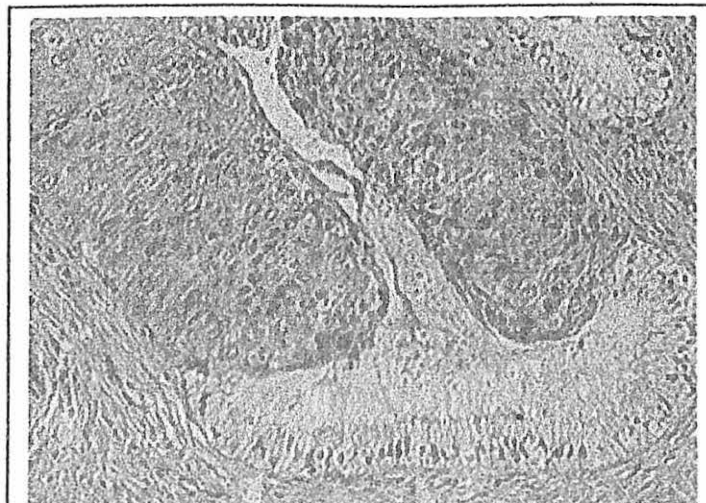
a) **Normal histology of the cervix**



b) Koilocytic atypia



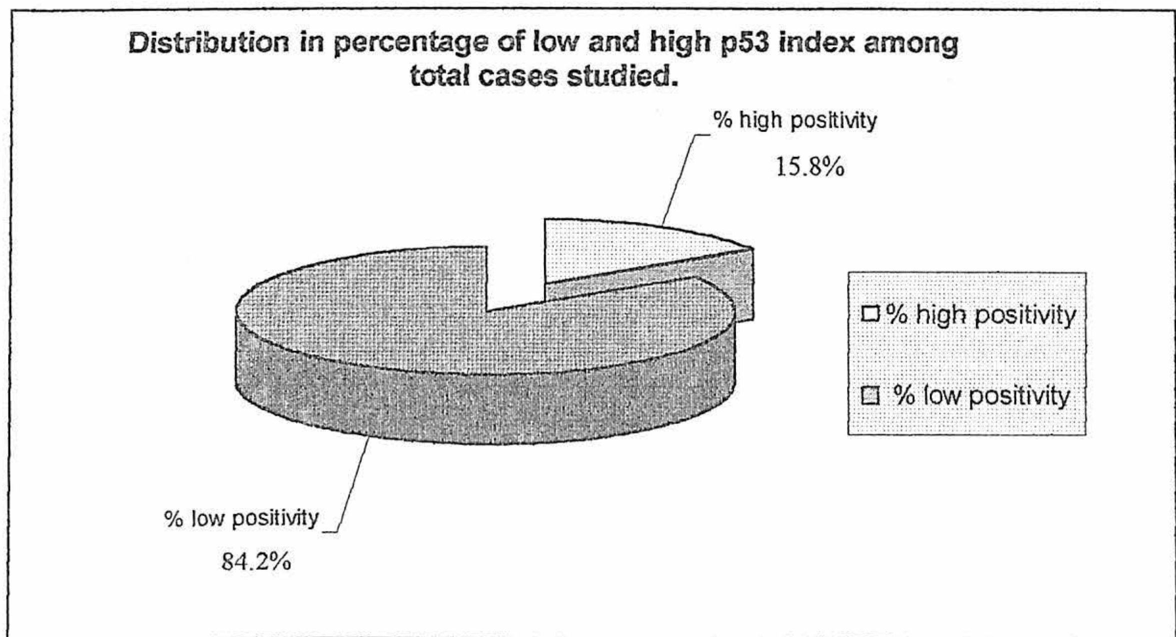
c) Severe dysplasia



Results for p53 immunohistochemical staining

In the evaluation of the total 152 cases studied, only 15.8% of the cases demonstrated high p53 index and 84.2% showed the low p53 index (Figure 9). On comparing each individual group with the control group, the SCCNK group was found to exhibit high positivity p53 index which is statistically significant (p = 0.001). In the Control group, 9.1% of them showed p53 positivity while 55.6% of the SCCNK showed p53 positive. There was no case of Adenocarcinoma exhibiting p53 positivity. The distribution of p53 index among all cases are shown in Figure 9.

Figure 9: The distribution of p53 index



Majority of the high p53 index belongs to the Squamous Carcinoma group; SCCNK(41.7 %) and SCCK (29.2%) (Figure 10 and Figure 11).

Figure 10 : Bar diagram displaying % p53 high positivity distribution among different case groups studied

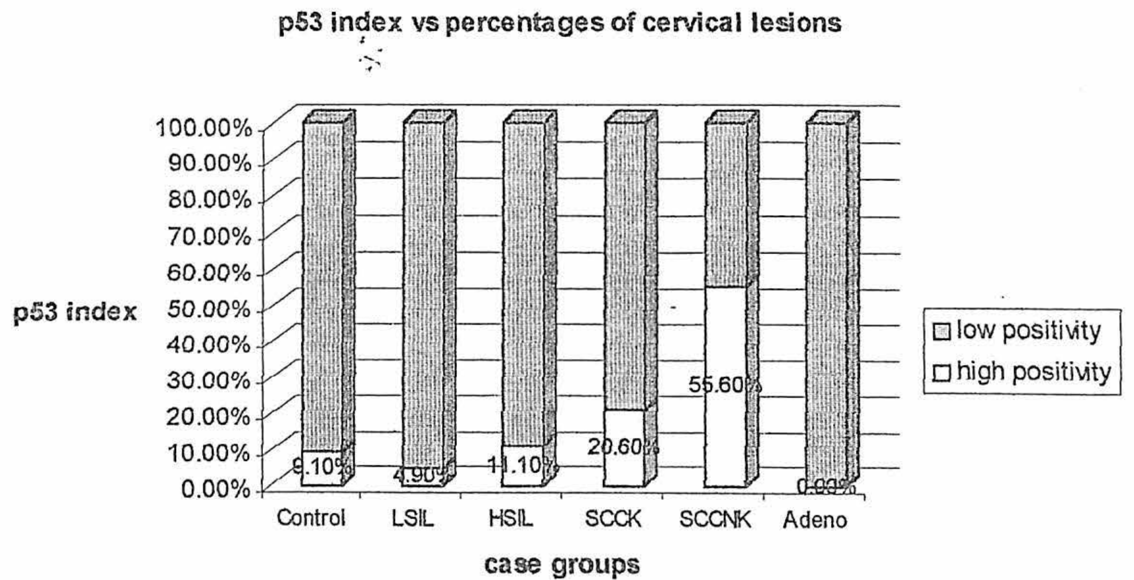


Figure 11: The percentage of high p53 staining

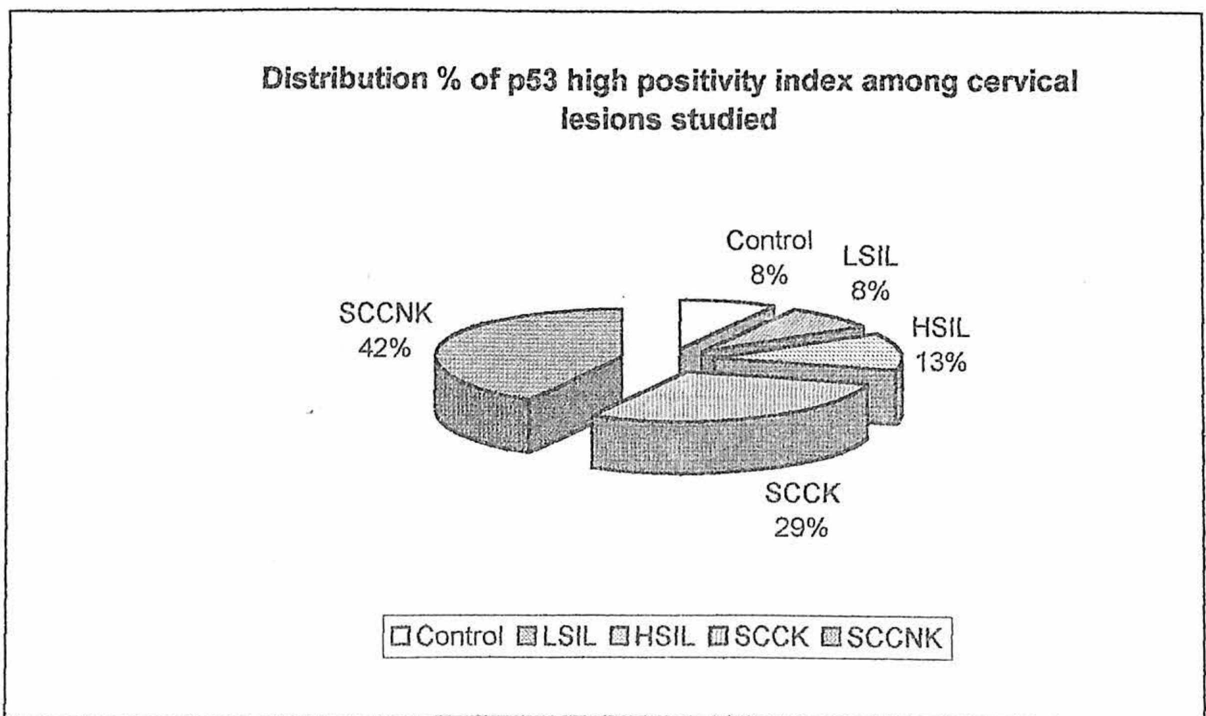


Table 7: Showing the percentage if p53 positivity According to Lesions And the p Value

Lesions	Percentage of p53 positivity	p value
Invasive cancer	71%	p= 0.005
Preinvasive cancer	21%	P> 0.005

The histology of p53 staining

Figure 12: The p53 staining – negative staining of normal cervix

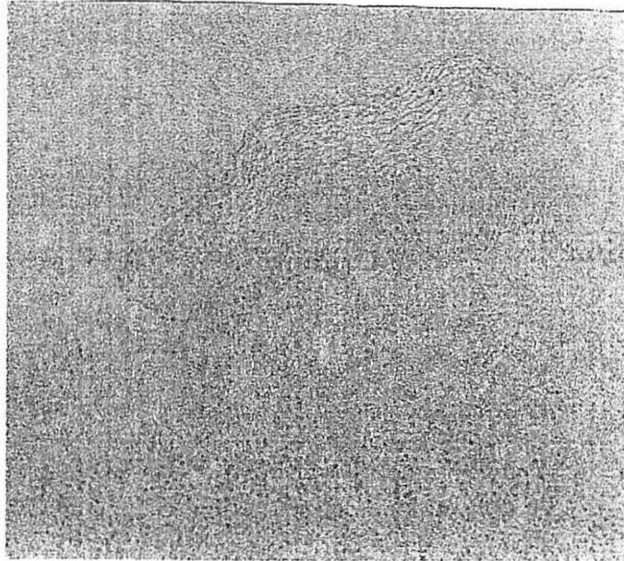
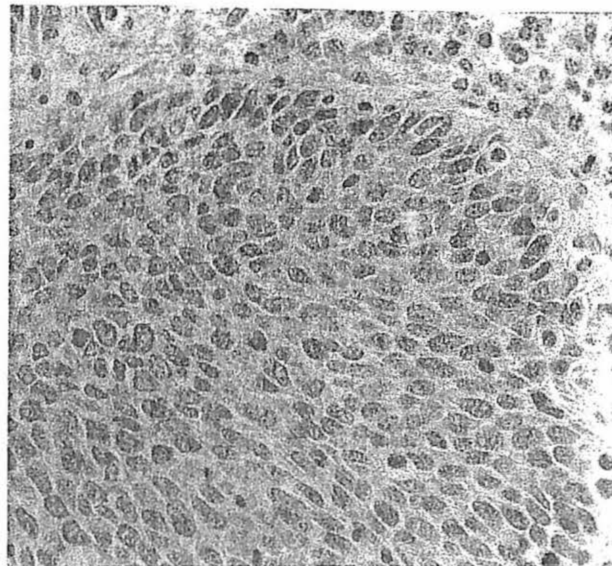
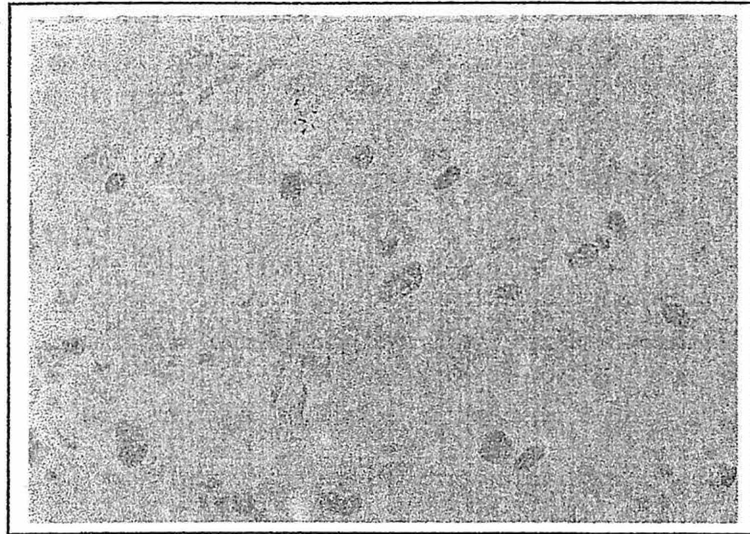


Figure 13: Positive staining of p53 in severe dysplasia (CINIII)



The positive cells are indicated by dark staining of the nuclei

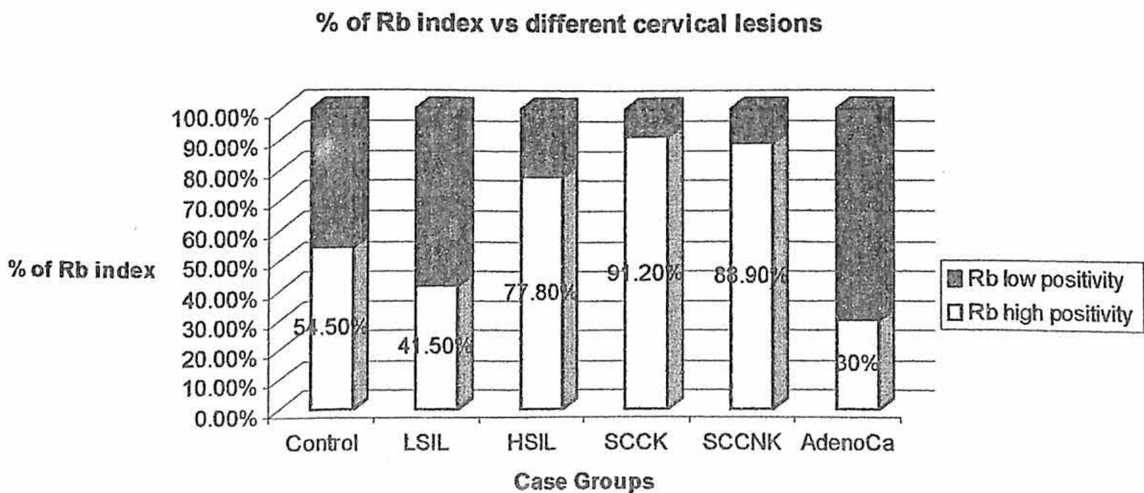
Figure 15: Positive P53 immunostaining in Large Cell Keratinising SCC (X40)



Results for Rb immunohistochemical staining

A total of 152 cases were subjected to Rb staining using immunohistochemsistry and scored in a similar manner as done to p53 staining. 65.8% of the cases were positve . The positive stained cells showed brown granular cytoplasmic staining. There was an upward trend of Rb high positivity index observed between each group. The Adenocarcinoma group showed lower Rb positivity compared to the Control group. The results is shown in Figure 12.

Figure 16 : The distribution of Rb positivity in the cervical lesions studied



The chi-square test was used and the comparison was valid among the Control group and the other 3 groups; LSIL, SCCK and SCCNK. The difference between Control and LSIL is significant (p=0.321). The high Rb positivity index for both is 54.5% and 41.5% respectively. Comparing the lesional groups and the control group, the p values are shown in table 8. The p values were statistically significant in SCCK (p=0.002) and SCCNK (p= 0.018).

Figure 17 : Proportions of Rb index in percentage among total cases studied.

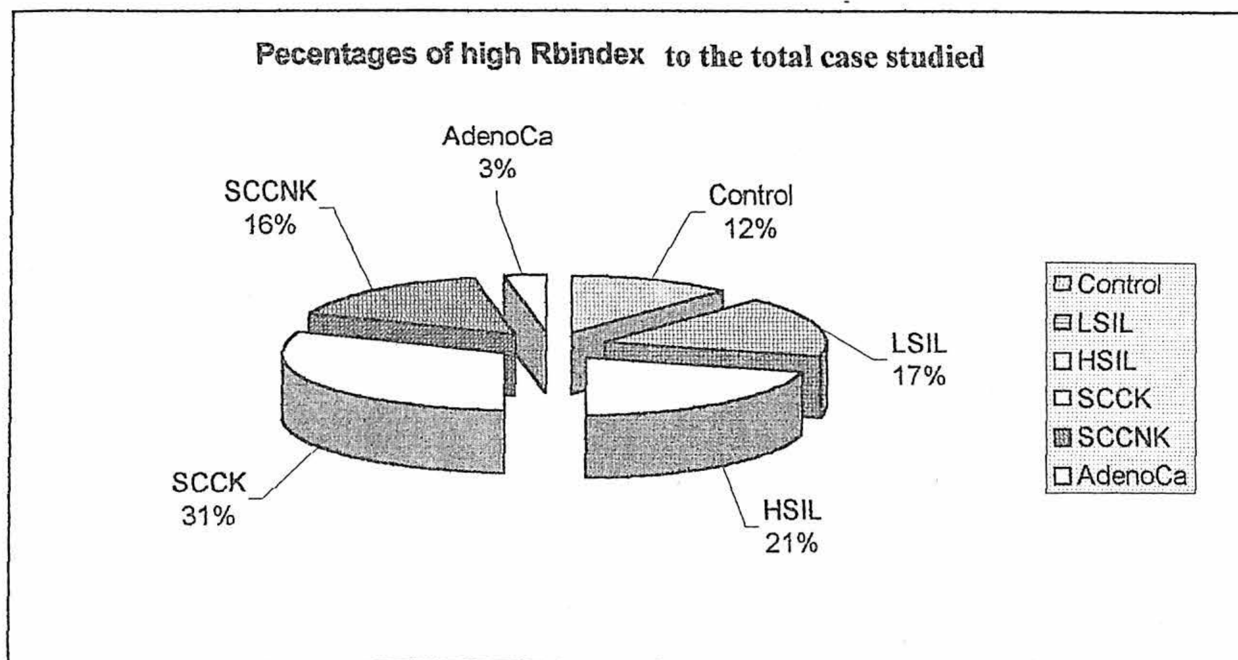


Table8: The percentage of Rb positivity According to Lesions And the p Value

Lesions	Percentage of Rb index	p value
LSIL	41.5%	p>0.005
Squamous cancers (SCCK and SCCNK)	>88%	p< 0.005
Adenocarcinoma	30%	p>0.005

Statistical correlation between p53 immunostaining and Rb protein immunostaining

Correlating between the two tumour suppressor gene markers, the proportion of cases (83.3%) demonstrating both high positivity index for p53 and Rb proteins were statistically significant with p values of 0.048 (Table 9).

Table 9 : The cross-tabulation below displays chi-square output correlating the proportion between % p53 index and % Rb index

	Retinoblastoma gene		Total
	Low positivity	High positivity	
p53 % low positivity (tumour suppressor gene)	48 **37.5%**	80 **62.5%**	128 **100%**
p53 % high positivity (tumour suppressor gene)	4 **16.7%**	20 **83.3%**	24 **100%**
Total cases with percentage positivity of p53	52 **34.2%**	100 **65.8%**	152 **100%**

Figure 18: Positive Rb staining in preneoplastic lesion (X40)

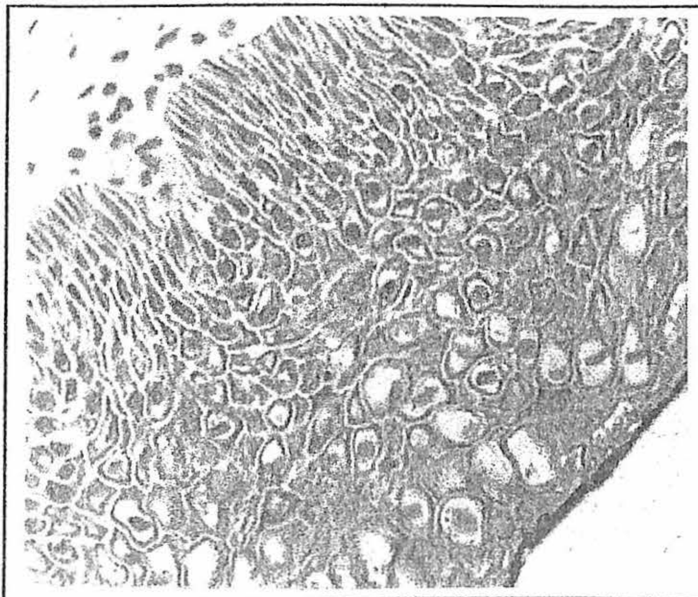


Figure 19: Positive Rb protein immunostaining in Lare Cell Non keratinising SCC (x10).

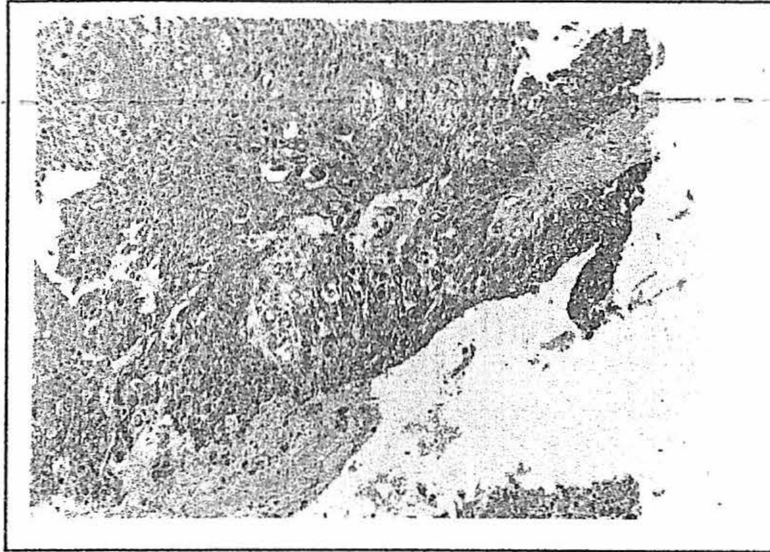
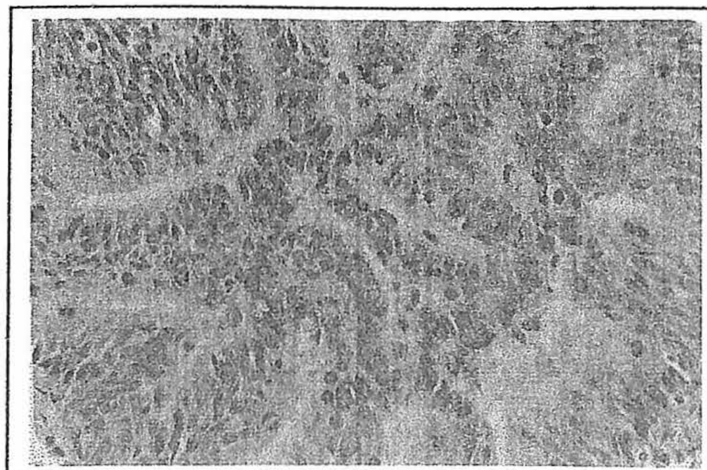


Figure 20: Rb immunostaining of large cell Keratinising SCC (x40)

Figure 21: Low Rb staining in adenocarcinoma (X40)



5. DISCUSSION

Cancer of the cervix is the second cause of cancer deaths among females in Malaysia after breast cancer. Penang, a state which has an up to date cancer registry records an annual incidence of 16 cases per 100,000 females. For the past twenty years Annual Reports of Ministry of Health recorded an average of 2200 new cases per year (Ministry of Health Annual Reports from 1980 to 2000). This number is higher than the speculated 16 cases per 100,000 females in this country where the population of females is about 10.5 million.

Cervical cancer is a disease of the economically disadvantaged countries (Sidawy, 1997). Masood (1999) estimated that approximately 440,000 new cases of cervical cancers occur annually worldwide, and 80% of these cancers occur in developing and undeveloped countries. Cervical cancer accounts for 6% of all malignant tumours and 4900 deaths in the United States each year (Sidawy, 1997). Cervical cancer is the first solid tumour shown to be directly induced by virus (Oncology Channel, Oct 2000). HPV is found in virtually all cervical carcinomas and their precursors. Deaths from cervical cancer account for 2% of all cancer deaths and 18% of all gynaecologic cancer deaths (Sidawy, 1997). In Mexico, death from cervical cancer accounts for 25% of all cancer – related deaths in females. The incidence rates of cervical cancer in several cities in Latin America, are among the highest worldwide, probably due to a high frequency of risks factors and a low screening coverage for the cancer (Eluf-Neto & Nascimento, 2001)

Socioeconomic factors play an important role in pathogenesis of the cancer the cervix. This is depicted by its high incidence in under developed or developing nations. The contributing factor is lack of proper and regular screening programs in these countries.

In our study, 90% of the invasive squamous cell cancers are seen in the Malays, an ethnic group known to be in the lower economic bracket than the Chinese. The total family income for the Malays is approximately 1000 Ringgit less than the Chinese. This finding is concurrent with a higher number of HSIL cases seen in the Malays (59%) compared to those seen in the Chinese (41%). The reverse is true for the lower category of preinvasive lesions, LSIL. Another possible inference is the Chinese having better awareness regarding the cervical screening programme, better accessibility to health care centers and has higher motivation for early screening. The Chinese community mostly resides in the urban or suburban areas as compared to the Malays.

15% to 70% of CIN cases would eventually develop into sinister invasive cervical cancer if left untreated (Oncology Channel, Oct 2000). Projecting this knowledge of tumour progression, the low incidence of invasive cervical cancer among the Chinese females in our study could be due to that the preinvasive stage was detected via the screening programme.

Conventional Adenocarcinoma, Adenosquamous Carcinoma and Neuroendocrine Carcinoma account for a very low percentage both among the Malays (5%) and Chinese (12%) patients. In this study Adenocarcinoma of the cervix were noted in the Chinese, however the number of cases were small for coherent statistical analyis. Adenosquamous Carcinoma, a variant which have a more aggressive biological behaviour (Wright *et al,* 1994) was seen in the Malays, nevertheless the number was also small.

Aggressiveness of cervical cancers depends on a number of factors. The most important of those is the HPV strains. Thomas *et al.* (2000) conducted a study on 75 women with invasive cervical cancer in Bangkok, Thailand between September 1991 and September 1993. Using PCR based assay, they typed specific HPV DNA in cervical scrapings. Seventy nine percent (79%) of the invasive cancers were HPV types 16 and 18 while only 57% of preinvasive cervical cancer lesions (CIN changes) had those strains, They did not find HPV strains of types 31/33/35/39 in any of those invasive lesions. The authors concluded that the invasive cancers are due to type 16 and 18 than due to other strains. In another study in Japan by Nagai *et al.* (2001), also utilizing PCR based assay, further enhanced the understanding that not only HPV 16 implicated in invasive cervical cancers, the DNA of this HPV strain was seen in Squamous Cell Carcinoma and Adeno-squamous Carcinoma. On the contrary, HPV 18 was proven to be the aetiological viral subtype for Conventional Adenocarcinoma. In our previous study using cocktail HPV detected by in situ Hybridization (Salina Awang and Nor Hayati Othman, 1997) we noted that HPV prevalence in invasive cervical cancers among female patients seeking treatment at Hospital Universiti Sains Malaysia was 24%. This figure is in marked discrepancy compared to the figure of 75.4% in a study done in Kuala Lumpur by Padmanathan et al using PCR essay (Padmanathan, Yadav Gregory 1996).

HPV infection is a sexually transmitted disease with a peak prevalence of infection in 22 – 25 year old women. The prevalence of infection decreases with age, suggesting that infection resolves through host immune responses (Sidawy, 1997). Stoler (1997) deduced from his epidemiological study that having the highest prevalence of LSIL is seen in women in early twenties and HSIL in late twenties and early thirties. In our study we do not see significant difference in the ages of women having LSIL and HSIL. The probable reasons of such findings could be due to lack of awareness for cervical smear screening programme, relatively low frequency in premarital sexual activity or commencing sexual

activity at a later age. In the west, there is a trend towards earlier age for all of these stages, supposedly related to early exposure of sexual activity (Stoler, 1997). The age of the patients with invasive cancers is in concordance with those seen by other authors. A sporadic case (one patient) of an 80 year old woman having LSIL in our series is an interesting observation. Weaker immune response among the elderly predisposes them to a greater risk of HPV infection (Oncology Channel, 2000). This is only possible provided they are sexually active. These women who lack post menopausal oestrogen have a higher than average risk for succumbing to HPV infection. Subsequently, exhibiting higher incidence of subsequent invasive cervical malignancy (Oncology Channnel, 2000).

Majority of the invasive cancers that we analyzed showed high p53 positivity (71%). They were the squamous cancers. This is an expected finding as p53 is a tumour suppressor gene, which is often expressed in many cancers. We expected a higher percentage of positivity. Prives and Hall (1999) confirmed that the abnormalities of the p53 tumour suppressor gene are among the most frequent molecular events in human and animal neoplasia. Unexpectedly we saw 9.1% of the control tissue showing p53 positivity, a finding higher than is seen in the LSIL cases and almost as high as those in the HSIL category.

The statistical analysis showed a significant difference ($p= 0.005$) for the invasive cancer when compared to the control group while the pre invasive lesions when analyzed together did not show a significant difference ($p<0.005$) compared to the control group. There is a good correlation between percentage of high p53 index and the presence of p53 mutation or degradation (Dowell, 1994).

Surprisingly none of the adenocarcinoma were p53 positive. Adenocarcinoma as a group is more invasive than the squamous carcinoma. It is unfortunate that we were not able to determine which HPV type was responsible to cause which particular cancer. Stoler in 1997 noted that p53 index is highly positive in the Squamous Carcinoma closely associated with HPV 16 infection while HPV 18 is more consistently associated with Adenocarcinoma of the cervix. HPV 18 rarely is associated with invasive Squamous Cell Carcinoma. Similar findings were confirmed by one study in China (Lo *et al*, 2001). Therefore, we could imply that the p53 index corresponds to the type specific HPV infections and the high p53 index is more pronounced in SCC than Adenocarcinoma. Conversely, Yang *et al.* (2001) using PCR amplification followed by restriction enzyme digestion technique, demonstrated that p53 polymorphism may have a role in the development of Adenocarcinoma but not Squamous Cell Carcinoma.

The high postivity of p53 in the control group (9.1%) is most probably due to technical false positivity. Other possible explanation is stabilization of p53 protein as a result from the changes in cellular environment other than mutation or due to over expression of p53 protein following cellular DNA damage resulting in defective p53/mdm-2 interaction.

Detection of p53 abnormality proved to be a valuable tool in selecting patients with an increased likelihood of poor survival (Prives and Hall, 1999). Due to the limitation of this study we were not able to follow the progress of our patients in terms of their survival period.

We do not think there is a coherent correlation between high p53 positivity and the ethic group. It is interesting to note that the majority of patients with high p53 index in our study were the Malays.

Retinoblastome (Rb) gene is also a tumour suppressor gene. In our study we noted an increasing trend of Rb positivity in the Control, Preinvasive and Carcinoma group. Generally the positivity seen is higher than those seen with p53 staining. The HSIL and the invasive cancers with the exception of adenocarcinoma displayed more than 77% high Rb index. This is understandable as the high risk HPV has E7 oncoprotein which has the ability to form inactivating complexes with the Rb protein. This is done when the virus protein competes the 'Rb pocket' protein (Stoler, 1997) causing functional inactivation of the Rb protein. As a consequence, there will be a release in host potent transcription factor, E2F, which is capable of activating a variety of host genes. Following which, there will be an exaggerated DNA synthesis and unperturbed cell cycle progression. This is as an influence from certain cell transcriptional factors such as Cyclin D_1 which will promote a neoplastic growth.

It is worrisome to note that the Control group in our series showed an unexpectedly high (54.5%) Rb index, higher than those seen in the adenocarcinoma group. It would appear that the Rb is not a sensitive predictor to differentiate precancerous from non neoplastic lesions. The p value of LSIL and the control group is >0.005. Among the two different types of cancers, the squamous and the adenocarcinomas, only the squamous cancers depicted a significant difference when compared to the control group ($p<0.005$).

Similar to p53 staining, the staining for Rb in the Adenocarcinoma group was the lowest in our series.

Comparing between p53 and Rb index, high Rb index is statistically significant in proportion for each cervical lesions studied. It could be implied that Rb index is a more sensitive tumour suppressor index for analyzing cervical lesions. Both tumour suppressor indices have a role in clinical practice. High risk HPV is consistently found in over 90% of cervical cancers and they possess transforming viral oncogenes (E6 and E7). Their main mechanism of action is reduction of tumour suppressor genes p53 and the Retinoblastoma (Rb) of the host cells. This is done by the E6 and E7 oncoproteins of the high risk HPV subtypes 16 and 18. The low risk HPV such as HPV6b or 11 do not exhibit such action. Tumour suppressor genes can be regarded as markers of aggressiveness and in predicting the prognosis. The application of the tumour suppressor index could be extended to cytology analysis especially in suspicious lesions whereby a clear diagnosis cannot be made such as ASCUS (Atypical Squamous Cells of Unknown Significance) and AGUS (Atypical Glandular Cells of Unknown Significance).

The knowledge of pathogenesis of cervical cancer is expanding. Herzog *et al.* (2001) documented the loss of heterozygosity (LOH) on chromosomes 3p which they claimed to be a common molecular event in invasive cervical cancer. This implies that more than 1 resident tumour suppressor gene is involved in the pathogenesis of cervical carcinoma. The invasive cancers were subjected to high density LOH analysis. Utilizing 25 microsatellite markers, LOH was detected in 7 out of 15 (47%) cervical carcinomas. Losses always included markers mapping to 3p22, implicating this is the site of cervical tumour suppressor genes. Further screening provides evidence that there are 2 known tumour suppressor genes, TbetaR-II and FHIT on chromosome 3p22 which are involved in cervical carcinogenesis. The LOH identification is our next step in research on cancer of the cervix.

Determination of HPV DNA via viral load study employing the second-generation hybrid capture test is a new and easier technique to identify HPV DNA in cervical tissues (Barlam, 2000). This is the plan for the very near future in Pathology Department, USM. Instead of identifying precursor cells CIN1,CIN ll and CIN lll, the move now is to use the presence of HPV DNA in cervical smear cells as the indicator whether the women is at risk or otherwise. This test may one day enable women to do self-test, improving the detection rates among women with limited access to health care or reluctance to undergo pelvic examination (Barlam, 2000).

In our opinion, screening women with the conventional Pap smear could be coupled with staining the cervical scrapes for tumour suppressor genes in order to enhance detection on the likelihood a woman will get cancer or otherwise.

11.

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