CHAPTER 2

LITERATURE REVIEW

2.1 Cancer

With 10.9 million new cases and 6.7 million deaths per year, cancer is a devastating disease, presenting an immense disease burden to affected individuals and their families as well as health care system (Parkin *et al*., 2005). Cancer is mass of abnormal cells that have lost, partially or fully, the normal regulatory growth control mechanism. This disorder disrupts the normal process of cell division, controlled by the genetic material (DNA) of the cells (Becker *et al*., 1995; Reddy *et al*., 2003).

Cancer stem from abnormal cell growth within the body, which results from damage to DNA. This damage could be due to a number of factors ranging, from environmental factors such as asbestos inhalation or smoking, to inherited damaged/mutated DNA. The DNA cannot be repaired in abnormal cells ultimately leading to uncontrollable proliferation. These abnormal cells rapidly outgrow normal cells, spreading to other parts of the body (Jiade, 2004).

Neoplasm can be classified as benign or malignant based on their likelihood of spreading to distant parts of the body. Benign tumours are typically encapsulated nodules of neoplastic tissue and therefore do not spread, whereas malignant tumours often invade neighboring tissue and damaging these tissue and organs. Cancer cells can break away and enter the blood stream to form new tumours in other parts of body. This type of spread of cancer is called metastasis (Becker *et al*., 1995). Table 2.1 shows the contrast between benign and malignant neoplasms.

Table 2.1: The characteristics of benign and malignant neoplasms

Characteristics	Benign	Malignant
Growth rates	Usually slow Low mitotic rate Normal mitoses Normal nucleoli	Usually rapid High mitotic rate Abormal mitoses Large nucleoli
Differentiation	Resembling normal Maintains normal functions	Often poor Loss or altered function
Spread	No invasion No metastases	Local invasion Metastases common
Boundaries	Often encapsulated Circumscribed	Non-encapsulated Irregular, ill-defined
Relationship to surrounding tissues	Merely compresses normal tissue	Invades and destroys normal tissue

(Adapted from Tanaka, 1997)

The patterns of cancer occurrence can be grouped in two categories. The first comprise of hormone-dependent cancers such as those of female breast, endometrium, ovary, prostate, testis colon and male lung cancer. The second comprised of cancers linked to biological agents such as cancers of the liver with hepatitis B and C which are associated to with viruses; nasopharyngeal cancer and Burkitt lymphoma which are associated with Epstein-Barr-virus; Kaposis sarcoma and non-Hodgkin lymphoma which are associated with human immunodeficiency virus (HIV) and cervical cancer which is associated with human papillomavirus. These tumours are common in Asia, Latin and South America as well as Africa (IARC, 1997). The occurance of most cancers such as breast, prostate, testis, colon, lung, and thyroid cancer, as well as brain tumours, non Hodgkin lymphomas are increasing worldwide. The occurance of other cancers such as stomach or cervical cancer is decreasing (Annie, 2008).

2.2 Carcinogens

Carcinogens are substances which may increase the risk of getting cancer by altering cellular metabolism or damaging DNA directly in cells, interfering biological processes, and inducing the uncontrolled, malignant division, ultimately leading to the formation of tumours. Usually DNA damage, if too severe to be repaired, leads to programmed cell death, but if the programmed cell death pathway is damaged, the damage persist causing transformation of the mutant cells (Parkin *et al*., 2005).

Carcinogens include both natural and man made or man-enhanced substances (Eric, 1993). They range from chemicals, viruses, hormone, ionizing radiation, solid materials to mineral fiber (Peter and Curtis, 1994: Reddy *et al*., 2003). Chemical carcinogens can be converted into highly reactive compounds that can damage DNA and other cell components, or they can be detoxified and thus prevented from causing cellular damage. The metabolic fate of chemical carcinogens is linked to the activities of particular enzymes, protein molecules in the body that help chemical reactions to occur but are not themselves changed in the reactions. The activities of these enzymes can differ among individuals because of the occurrence of genetic polymorphisms (different forms of the genes that code for the enzymes) and the differing activities can either increase or decrease a person's susceptibility to environmental carcinogens (Holland *et al*., 1997).

Carcinogens may be divided into several classes (Table 2.2). Genotoxic carcinogens react with nucleic acids and directly affect cellular constituents. Ionizing radiation is a genotoxic carcinogen. Procarcinogens on the other hand, require

metabolic activation to induce carcinogens. Epigenetic carcinogens are those that are not genotoxic. Other carcinogens may change DNA expressesion without affecting its structure directly, or may increase susceptibility to DNA damage from other causes. These are known as nongenotoxic carcinogens or promoters. Arsenic and estrogen are nongenotoxic carcinogens. Still other carcinogens, such as nickel, may interfere with cell division and changing the number or structure of chromosomes in new cells after cell division (Zur Hausen, 2001; Parkin, 2006).

Susceptibility to the action of carcinogens is very complex and is affected by genetic heritage, behavior, physiology, nutrition, external exposures, and other factors of the host. For example, some chemicals are carcinogenic in their original form (direct carcinogens), while some must be metabolized in the body to their active form (indirect carcinogens). In such cases, individual susceptibility to a chemical carcinogen is affected by the rate at which the chemical metabolizes in the body into a cancer-causing form or into a harmless form. This rate varies from person to person (Holland *et al*., 1997)**.**

Table 2.2: Types of carcinogens

Type	Example
Genotoxic carcinogens	
Primary, direct-acting alkylating agents	Dimethysulfate, ethylene imine, 3-propiolactonel
Procarcinogens	
Polycyclic aromatic hydrocarbons, nutrosamines, hydrazine, inorganic	benzo[α]pyrene, dimethynitrosamine, 1,2-dimethylhydrazine, cadmium, plutonium
Epigenetic carcinogens/nongenotoxic	
Promoters, solid state, hormones, immunosuppressant, cocarcinogens	phorbol ester, saccharin, bile acids, asbestos, plastic, estrogens, purine analogues, catechol
Unclassified	peroxisome proliferators clofibrate, phthalate ester

(Adapted from: Reddy *et al*., 2003)

2.3 Carcinogenesis

Carcinogenesis refers to the development of neoplasia (Zur Hausen, 2001; Parkin, 2006). Once inside the body, most chemical carcinogens are metabolized. They are transformed by the physical and chemical processes in the body (Holland *et al*., 1997). Although there are many different forms of cancer, the basic multistage process by which various tumours develop is similar for all cancers.

Carcinogenesis is a multistage process driven by genetic damage and apigenetic change (Zur Hausen, 2001; Parkin, 2006). Recently, multistage carcinogenesis processes have been advocated, but the two-stage model is considered very convenient for the detection of carcinogenic and anticarcinogenic substances. Since the time Berenbelum describe two-stage mouse skin carcinogenesis model in 1994, the secrets of chemically induced carcinogenesis in several animal models have been well studied and the carcinogenesis process has become clearer, especially in the past decade (Tanaka, 1997). Cancer develops through four definable stages: initiation, promotion, malignant conversion and progression (Figure 2.1). These stages may progress over many years.

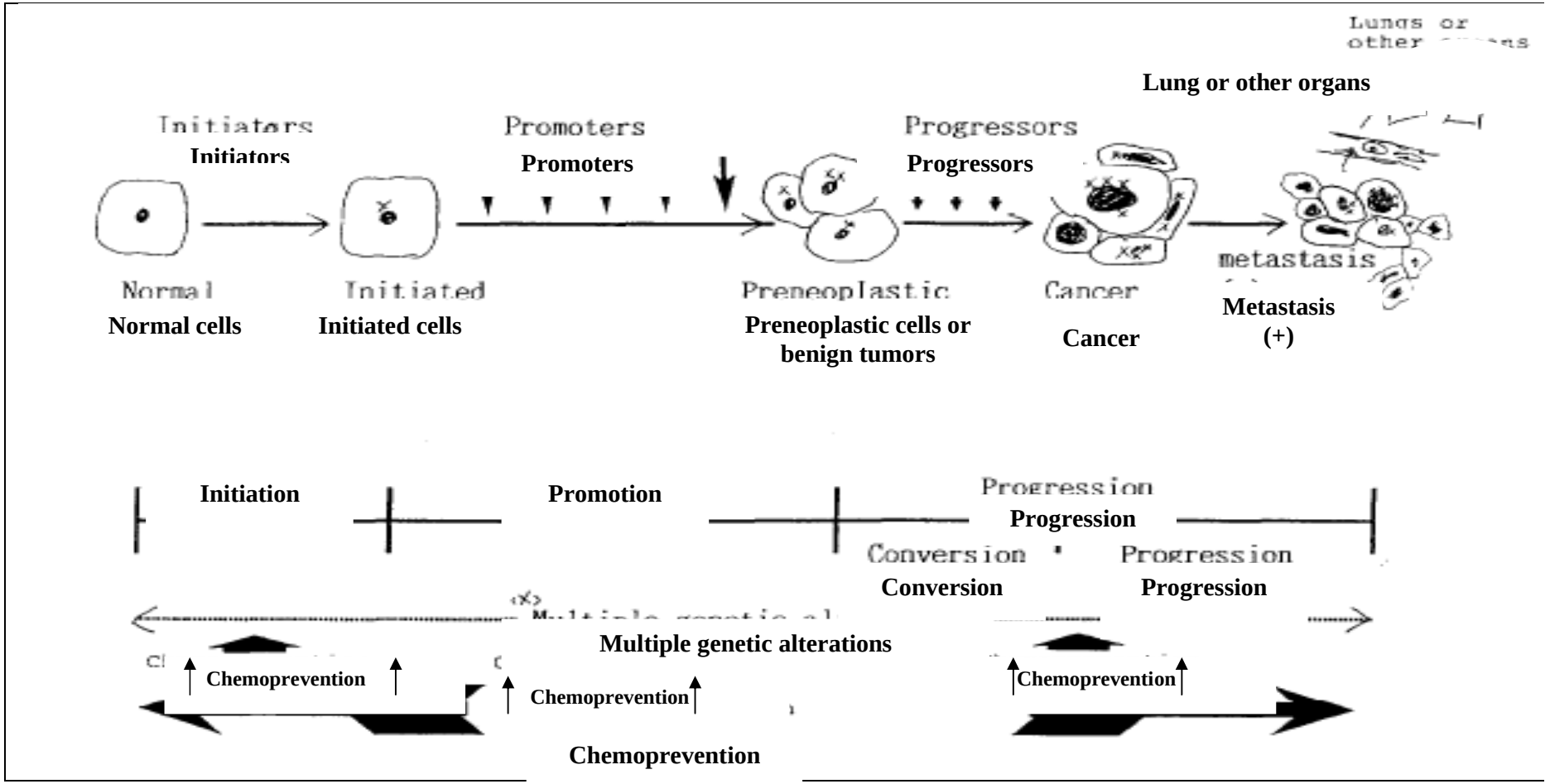

Figure 2.1: Multistage process of carcinogenesis (Tanaka, 1997)

Initiation

The first stage, initiation can result from a single application of a sub-carcinogenic dose of a carcinogen and it is a direct action on the target cell. Tumour initiation begins in cells through mutations from exposure to carcinogens (Peter and Curtis, 1994). An initiating agent is defined as chemical, physical or biological agents capable of directly altering in an irreversible manner, the native molecular structure of the genetic component (DNA) of the cells (Vimala, 1995).

Initiation process is divided into three substages. First, macromolecular binding and DNA adducts. Administration of chemical carcinogens to living animals or cells, whether by topical, subcutaneous, intragastric, intraperitoneal or any other route, results in absorption, transport, entry into cells and metabolism, generally with excretion of detoxified products (Laconi *et al*., 2008). The genotoxic groups of chemical carcinogens can be divided into direct and nondirect acting species, depending on whether not they require metabolism for reactive species to be generated. The enzymes involved include the phase I and phase II drug metabolizing species (Ito *et al*., 1995). The products of phase I may be more electrophilic then the parent and will bind to macromolecules in the cell. Hormones, irradiation, UV light and physical agents or procedures such as *in situ* freezing know to be capable of initiation.

Second, DNA repair and cell proliferation. From oxidative process alone, production of adducts by endogenous sources is exceedingly high and evoluttion has

presumably demanded and ability of cells to remove or repair damage to their DNA (Laconi *et al*., 2008). A number of repair systems have been generated. If cell devision occurs before repair can take place then any changes in one DNA strand will be replicated and no longer recognizable as and error. In fact it has even been postulated that proliferation is a requirement for initiation to occur (Ito *et al*., 1995).

Third, specific mutation / tissue character. Specific mutation has been linked with specific carcinogens and in the N-Methyl-N-nitrosourea (MNU) case. H-ras is apparently activated in 100% of induced mammary carcinomas. Transplancental induction of a specific H-ras mutation is described as critical for natal carcinogenesis and the same oncogen maybe involved in generation of esophageal papillomas by methylbenzylnitrosamine. Similarly, Ki-ras has been implicated in gastrointestinal dysplasias. For initiation of skin carcinogenesis covalent binding of adenine residues for 3,2-dimethyl-4-aminobiphenyl (DMBA) and guanine for benz[a] pyrene have been described to be of particular importance (Ito *et al*., 1995).

Promotion

The second stage known as the promotion stage is the longer stage. This stage can be divided into cell proliferation and altered phenotypic expression (Ito *et al*., 1995). The cell proliferation involves repeated application of irritating and promoting agent. A promoting agent is an agent that alters the expression of genetic information of the cells (Pitot *et al*., 1982). Examples of such agents include hormones, drugs and plant

products, which themselves do not directly react with the genetic material but rather affects its expression by a variety of mechanism including their interaction with cell surface receptors, with cytoplasmic and nuclear protein receptors or by alteration of other cellular components and function (Ito *et al*., 1995 and Laconi *et al*., 2008).

On the other hand, in alterated phenotypic expression, the process of promotion is mainly a quantitative phenomenon (many cells arising from a single cell). While no qualitative changes are necessarily implied, it is a fact that cell populations in early nodules, papillomas, or polyps are rather homogenous in size and shape or in the expression of specific biochemical markers (Ito *et al*., 1995).

The mutated cell is stimulated to grow and divide faster and becomes a population of mutated cells. The lesions are known as premalignant lesions (Holland *et al*., 1997). Eventually a benign tumor becomes evident. In human cancers, hormones, cigarette smoke, or bile acids are substances involved in promotion (Schottenfeld and Fraumeni, 1996). This stage is usually reversible as evidenced by the fact that lung damage can often be reversed after smoking stops (Holland *et al*., 1997).

Conversion

The process that transforms a preneoplastic cell into one that possesses malignant phenotype is known as the conversion process. This process starts with the

invasion of cells outside their normal tissue boundaries into surrounding tissues and often show changes in nuclear morphology (Kulesz-Martin, 1997). If the tumour does not break through the boundaries between tissues, it is known as "in situ" cancer (Holland *et al*., 1997). Increasing rate of exposure to DNA damaging agents and cell division can be mediated by the activation of proto-oncogens and inactivation of tumour suppressor genes.

Progression

During progression, there is further growth and expansion of the tumour cells over normal cells. The genetic material of the tumour is more fragile and prone to additional mutations (genetic alteration). A variety of consequences for the tumour cells ensue, including chromosome or gene amplification, translocations and rearrangement, deletion, and possibly also proto-oncogene activation and tumour suppressor inactivation (Ito *et al*., 1995). Karyotypic instability appears unique to the progression stage.

In situ tumours can develop further mutations, break through tissue boundaries, and invade surrounding tissues; at this stage, they become malignant tumours that can send cells throughout the body to establish new tumours (metastasis). During the development of a malignant tumour, DNA damage occurs as an accumulation of mutations in as many as six or more genes (Holland *et al*., 1997).

2.4 Cervical Cancer

Epidemiology

Cervical cancer is the second most frequent cancer type in women with yearly 450, 000 newly diagnosed women and 230,000 deaths worldwide. Approximately 75-80% of cancer cases are found in developing countries (Chris *et al*., 2009). The countries of Western Asia (2.9%), China (3.8%) and Japan (2.8%) have similar rates to those of European countries (4.4%) (Simcock and Shafi, 2007). On the other hand, sub-Saharan Africa, most of central and southern Asia, and south and Central America have higher incidence and mortality rates for cervical cancer (IARC, 2005).

Histological Types of Cervical Cancer

Invasive and preinvasive cervical lesions are classified by appearance of cells morphology. There are two main types of cervical cancers, namely *squamous cell carcinoma* and *adenocarcinoma*. About 80% to 90% of cervical cancers are squamous cell carcinoma (Jiade, 2004). Development of squamous cervical carcinoma is preceded by cervical intraepithelial neoplasia (CIN) of which CIN 3 in the most advanced (Bosch *et al*., 2002; Wright *et al*., 2002; Chris *et al*., 2009). Squamous cell carcinoma comprise of squamous cells that live the surface of the exocervix. Squamous cell carcinomas most often begin where the exocervix joins the endocervix (Kim, 2008).

The remaining 10% to 20% of cervical cancers are adenocarcinomas. Adenocarcinomas are becoming more common in women born in the last 20 to 30 years (Kim, 2008). Cervical adenocarcinoma develops from the mucus-producing gland cells of the endocervix. Less commonly, cervical cancers have features of both squamous cells carcinomas and adenocarcinomas. These are called adenosquamous carcinomas or mixed carcinomas (Jiade, 2004).

International Classification of Cervical Carcinoma

The activity of anticancer drugs is evaluated by measuring changes in tumour size in response to treatment (Therasse, 2002; Park *et al*., 2003). Tumour size has traditionally been estimated from bidimensional measurements (the product of the longest diameter and its longest perpendicular diameter for each tumour) (James *et al*, 1999; Park *et al*., 2003). In the early 1980s, the World Health Organization (WHO) developed recommendations in an attempt to standardize criteria for response assessment, and the WHO response criteria were adopted as the standard method for evaluating tumour response (Miller, 1981; Park *et al*., 2003). Table 2.3, shows the International Federation of Gynecology and Obstetrics (FIGO) staging of cervical cancer and have four satges in this classification. There are stages I, stage II, stage III and stage IV.

Table 2.3: International Federation of Gynecology and Obstetrics (FIGO) staging of cervical cancer (Simcock and Shafi, 2007).

Stage I: carcinoma strictly confined to the cervix - Stage IA Invasive cancer identified only microscopically; all gross lesions, even with superficial invasion - Stage IA1 Measured invasion of the stroma ≤ 3 mm depth and ≤ 7 mm diameter - Stage IA2 Measured invasion of stroma > 3 mm but ≤ 5 mm depth and ≤ 7 mm diameter - Stage IB cancers; invasion is imited to measured stromal invasion with a maximum depth of 5 mm and no wider than 7 mm - Stage IB Clinical lesions confined to the cervix or preclinical lesions greater than stage IA - Stage IB1 Clinical lesions ≤ 4 cm - Stage IB2 Clinical lesions > 4c m
Stage II: carcinoma that extends beyond the cervix but has not extended onto the pelvic wall; the carcinoma involves the vagina, but not as far as the lower-third section - Stage IIA No obvious parametrial involvement; involvement of as much as the upper two-thirds of the vagina - Stage IIB Obvious parametrial involvement, but not onto the pelvic sidewall
Stage III: carcinoma that has extended onto the pelvic sidewall and/or involves the lower third of the vagina; all cases with hydronephrosis or a non-functioning kidney should be included, unless they are known to be due to other causes - Stage IIIA No extension onto the pelvic sidewall, but involvement of the lower third of the vagina - Stage IIIB Extension onto the pelvic sidewall, or hydronephrosis or non-functioning kidney
Stage IV: carcinoma that has extended beyond the true pelvis or has clinically involved the mucosa of the bladder and/or rectum - Stage IVA Spread of the tumour onto adjacent pelvic organs - Stage IVB Spread to distant organs

Symptoms

Early-stage disease may be asymptomatic and detected on a smear or large loop excision of the transformation zone procedure. The classical symptoms are irregular vaginal bleeding, especially post-coital but symptoms may be absent until the cancer is in the advanced stage. Invasive cancer is rare in women with post-coital bleeding i.e vaginal bleeding that occurs after sexual intercourse, but assessment is merited as it much more common in this group than in the general population. Discharge and pain are often associated with more advanced disease (Simcock and Shafi, 2007).

2.4.1 Risk Factor for Cervical Cancer

Epidemiologic studies have identified a number of factors that play a significant role in the development of cervical intraepithelial neoplasma (CIN), a precursor to cervical cancer. There are a sexually trasmited diseases (STDs), sexual behaviour, having blood relative, aging and tobacco (Munoz, 2006).

Infection by HPV is basically a sexually transmitted disease (STDs). As such, both man and women are involved in the epidemiological chain of infection and at the same times are able to be asymptomatic carriers, transmitters and also victims of the infection by HPV and other viruses such as *Chlamydia trachomatis*, *Trichomonos* and *Cytomegalovirus* which are also associated with cervical cancer (Castellsague *et al*., 2003; Muñoz, 2006).

Women also increase their risk for CIN by engaging in other behaviors known to suppress the immune system. Suppression of the immune system due to HIV infection also is an important risk factor because it makes the cells lining the lower genital tract (vulva, vagina and cervix) more easily infected by the cancer-inducing types of HPV. There is substantial evidence that HIV-positive women are at increased risk of developing cervical cancer as well (Munoz, 2006). In two studies, both from high HIV prevalence areas, a statistically significant association between HIV and CIN was reported. Because the number of adolescents, as well as adults, with HIV is rising in most countries where cervical cancer is largely untreated, it can be expected that cervical cancer rates will continue to increase, especially in areas where STDs and HIV/AIDS rates are high. Other less common conditions that cause immunosuppression include those requiring chronic corticosteroid treatments, such as asthma (Castellsague *et al*., 2002 & 2003; Munoz, 2006).

An analysis stratified by HPV status in Colombia and Spain studies showed that risk factors of cervical cancer are associated with the individual's sexual behavior. The most important are early age at the start of the first sexual relationships, high number of sexual partners throughout life, sexual contacts with high risk individuals (in men, frequent contact with women that practice prostitution and in women, frequent contacts with men with multiple sexual partners) (Castellsague *et al*., 2002; Hogewoning *et al*., 2003; Bleeker *et al*., 2003). It is important to emphasize that at young ages and at the most sexually active ages in spite of very frequent infection by HPV, the great majority of infected women (more 90%) resolve the infection spontaneously and the infection persists in only a small fraction of women (Bosch *et al*., 2003; Elfgren *et al.*, 2000).

In Malaysia, a cross-sectional school survey of 12-19 year old adolescents is 5.4% (of which 8.3% were males and 2.9% were females) reported having had sexual intercourse (Lee *et al*., 2006). Median age at first sexual intercourse was 15 years; however, this estimate may be underreported given that talking about sex is culturally taboo subject in Malaysia.

Another risk factor is having a blood relative (mother or sister) with cervical cancer. Magnusson, *et al*., (1999) compared the incidence of dysplasia and carcinoma *in-situ* (CIS) in relatives of women with disease and in age-matched controls (Munoz, 2006). They found a significant familial clustering among biological, but not adoptive, relatives.

In many developing countries, women who have abnormal Pap smears frequently do not receive treatment at an early stage when cervical cancer could be prevented because of there are long delays in reading and reporting the results. Furthermore it is difficult to locate the patient once the report becomes available. Besides that the cost of treatment is not affordable for many women, even when simple outpatient procedures are used and there is a lack of equipment as well as service providers trained to use and maintain it. As a consequence, even in countries where Pap smears are available, many women may not get the treatment they need in a timely manner. According to the World Health Organization (WHO) Health Survey 2001/2002, Pap smear coverage was only 23%. The highest Pap smear uptake was among women aged 30-39 years (36.6%) as compared to women in other age group: 18-29 years (14.6%), 40-49 years (28.8%), 50-59 years (20.9%) and 60-69 years (5%) (WHO, 2002).

The number of women who smoke has increased dramatically in recent years especially among teenage girls. A survey carried out in 2005, showed that in Malaysia current smoker prevalence rate was 18.6% (Rosliza and Khadijah). Nicotine and the byproducts of smoking are thought to increase a woman's relative risk for cervical cancer because they concentrate in the cervical mucus and decrease the immune capability of Langerhan's cells to protect cervical tissue from invading oncogenic factors, such as HPV infection (Munoz, 2006).

2.5 Human Papillomavirus

Papillomaviruses consists of small double-stranded circular DNA genomes of approximately 8-kilobase pairs (Rapp and Chen, 1998; Castellsague, 2008). The human papillomavirus (HPV) is a non-enveloped virion with an icosahedral capsid. The HPV genome encodes eight early genes products, two late genes products (Table 2.4) and upstream regulatory region (URR, non-coding) (Soliman, 2004). These viruses infect squamous epithelial of a variety of species. Approximately 200 human papillomavirus (HPV) types have been described. HPVs cause a range of epithelial hyperplastic lesions and can be classified into two groups: mucosal and cutaneous. These groups can be further divided into low-risk and high-risk depending on the associated lesion's propensity for malignant progression (Margaret *et al*., 2008).

Table 2.4: Functions of the products of HPV early region open reading frames (Anderson, 2003).

Early region open reading frame	Protein function
E1	• Two proteins required for extrachomosomal DNA replication and completion of viral life cycle • Work with E2 products
E2	• Two proteins required for extrachromosomal DNA replication • Work with E1 products • Full length protein acts as a transcriptional activator and binds to DNA in the URR to increase transcription of the early region • Smaller protein inhibits transcription of the early region
E4	• Important for the maturation and replication of the virus • Expressed in later stages of infection, when complete virions are being assembled
E5	• Interacts with cell membrane receptors, such as EFG and PDGF • Might stimulate cell proliferation in infected cells
E6	• Crucial for viral replication, host cell immortalization and transformation • Binds to p53 and stimulates p53 degradation through ubiquitin-dependent proteolytic pathway
E7	• Crucial for viral replication, host cell immortalization and transformation • Binds to Rb proteins and dissociates E2F-Rb complex stimulating transcription of cellular genes

Figure 2.2 shows the electron micrograph of purified virus from a human skin wart in a cutaneous or mucosal epithelial cell negatively stained with potassium phosphotungstate (Howley, 1996).

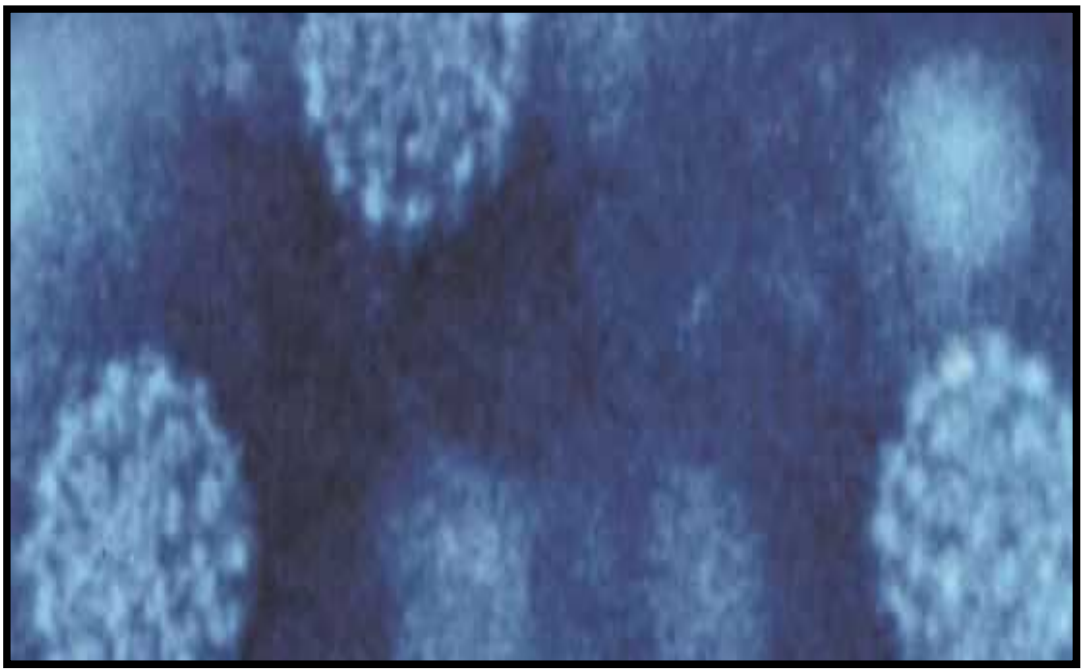

Figure 2.2: Electron micrograph of purified free (HPV) from a human skin wart negatively stained with potassium phosphotungstate (Howley, 1996).

2.5.1 Diversity Amongst Human Papillomavirus

Over 100 different HPV types have been described, with new types classified based on nucleotide and amino acid sequence alignment (Bernard, 2005; Castellsague, 2008). Intratypic variation may also occur with a specific HPV type, and these natural variants may demonstrate different biological properties. The distribution of these variants shows geographic variability and non-prototypic variants have been shown in some studies to increase the risk of cervical lesions and cancer. Although papillomavirus classification is based on nucleotide sequence homology, the differences between evolutionary groups are reflected to some extent in the differences that exist in the biology of the different viruses (Meyers *et al*., 1994). Approximately 35 HPV types have been isolated from the anogenital epithelium. The remaining HPV types have been associated with benign lesion (i.e. hand and plantar warts) and malignant skin lesion

that occur in a rare condition known as epidermodysplasia verruciformis. Table 2.5 shows the association of HPV types with lesion categories (Anderson, 2002).

Table 2.5: Association of HPV types with lesion categories

Lesion	HPV types
Common warts	1, 2, 3, 4
Plantar warts	1, 4
Flat warts	3, 10
Butcher's warts	3, 7
Epidermodysplasia verruciformus	3, 5, 8, 9, 10, 12-15, 17, 19, 20-25, 28, 29
Respiratory	6, 11, 30
Genital	6, 11, 16, 18, 30, 31, 33-35, 39, 40, 42, 43-45, 51-59, 66, 68, 70

(Adapted from Anderson, 2002)

Anogenital HPV types are often classified as low-risk or high-risk depending on their association with lesion types (Margaret *et al*., 2008). Low-risk viral types such as HPV 6 and HPV 11 are associated with low grade lesion, primarily with benign lesion such as condylomas which rarely progress to cancer. The high-risk HPV types, including HPV type 16 (HPV-16) and 18 (HPV-18) are commmonly associated with lesions that can progress to high-grade cervical intraepithelial neoplasia (CIN) and ultimately to cervical carcinoma and detected in almost 100% of squamous carcinomas and adenocarcinomas of the uterine cervix (Tahir, 1994; Rapp and Chen, 1998; Walboomers *et al*., 1999 and Pirog *et al*., 2000; Castellsague, 2008). The stratification of HPV types into low-risk and high-risk group is summarized in Table 2.6.

Table 2.6: Classification of HPV into low-risk and high-risk groups

Risk group	Type
Low	6, 11, 42, 43, 44
High	16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68, and 70

(Adapted from Margaret *et al*., 2008).

The progression of high risk HPV-induce lesions is depicted in Figure 2.3 and is mainly based on experimental findings within various stages of progression (De Villiers, 1992). This figure has shown the stepwise progression of high risk HPV induce lesion that can progress to low grade CIN, high grade CIN, carcinoma *in situ* and ultimately to invasive cancer. The inner circles schematically represent initially infected cells. The subsequent circle symbolized progressively modified clones with increased and growth potential.

Genetic modification	Clinical effect
Induction of chromsomal instability	No visible modification
Disruption of a signaling cascade (CIF I) interfering with viral oncogen function	Low grade CIN
Integration of viral DNA and partial deregulation of E6/E7 oncoprotein expression	High grade CIN
Disruption of signaling cascade under paracrine control (CIF II) suppressing viral oncogens transcription	Carcinoma *in situ*
Failing immunological control of HPV-modified cells	Invasive cancer

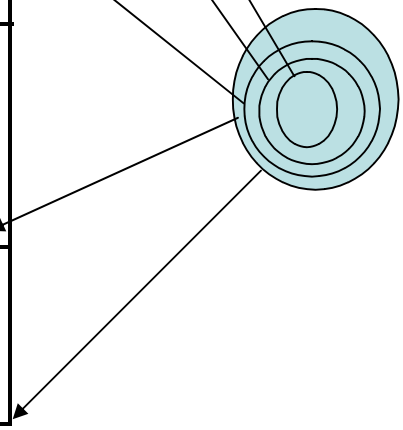

Figure 2.3 Stepwise progression of high risk HPV-induce lesion. The inner circle schematically represent initially infected cells, subsequent circles symbolize progressively modified clones with increased and growth potential.
(de Villiers, 1992).

2.5.2 Human Papillomavirus and Cervical Cancer

Human papillomaviruses (HPVs) are seen as the primary cause of cervical cancer because HPV genomes are detected in about 90% of all cervical cancer and encode proteins with molecular properties required for cellular transformation in cell culture and *in situ*. The development of cervical cancer intraepithelial neoplasm (CIN) and cervical cancer was reported be asociated with HPV 16 and frequently detected in cervial intraepithelial neoplasia (CIN) and invasive cervical carcinoma (ICC) (Igor *et*

al., 2003). Invasive cancer is preceded by a progressive spectrum of abnormalities of the cervical epithelium which are considered cancer precursors and classified as CIN 1 (mild dysplasia), CIN 2 (moderate dysplasia) and CIN 3 (severe dysplasia and carcinoma *in situ*). Flat cervical condylomas are considered part of the spectrum of CIN 1.

Progression of cervical intaepithelial neoplasia (CIN) to invasive disease is shown in Figure 2.4. CIN is classified in grades namely low grade squamous intraepithelial lesion (CIN 1) and high grade squamous intraepithelial lesions (CIN 2 and 3). CIN can start in any of the three stages, and can either progress to the next grade, or regress (Kumar *et al*., 2007). Most cases of CIN remain stable, or are eliminated by the host's immune system without intervention. However a small percentage of cases progress to become cervical cancer, usually cervical squamous cell carcinoma (SCC), if left untreated. However most of CIN spontaneously regress. About 50% of CIN 2 will regress within 2 years without treatment (Agorastos *et al*., 2005).

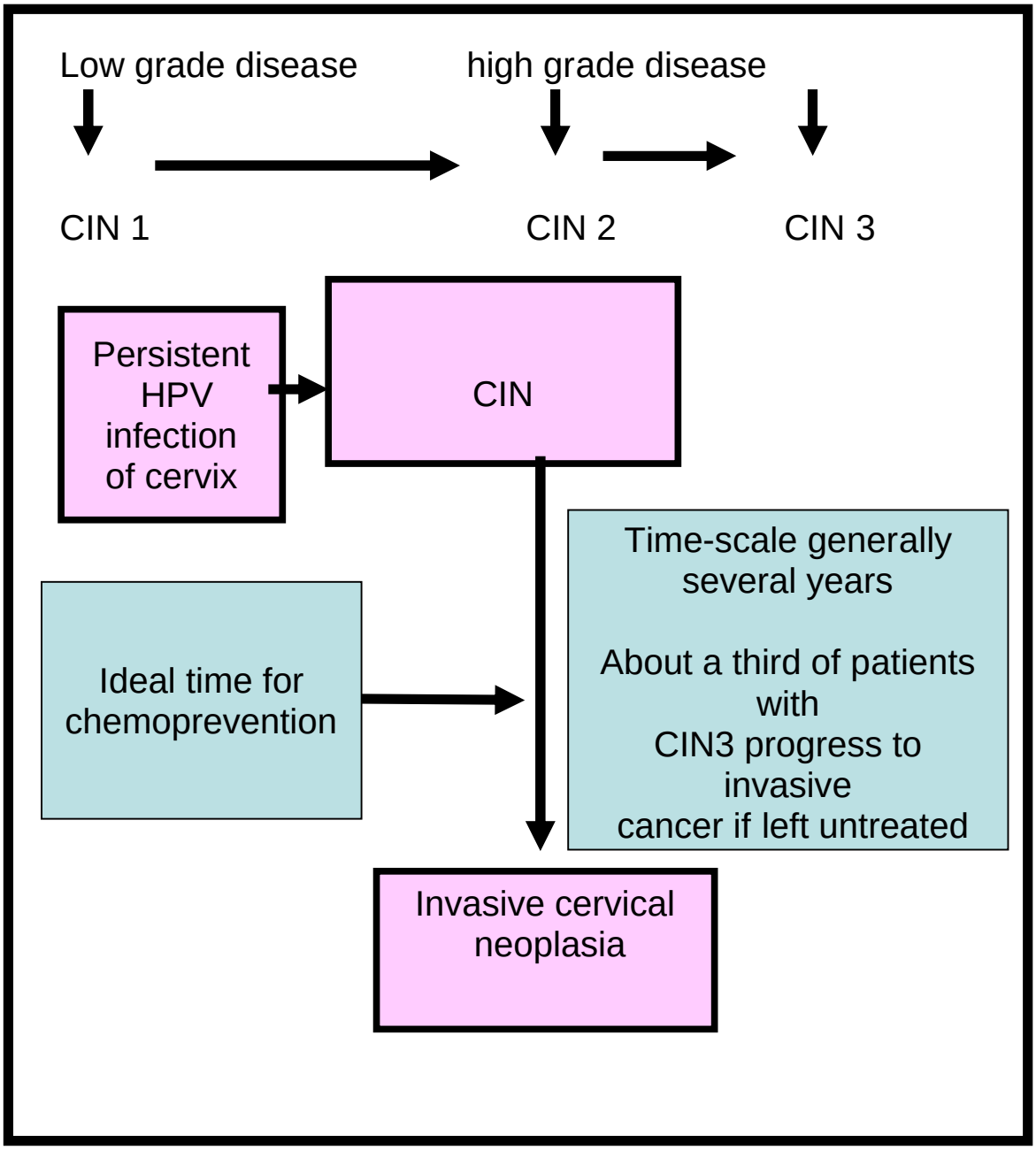

Figure 2.4: Progression of cervical intraepithelial neoplasia (CIN) to invasive disease (Adapted from Stanly and Margaret, 2003)

Figure 2.5 shown annual numbers of cases of various cancers worldwide. Gray bars show the total annual number of cases of various cancers worldwide. The fraction of cancers estimated to be induced by HPV types is shown in red. For example, nearly

all cases of cervical cancer and a substantial majority of anal cancers are believed to be the caused by HPV (Parkin 2006).

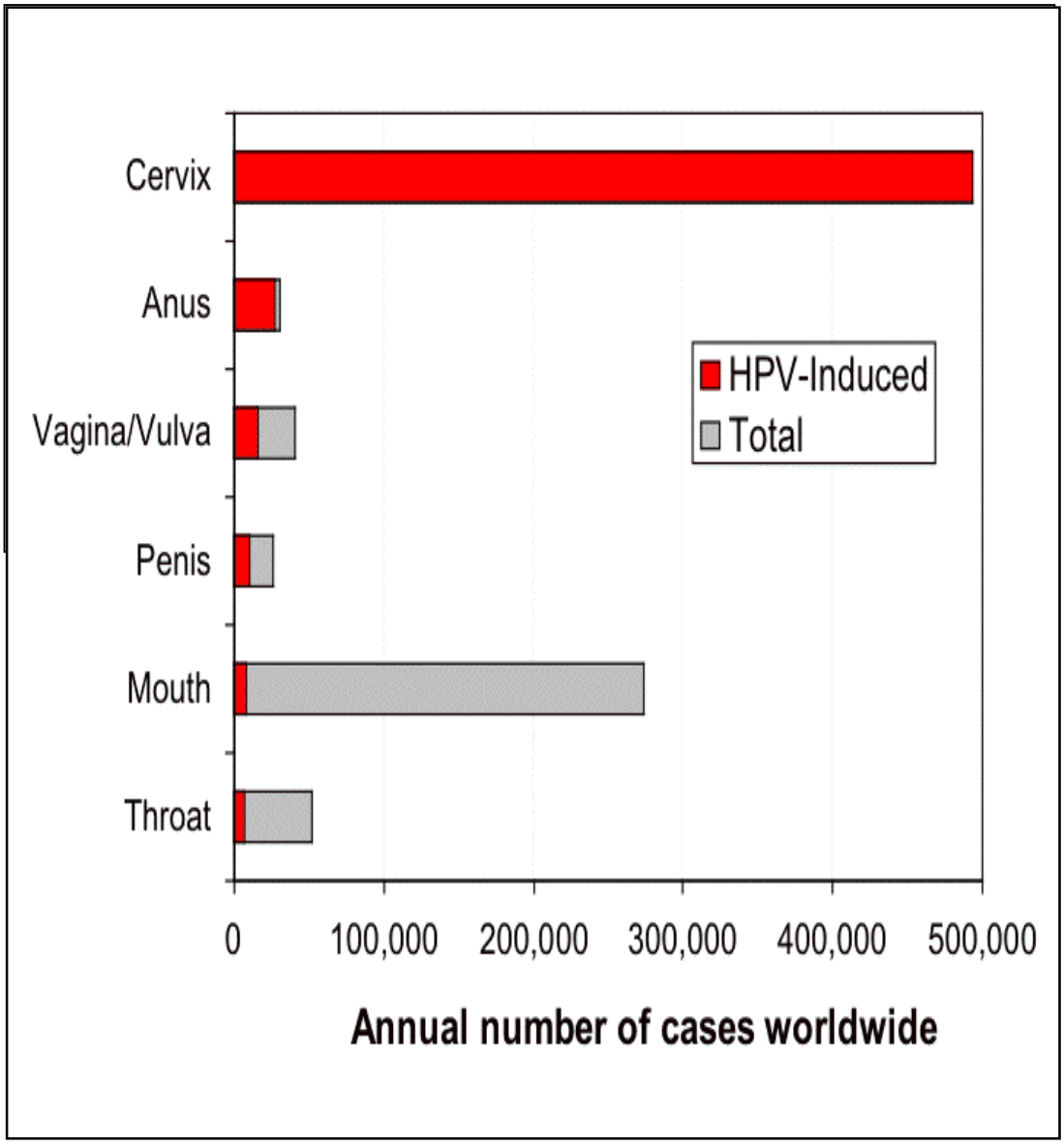

Figure 2.5 Annual numbers of cases of various cancers worldwide (Adapted from Parkin, 2006).

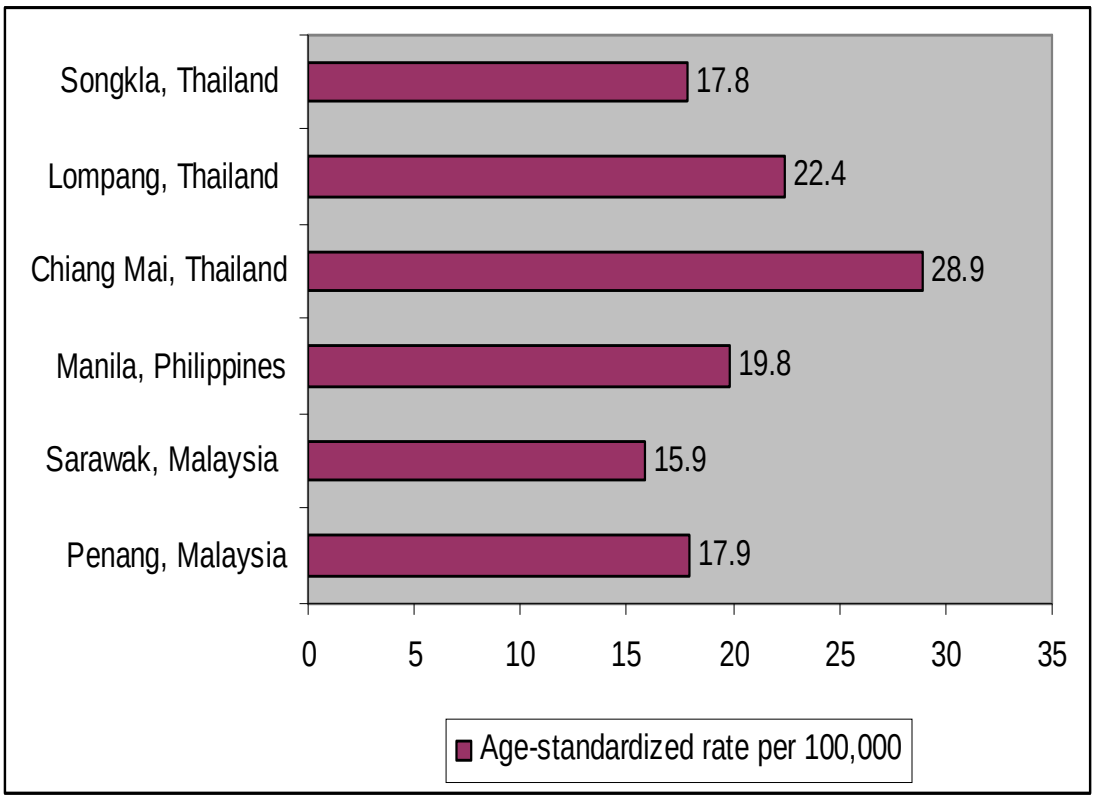

Figure 2.6: Age-standardized (world) incidence rates of cervical cancer by cancer registries (1998-2002) in Malaysia, the Philippines, and Thailand (Curado *et al.*, 2007).

Figure 2.6 shows age-standardized (world) incidence rates of cervical cancer by cancer registries (1998-2002) in Malaysia, the Philippines, and Thailand (Curado *et al.*, 2007). Malaysia has a population of 8.49 millions women ages 15 years and older who are at risk of developing cervical cancer. Current estimates indicate that every year 1492 women are diagnosed with cervical cancer and 766 die from the disease. Cervical cancer is the second most frequent cancer among women between 15 and 44 years of age (WHO, 2007). In Malaysia, the overall incidence rate in 2002 is 19.7 per 100,000 women, however differs by ethnic group. Ethnic Chinese women have the highest ASR of 28.8 per 100,000, followed by ethnic Indians with 22.4 and ethnic Malays (includes Peninsular Malaysia but not East Malaysia) with 10.5 per 100,000 women (Second reports of National Cancer Registry, 2004; Ferly *et al*., 2004).

2.5.3 Biochemical Properties of HPV E6 and E7 Oncoproteins

HPV 16 is a virus that infects several hundred thousand patients per year. Walter *et al*., 1999; was reported the E6 protein contains 158-amino-acid residue and contains two hypothetical Cys-X2-Cys-X29-Cys-X2-Cys (where X represents any amino acid and the number represents the number of residues) zinc fingers.

The oncogenic activities of papillomavirus E6 proteins have been reflected in many biological assays. These include immortalization of primary cells, transformation of established cell lines, resistances to terminal differentiation, tumorigenesis and abrogation of cell checkpoint (Rapp and Chen, 1998). The E6 protein by itself or in cooperation with HPV 16 E7 gene product can immortalize primary keratinocytes, fibroblast and epithelial cells (Halberd *et al*., 1991).

A Patscan search reveals that this sequence motif is unique for papillomavirus E6 as well as for E7 proteins (Rapp and Chen, 1998). E6 binding protein (E6BP) (also known as ERC-55) is a calcium binding protein localized in the endoplasmic reticulum, with possible consequences for intracellular signaling. E6 has also been described to activate or, alternatively to repress transcription, stimulate telomerase, immortalize primary cell culture and interfere with the differentiation of human keratinocytes (Walter *et al*., 1999).

The E6 protein promotes the degradation of the p53 tumour suppressor protein, whereas the E7 protein inactivates the Rb protein and related pocket protein, p107 and p130 (Munger *et al*., 2001). Alteration in the p53 gene including deletion, insertation and point mutation are the most frequent genetic events in many different carcinomas

(Takashi, 1992). However, the tumorigenic properties of the E6 and E7 proteins may not necessarily be limited only to the Rb and p53 to related pathway (Igor *et al*., 2003).

The E6 and E7 products from oncogenic viral types like HPV 16 and HPV 18 can binds to inactivate and cause the degradation of cellular tumours suppressor gene products p53 and Rb. This process plays an important role in the development of cervical cancer by a variety of mechanisms including altering host gene expression, releasing cell from cell cycle checkpoints, affecting the DNA repair process and/or activating the expression of telomerase (Rapp and Chen, 1998 and Anderson, 2003).

The viral E6 and E7 oncoproteins can alter the keratinocyte terminal differentiation phenotype and abrogate negative cell cycle controls to induce cells to enter S-phase. In doing so, the E6 and E7 proteins are establishing conditions in which viral DNA synthesis can occur in the differentiating epithelial cells. Cells constitutively expressing E6 and E7 proteins can pass by normal cell cycle checkpoints resulting in the accumulation of genetic damage which could ultimately result in malignant progression (Rapp and Chen, 1998).

P53 mutation is the most commonly reported change in human oncogenesis. Wild-type p53 has distinct DNA binding and transcription factor properties. The p53 protein levels and DNA binding activity are inducible by DNA damaging agents such as actinomycin D and irradiation. Induction of p53 is followed by the transcriptional activation of genes involved in DNA repair. Wild-type p53 also block cell cycle progression in response to DNA damage and has been implicated in apoptosis. Mutation or inactivation by viral oncogenes inhibit the transactivation function of p53 and subsequently affects its control on cell growth, cell cycle progression and apoptosis.

The major mechnanism of control of p53 expression appears to be through protein stability (posttranslational modification) and several observations support the hypothesis that HPV E6 modulates p53 function by targeting the protein for ubiquition and rapid degradation (Maria *et al*., 1999).

2.6 Treatment and Prevention of Cervical Cancer

The last few decades have seen a significant progress in the management of various cancers. In addition to traditional treatments such as radiation, surgery, and chemotherapy, several new approaches based on knowledge of biological processes have been developed and tested and are being constantly refined.

Radiation therapy also called radiotherapy, x-ray therapy, or irradiation is the use of a certain type of energy (called ionizing radiation) to kill cancer cells and shrink tumours. Radiation therapy injures or destroys cells in the area being treated (target tissue) by damaging their genetic material, making it impossible for these cells to continue to grow and divide (Bucci *et al*., 2005). Although radiation damages both cancer cells and normal cells, most normal cells can recover from the effects of radiation and function properly. The goal of radiation therapy is to achieve maximum toxicity in the tumour while limiting injury to the surrounding normal tissues. On the other hand, radiation sensitivity of normal tissues is affected by different tumour and patient factors, principally vascular co-morbidities and genetic susceptibility (Jiade, 2004).

Historically, the three main divisions of radiotherapy are external beam radiotherapy or teletherapy, brachytherapy or internal radiotherapy, and systemic radioisotope therapy or unsealed source radiotherapy (Bucci *et al*., 2005). The differences relate to the position of the radiation source; external is outside the body, brachytherapy uses sealed radioactive sources placed precisely in the area under treatment, and systemic radioisotopes are given by infusion or oral ingestion (Jiade, 2004). The radiation used in external radiation therapy can come from a variety of sources, including an x-ray, electron beam or gamma rays (Galvin *et al*., 2004).

Chemotherapy is a systemic therapy with anticancer drug in the treatment of cancer. Chemotherapy destroys cancer cells anywhere in the body. It even kills cells that have broken off from the main tumour and traveled through the blood or lymph systems to other parts of the body (Fischer *et al*., 2003). Chemotherapy can cure some types of cancer. In some cases, it is used to slow the growth of cancer cells or to keep the cancer from spreading to other parts of the body (Skeel *et al*., 2003). When a cancer has been removed by surgery, chemotherapy may be used to keep the cancer from coming back (adjuvant therapy). Chemotherapy also can ease the symptoms of cancer, helping some patients have a better quality of life (Fujiki *et al*., 1997; Morrow and Cowman, 2000).

Robotic surgery is the use of robots in performing surgery. Three major advances aided by surgical robots have been remote surgery, minimally invasive surgery and unmanned surgery. Some major advantages of robotic surgery are precision, miniaturization, smaller incisions, decreased blood loss, less pain, and quicker healing time (Pott *et al*., 2005). Further advantages are articulation beyond

normal manipulation and three-dimensional magnification. Robotic surgery is a minimally invasive alternative to laparoscopy for the surgical treatment of endometrial cancer and cervical cancer (Pott *et al*., 2005).

The Papanicolaou test (also called Pap smear, Pap test, cervical smear, or smear test) is a screening test used in gynecology to detect premalignant and malignant (cancerous) processes in the ectocervix. Significant changes can be treated, thus preventing cervical cancer. The test was invented by and named after the prominent Greek doctor Georgios Papanicolaou. Prior to the introduction of the Pap test, carcinoma of the cervix was a leading cause of death in women (Coste *et al*., 2003). Since the introduction of the Pap test, deaths caused by carcinoma of the cervix have been reduced by up to 99% in some populations wherein women are screened regular (DeMay, 2007).

2.7 Natural Products and Anticancer Drug

Natural products from plants are used for centuries to cure various ailments. Today, the use of bioactive plant-derived compounds is on the rise worldwide. This is due to the main apprehension in side effects from the use of synthetic drugs developed by pharmaceutical industries. There side effects can be more dangerous than the diseases, these drugs are claimed to cure. The demonstration of the presence of natural products such as polyphenols, alkaloids, flavonoids and other secondary metabolites in medicinal plants will provide a scientific validation for their popular use and may serve

as guide to assist in the selection of the plants with anticarcinogenic activity (Swayamjot *et al*., 2003).

Plants have played an important role as a source of effective anticancer agents, and it is significant that over 60% of currently used anticancer agents are derived in one way or another from natural sources, including plants, marine or organisms and microorganism (Cragg *et al*., 2005; Newman *et al*., 2003). The search for anticancer agents from plant sources started in earnest in the 1950s with the discovery and development of the vinca alkaloids, vinblastine and vincristine, and the isolation from the Madagascar periwinkle, *Catharanthus roseus* (Apocynaceae) which was used by various cultures for the treatment of diabetes (Cassady and Douros, 1980; Gueritte and Fahmy, 2005).

Each of these points should be considered when developing a new protocol or in using an unfamiliar protocol for the first time (Bruce, 1993). The process of drug discovery and development of anticancer agents involve substantial time, effort and resources. The approaches to identify the new therapies are constantly being evaluated and modified.

Plant alkaloids are antitumour agents derived from plants. These drugs act specifically by blocking the ability of a cancer cell to divide. Although they act throughout the cell cycle, some are more effective during the S-and M phase. Examples of plant alkaloids used in chemotherapy are actinomycin, doxorubicin and mitomycin.

The literature indicated that many natural products are available as chemoprotective agents against commonly occurring cancers worldwide. A major group

of these products are the powerful antioxidants, others are phenolic in nature and remainder includes reactive groups that confer protective properties. These natural products are found in vegetables, fruits, plant extracts and herbs. Although the mechanism of the protective effect in unclear, the fact that the consumption of fruit and vegetables lowers the incidence of carcinogenesis is widely reported (Reddy, 2003).

The antioxidants, vitamin C, E and the provitamin β-carotene from vegetables and herbs exert chemopreventive properties. Antioxidants are substances or nutrients in foods which can prevent or slow the oxidative damage to body. When the body cells use oxygen, they naturally produce free radicals (by-products) which can cause damage. Antioxidants act as "free radical scavengers" and hence prevent and repair damage done by these free radicals (Gueritte and Fahmy, 2005).

Chemoprevention trials investigate the abilities of specific dietary constituents or synthetic compounds to block or suppress the initiation or progression of carcinogenesis. Chemoprevention trials test potential cancer inhibitory agents in initial phase I (pharmacological and toxicological profile) and phase II (biomarker endpoint) trials to determine which agents have the most potential with regard to high efficacy and low toxicity. If findings in phase I and II clinical studies of a chemoprevention agents support the initial hypothesis, a phase III study is conducted to determine specific clinical outcomes (Greenwald *et al*., 1990).

Numerous epidemiological studies have demonstrated a protective effect of vegetable and fruit consumption on cervical cancer risk. Increase in vegetable intake up to 50% of all dietary products will decrease up to 50% risk of cancer (Ong, 2003) and

eating at least five fruits and vegetables a day may also help prevent cervical cancer (Berkely, 2003).

Amaranthus gangeticus have been found to be one of diserable source of effective cancer preventive agents because high frequency of inhibitory activity towards liver cancer (HepG2) and breast cancer (MCF7) cell lines (Sani *et al*., 2004). *A. gangeticus* extracts exhibited strong activity toward tumour promoter 12-O-hexadecanoylphobol-13-acetate (HPA)-induced Epstein Barr virus activation in Raji cells (Mukarami *et al*., 2000). The inhibitory effects were also observed in colon cancer cell line (Caco-2) but at a lower percentage compared to HepG2 and MCF7. In normal cells line (Chang liver), there are no inhibitory effects were observed (Sani *et al*., 2004).

Asparagus (*Asparagus oficinalis*) is a popular vegetable consumed in most parts of the world. Asparagus shoots which are frequently used in salads. The crude saponins from the shoots of asparagus (asparagus crude saponins; ACS) were found to have antitumour activity. The ACS inhibited the growth of human leukemia HL-60 cells in culture and macromolecular synthesis in a dose and time dependent manner (Shao, 1996). *A. officinalis* extracts have been found to exhibit moderate activity toward tumour promoter 12-O-hexadecanoylphobol-13-acetate (HPA)-induced Epstein Barr virus activation in Raji cells (Mukarami *et al*., 2003).

Citrus aurantifolia have been found showed inhibition of human colon cancer (SW-480) (Patil, 2009) and human lymphoblastoid B cell line (RPMI-8866). The citrus extracts also inhibited the Epstein-Barr virus early antigen (EBV-EA) activation induced by 12-O- tetradecanoylphorbol 13-acetate (TPA) as a useful screening method for antitumour promoters. Other researcher found that *C. aurantifolia* extracts exhibited

weakly activity toward tumour promoter 12-O-hexadecanoylphobol-13-acetate (HPA)-induced Epstein Barr virus activation in Raji cells (Mukarami *et al*., 2003).

Sesbania grandiflora extracts displayed cytotoxic activity against the HeLa, human cervical carcinoma cell lines (Mackeen, 2007). Other researchers showed that *S. gandiflora* extracts exhibited no activity toward tumour promoter 12-O-hexadecanoylphobol-13-acetate (HPA)-induced Epstein Barr virus activation in Raji cells (Mukarami *et al*., 2003).

2.8 Antitumour Compounds of Vegetables

Glycosinolates

Cruciferous vegetables are a rich source of glucosinolates and their hydrolysis products, including indoles and isothiocyanates, and high intake of cruciferous vegetables has been associated with lower risk of lung and colorectal cancer in some epidemiological studies (Fedrick *et al*., 2003). Glucosinolate hydrolysisproducts alter the metabolism or activity of sex hormones in ways that could inhibit the development of hormone-sensitive cancers. Isothiocyanates and indoles derived from the hydrolysis of glucosinolates, such as sulforaphane and indole-3-carbinol (I3C), have been implicated in a variety of anticarcinogenic mechanisms. Epidemiological studies indicate that human exposure to isothiocyanates and indoles through cruciferous vegetable consumption may decrease cancer risk, but the protective effects may be influenced by individual genetic variation (polymorphisms) in the metabolism and elimination of isothiocyanates from the body (Greenwald, 2001)

Carotenoids

Epidemiological studies have shown that people with high β-carotene intake and high plasma levels of β-carotene have a significantly reduced risk of lung cancer. However, studies of supplementation with large doses of β-carotene in smokers have shown an increase in cancer risk (possibly because excessive β-carotene results in breakdown products that reduce plasma vitamin A and worsen the lung cell proliferation induced by smoke (Alija *et al*., 2004).

Carotenoids were present in *Daucus carota* (carrot) and *Lycopersicum esculentum* (tomato). Blood levels of micronutrients beta-carotene (precursor vitamin A) have been inversely correlated with the development of cancer (Greenwald *et al*., 1990; Fredrick *et al*., 2003). Carotenoids incuding ß-carotene may affect cell transformation and differentiation, enhance cell to cell communication, and enhance immune responeses. ß-carotene is simply a marker for the actual substances in vegetables and fruits that may inhibit cancer development (Greenwald *et al*., 2001). Consumption of carotenoid juice reduce oxidative DNA damage in human lymphocytes by various mechanisms, possibly reduceding cancer risk (Greenwald *et al*., 2001).

Vitamin E

Vitamin E consists of 8 different molecules. There are 4 tocotrienols and 4 tocopherols. The vitamin E molecules all have right-handestereochemistry (McVean, 1999). Vitamin E has been associated to decrease risk for lung and cervical cancers (World Cancer research Fund, 1997). Vitamin E succinate (VES), a drivate of vitamin E, has been shown to trigger apoptosis of human prostate carcinoma cells *in vitro* (Israel, 2000). Report shows that vitamin E inhibited cell proliferation (Azzi, 2000).

Vitamin C

Early epidemiologic evidence indicated that high intakes of vitamin C, rich fruit and vegetables and a high vitamin C concentration in serum are inversely associated with the risk of some cancers (Lee e*t al*., 2003). A recent epidemiologic study by Khaw *et al*., 2001, showed that a high vitamin C concentration in plasma had an inverse relation with cancer-related mortality. In 1997, expert panels at the World Cancer Research Fund and the American Institute for Cancer Research estimated that vitamin C can reduce the risk of the stomach, mouth, pharynx, esophagus, lung, pancreas, and cervical cancers (World Cancer Research Fund, 1997).

Selenium

Selenium is effective in reducing cancer incidence in animal models, and epidemiologic data, as well as supplementation trials, have indicated that selenium is likely to be effective in humans (Holben, 1999). Experiments in variety of animal models have demonstated that selenium can inhibit carcinogenis. For example, selenium supplied as high-selenium broccoli significantly decreased the incidence of chemically-indicative of colon cancer, in rat (Finley, 2000). Selenium is a component of numerous selenoproteins (e.g. glutathione peroxidase, thioredoxin reductase) that function as enzymes in redox reactions that may affect cancer risk (Holben, 1999).

Retinoids

Apart from chemoprevention, retinoids could have a place in cancer chemotherapy or as radiation sensitizers. Radiation sensitizers act in a number of ways

to make cancer cells more susceptible to death by radiation than surrounding normal cells, and several such compounds are now available for the treatment of solid tumours (Park *et al*., 2003). In human cervix, retinoid have been shown to interact with the HPV proteins E6 and E7, thus restoring the tumour suppressive roles of pRB and p53 proteins. The oncogenicity of HPV is attributable to the E6 and E7 proteins and expression of these proteins is higher in tumours than in healthy tissue. Increased expression of E6 and E7 has also been shown in high grade cervical lesions such as CIN 3. Clearly, the retinoids are promising class of drugs that will potentially aid in combating cancer (Dianne, 2007).

2.9 Bioactivities of Selected Vegetables

Antigenotoxic Activity

Centella asiatica has been reported to exert antigenotoxic properties (Siddique *et al*., 2007). In, 2008 Siddique found that *C. asiatica* plant extract can modulate the genotoxicity of cyproterone acetate (CPA) of human lymphocytes *in vitro.* CPA is not only a tumour promoting agents but also a genotoxic chemical by generating free radicals and the excess of reactive oxygen species leads to the DNA damage. Therefore it can be concluded that *C. asiatica* extracts has the potential to reduce the genotoxic damage induced by CPA in cultured human lymphocytes (Siddique *et al*., 2008).

Apoptosis activity

Allium tuberosum contains thiosulfinates, which is used as folk medicine as well as to flavor foods. Thiosulfinates are known in the Allium species as unstable intermediates in the enzymatically initiated degradation of S-alk(en)yl-L-cysteine

sulfoxide (Kim, 2008). Thiosulfinates significantly induced cell death in dose-and time-dependent manners in HT-29, colon cancer cells and PC-3 cells, human prostate cancer cells which is associated with apoptosis. Thiosulfinates activated the initiator caspase-8, and-9 and the effector caspase-3. Thiosulfinates were found to increase the expression of AIF, a caspase-independent mitochondrial apoptosis factor and induced DNA fragmentation and chromatin condensation in HT-29 cells (Leea *at al*., 2009).

Antioxidant activity

Recently, there has been increasing interest in the protective biochemical function of phytochemical, especialy flavonoids from *Pisum sativum* and their related compounds for the prevention of oxidative damage to an organism caused by reactive oxygen species (ROS). Imbalance between ROS and antioxidant defence systems may lend to chemical modification of biologically relevant macromolecules like DNA. This pathobiochemical machanisme involved in initiation or development of various diseases including cancer (Troszyrisa *et al*., 2002).

Kudo were reported that *Solanum tuberosum* have antioxidant activities (Kudo *et al*., 2009). Similar result were observed in *Asparagus officinalis* where the composition of bioactive compounds and the antioxidant activity of a fibre-rich product obtained from asparagus by-product, which may be used as an ingredient in the preparation of additional food products (Alventosa, 2009).

Antifungal activity

An antifungal drug is a medication used to treat fungal infectin. A drug that has been used for 40 years for the treatment of skin fungus has been found to be a possible

cancer treatment. The antifungal drug, griseofulvin, has been shown to inhibit the growth of cancer cells in laboratory (Wilson, 2005).

Brassica oleracea var *alboglabra* were contain napin-like polypeptide (Ngai, 2004) were showed inhibited mycelial growth in number of fungal species including *Fusarium oxysporum, Helminthosporium maydis, Mycosphaerella arachidicola and Valsamali* and exhibited pronounced thermostability and pH stability (Lin and Ng, 2008).

Antiproliferative

Antiproliferative was used or tending to inhibit cell growth and effect on tumour cells and cruciferous vegetables are an important source of compounds that may be useful for chemoprevention (**Brandi** *et al*., 2005). *Brassica oleracea* var *alboglabra* inhibited proliferation of hepatoma (HepG2) and breast cancer (MCF7) cells with an IC_{50} of 2.7 mM and 3.4 mM and the activity of HIV-1 reverse transcriptase with an IC_{50} of 4.9 mM (**Brandi** *et al*., 2005; Lin and Ng, 2008). Antiproliferative activities were also studied *in vitro* using HepG(2) human liver cancer cells in *Amaranthus* sp (Spinach), *Capsicum annum* (red pepper) and *Amaranthus* sp showed the highest inhibitory effect, than the Capsicum annum (**Brandi** *et al*., 2005).

Antibacterial activity

Investigation of the traditional uses of *Momordica charantia* (Cucurbitaceae) in Togo (West Africa) showed that it is one of the most important local medicinal plants both for ritual and ethnomedical practices. There was a high degree of consensus

(>50%) for use in the treatment of gastrointestinal and viral disease (Beloin *et al*., 2005).

Leaf extracts (water, ethanol and methanol) of *Momordica charantia* have demonstaretd broad-spectrum antimicrobial activity (Khanna *et al*., 1998). *In vitro* antimicrobial activity of leaves extracts was seen against *E. coli*. An extracts of entire plant was also shown to have antiprotozoal activity against *Entamoeba histolitica* (Grover *et al*., 2004). Saeed and Tariq, 2005 were reported that the skin and seeds of *Pisum sativum* (green pea) exhibited good antibacterial activity with average zone of inhibition (Saeed and Tariq, 2005).

Antiviral activity

Medicinal plants have been traditionally used for different kinds of ailments including infectious diseases. There is an increasing need for substances with antiviral activity since the treatment of viral infections with the available antiviral drugs often leads to the problem of viral resistance (Vijayan *et al*., 2003).

Momordica charantia and several of it isolated phytochemicals, eg. alpha and beta-momorcharin, have been documented to have *in vitro* antiviral activity against Epstein-Barr, Herpes and Human immunodeficiency **virus** (HIV) promising anti Human immunodeficiency **virus** activity has been attributed to a isolated protein known as MAP 30 (Grover *et al*., 2004). Beloin *et al*., (2005) reported that lyophilized extracts of *M. charantia* plants were exerted strong antiviral activities against Herpes simplex virus-1 (HSV-1) and **Sindbis virus** (SINV).

Analgesic activity

Many ethnic groups have used different species of *Portulaca oleracea* (Beremi), a member of the Family Portulacaceae, as vegetable and also herbal medicine against several diseases for many centuries. The 10% ethanolic extract of the aerial parts (dried leaves and stem) showed significant analgesic after intraperitoneal and topical but not oral administration when compared with the synthetic drug, diclofenac sodium as the active control (Chan *et al*., 2000).

Other researcher found that *Portulaca oleracea* were evaluated for their analgesic activity using the hot-plate and tail-flick respone on albino mice and wistar rats, respectively. Results showed that 10% ethanolic extracts showed significant analgesic effects and was comparable with that of sodium diclofenac solution (Chan *et al*., 2000).

2.10 List of Vegetable

Table 2.7 shows the distribution, medicinal uses and phytochemistry of selected vegetables studies. In the present study, all the vegetables were evaluated for their cytotoxic and anti-HPV activities. All the vegetables are commonly found in Malaysia and have been documented for their medicinal uses and phytochemical conten

Table 2.7: The distribution, medicinal uses and phytochemistry of selected vegetable

No	Scientific name	Family name	Common name	Distribution	Medicinal uses	Phytochemical contents
1	Liliaceae	*Asparagus officinalis*	Asparagas	Not naturalized in any country and widely distributed in the Mediterranean countries	In India the root has uses as astimulant, restorative, demulcent, diuretic and antidysentric	Green shoots contains an abundance of amino-succinamic acid
2	Liliaceae	*Allium fistulosum*	Spring onions	Its original country is unknown. It has been also commonly cultivated in India, China and Japan	The bulb is antibacterial, antiseptic, diuretic, galactogue, stamachic, vermifuge and vulnerary	The leaves contains protein, fat, carbohydrate, vitamin B1 and vitamin C
3	Liliaceae	*Allium tuberosum*	Chinese chive	It has been cultivated in East Asia	The plant is antibacterial, digestive and stimulant	Both leaves and flowering clums contains of vitamin A, B and C, Protein, fat and carbohydrate
4	Solanaceae	*Capsicum annum*	Chili	It is know from prehistoric in Peru and widely cultivated in Central and South America	Fruits use for circulatory stimulant and pain reliever. Roots in a compound decoction given for gonorrhoea.	Fruits of chilies rich of solanine and capsaicin and vitamins A and C
5	Solanaceae	*Capsicum frutescens*	The bird pepper	Occurs as weed from northern South America to Southern USA	To treat digestive complaints, relieving colds and headaches	Fruits of chilies contains oleoresins and capsaicin
6	Solanaceae	*Lycopersicum esculentum*	Tomato	Native to Central and South America and have spread as far north as Mexico	Fruit is beneficial skin wash for people with oily skin. Sliced fruits used treatment for buns, scalbs and sunburns	The seed contains oil and solanine. Solanine and saponin are present in the green parts of the plant
7	Solanaceae	*Solanum melongena*	Brinjal	Have been domesticated in India.	Fruits are prescribed for use haemorrhoids. The root is applied for ulcerationFruits contains carbohydrates and proteins	Fruits contains carbohydrates and proteins

Table 2.7 continued

8	Solanaceae	*Solanum tuberosum*	The potato	It is native of the Andes. Ireland was the first country to cultivate potatoes	The fruits were reported for buns, corns, cough, cystitis, fistula, prostates, scurvy, spasm, tumour and warts	Potato contains the alkaloid solanine. The leaves and other green parts of the plant also contain solanine
9	Cucurbitacea	*Cucumis sativus*	Cucumber	The species was cultivated in France by the ninth century	The fruit is depurative, diuretic, emollient, purgative, and resolvent	Oil from seed contains linoleic acid, oleic acid, palmitic acid and stearic acid
10	Cucurbitacea	*Cucurbita moschata*	Muskmelon	The plant has been growing in America and it might be naturalized in Asia, Africa and America	The seed is vermifuge. It is eaten fresh or roasted for the relief of abdominal cramps	Fruits contains antioxidant, vitamin A, C, mineral and carbohydrate
11	Cucurbitacea	*Lagenaria sceraria*	The bottle gourd	Bottle gourd was carried from Africa to South America	The leaves have a purgative action. The bottle fruit is eaten for colic with fever. Externally, juice of the fruit with limejuice is an application for pimples. The pulp is applied to the head in delirium in India	The seeds contain saponin
12	Cucurbitacea	*Momordica charantia*	Bitter gourd	*M. chanrantia* is a genus about 42 species, mainly Africa, and several species are cultivated for their edible fruits	As a medicine, the vegetative parts a chiefly used. The fruits common to apply for skin. They are applied for buns, scalds, and to the abdomens of children for stomachache.	The bitter substance in the tissues is a glucoside. Along with it, in the leaves have small quantities of a oil and resin

Table 2.7 continued

13	Cucurbitacea	*Luffa acutangula*	Loofah	Angled loofah is probably native to India where the wild from with bitter fruits occur	A tea of leaves is used as a diuretic, while juice of the fruits is used against internal hemorrhage	Fruits contain colocynthin. The seed contains oils nearly 50% count.
14	Compositae	*Cosmos caudatus*	Ulam rajah	Native to scrub and meadow areas in Mexico, the southern United States, Central America and northern South America	The leaves recommended in the traditional medicine system for improving blood circulation	The leaves contains protein, calcium, fibre, vitamin A and essential oil
15	Compositae	*Lactuca sativa*	lettuce	It is through temperate and warm Europe, in Mediterranean and through the warmer temperate parts of western Asia	The seed is anodyne and galactogogue lettuce has acquired a folk reputation As an aphrodisiac, anodyne, carminative and diuretic	The seed of lettuce contains bland oil. It is rich in vitamin and minerals eg. sodium, phosphorus, calcium and magnesium
16	Leguminosae	*Archidendron jiringga*	Black pot	Native in Bangladesh, Borneo, Brunei, Burma, Indonesia, Kalimantan, Malaysia, Myanmar, Singapore, Sumatra and Thailand	The jering fruit is used to treat hypertension. How ever, an overdose can result in toxicity that can cause kidney hyperemia and difficulty in urinating	The seeds contain volatile oil consisting of an allyl sulphur compound an alkaloid and toxic jenkolic acid
17	Leguminosae	*Neptunia prostrata*	Winter mimosa	It is found almost as widely as the genus and in the Malay Peninsula	The Malays use the root in the late stages of Syphilis	Leaves contains carbohydrate, starch and vitamins

Table 2.7 continued

18	Leguminosae	*Pachyrrhizus erosus*	Yam bean	Native to Central America. It was brought to the Philippines and later become widely cultivated in India, China and east Africa.	The seeds are used in Java for applying to skin affection	From analysis, tubers contain 10% had carbohydrates, 1% protein, fat, albuminoids, and starchy matter
19	Leguminosae	*Psophocarpus tetragonolobus*	Botor bean	It came from the African side of the India Ocean, probably from Madagascar. The plant is now widely cultivated from India to New Guinea	Leaves used in a compound lotion for smallpox. The root is used in the Shan States for poulticing to cure vertigo	Seeds contain albuminiods, carbohydrates, oil, fat, starch and protein
20	Leguminosae	*Parkia speciosa*	Foul-smelling edible seeds	Native of Malaysia and often cultivated by the Malays	The petai seeds are used to treat diabetes and intestinal worms. The bark is considered to be tonic	Petai seeds contain β-carotene, thiamine, riboflavin, tannin, nitrates, nitrites, alkaloids, fatty acids and amino acids
21	Leguminosae	*Sesbania grandiflora*	Turi	It is a native of tropical north eastern Asia and planted widely from Africa to Pacific.	Bark, leaves and gums are considered medicinal. Resorted to be aperients, diuretic, emetic, emmenagouge, febrifuge, laxative and tonic	Leaf contains protein, fat, fiber, calsium, magnesium, thiamin, riboflavin, niacin and ascorbic acid
22	Leguminosae	*Vigna sinensis*	Long bean	It is probably a native of India and China and originally either Asiatic or African, and was spread to Europe early enough for the Greeks and Latins to grow it.	The juice of leaves is dropped hot into the ear forearm-ache	Bean contains fats and albuminoids

Table 2.7 continued

23	Leguminosae	*Pisum sativum*	Snow pea	Its origin can be traced to the stone age. The plant probably originated in Southwest Asia	Seed is contraceptive, fungistatic and spermicidal	Tender pods and peas are highly nutritive and contain a percentage of digestible protein, carbohydrates, vitamin and minerals
24	Leguminosae	*Phaseolus vulgaris*	French bean	Widely cultivated of all beans in temperate regions and widely cultivated in semitropical regions	Beans are said to be used for acne, bladder, buns, cardiac, carminative, depurative, diabetes, diarrhea, diuretic	Beans contains 20% proteins and at least 50% carbohydrates
25	Amaranthaceae	*Amaranthus gangeticus*	Spinach	Its original home is India. The Chinese cultivate it throughout a large part of China and its cultivation is nothing new to them when undertaken in Malaysia	A decoction made from the roots of plant and of *Cucurbita pepo* is used to control the resulting haemorrhage	The whole plant contains 62 per cent of starch in it, with 6 per cent of fat. It contains substantial amounts of vitamins A, B, C and double the amount of iron found in spinaches
26	Amaranthaceae	*Amaranthus viridis*	White bayam	Virgin in Hawaii and Island	The decoction of the plant is used to dysentery and inflammation. The plant is emollient and vermifuge	The seed contains protein and fat. Leaves contains calories, protein, carbohydrate, fiber and calcium
27	Malvaceae	*Hibiscus esculentus*	Ladies finger	The plant originated from west or central Africa and spread to Europe and the far east in Christian times. It was brought to the new world in the 1600s	The fruits is used for gonorrhoea, dysuria, catarrh and urinary troubles	The fruit contains sodium, potassium, calcium, magnesium, phosphorus, iron, copper, zinc, carbohydrate, protein, carotene, thiamin, riboflavin and niacin

Table 2.7 continued

28	Oxalidaceae	*Averrhoa carambola*	Starfruits	The carambola is believed originated in Cylon and moluccas but it has cultivated in Southeast Asia and Malaysia for many centuries	The ripe fruits is administered to relieved bleding hemorrhoids and the dried fruits of the juice may be taken to counteract fevers	Fruits contains protein, fat, carbohydrate, fiber, iron, carotene, thiamin, riboflavin and ascorbic acid
29	Euphorbiaceae	*Manihot esculenta*	Tapioca	It was first domesticated in central or south America. The cassava was taken to west Africa by the Portuguese	Fresh rhizome made into poultice is applied to sores, cancerous affections, condylomata, excrescences of the eye and tumour	The tubers contain starch with an average at about 26%with the starch, there are 1.5 to 2% of proteins, fat, carbohydrate, calcium, manganese, and vitamins A, B and C
30	Portulacaceae	*Portulaca oleracea*	Beremi	A succulent herb, found as a weed throughout the warmer parts of the world, in the Peninsular Malaysia in all the more inhabited parts	The leaves are used as a remedy for rheumatism. The leave juice is given to children for relief in bronchitis and diarrhea	Both steam and leaves contains Bacosides A and B, Stigmasterol beta-sitosterol, sapogenins, flavonoids, triterpinoid, saponins, alkaloids, brahmine and herpertine, D-manitol, betulic acid, octacosane, nicotine and amino acids
31	Convulvulaceae	*Ipomoea batatas*	Sweet potato	The plant is native to tropical America. It spreads to the Pacific Island	The tops are used for poulticing	The root tubers are contains 65% of water, 20% of starch, sugar, 25% of fermentable matter

Table 2.7 continued

32	Convulvulaceae	*Ipomoea reptans*	Kangkung	Probably originated in India and distributed throughout the tropics of the world. It is very abundant in Asia and Malaysia	The young plants is laxative and recommended for piles, and fever and applies buds to ringworm	leaves and shoots contains high amounts of iron
33	Umbeliferae	*Apium graveolens*	Celery	Its arrival in western Malaysia was from Europe. Celery is more cultivated in Java than in other parts of Malaysia	It is aromatic bitter tonic herb that reduces blood pressure, relieves indigestion, stimulates the uterus and is anti-inflamatory	Seeds contains glycoside-apiin and volatile oil
34	Umbeliferae	*Centella asiatica*	Pegaga	Native to Asia	Pegaga have been used for treating bronchitis, asthma and excessive secretion of gastric juice, dysentery, leucorrhoea, kidney trouble, urethritis and dropsy. This herb is said to have a direct action in lowering blood pressure	Pegega is reported to possess Asiatic acid, asiaticoside, madessiec acid, madecassoside, acorbic acid, β-sitosterol, stigmasterol, 3-glocosylkaempferol, hydroccotyline, riboflavin, carbohydrate, protein, calcium phosphorus and iron

Table 2.7 continued

35	Umbeliferae	*Daucus carota*	Carrot	It is ancient cultivation in the Mediterranean. It is native to Europe, Asia and North Africa	Roots are refrigerant and used in infusion for threadworm. The juice of the roots is applied to ulcers of the neck and uterus, cancer of bowels and stomach cancer	Carrots contains calcium and carotene (source of vitamin A)the leaves contain a volatile oil in small quantities
36	Umbeliferae	*Oenanthe javanica*	Shelum	An erect herb, which in the marshes of Java and is cultivate in Indo-China, Sumatra, and the Malaya Peninsula	The whole plant is depurative, febrifuge and styptic. A decoction is used in the treatment of epidemic influenza, fever and discomfort, jaundice, haematuria and metrorrhagia	The leaves a rich source of vitamins and minerals. The seed contains 3.5% essential oil
37	Chenopodiaceae	*Beta vulgaris*	Beetroot	Native to the Mediterranean but spread east world into west Asia but was only introduce into Germany and Britain around the sixteenth century	the roots as treatment for fever, constipation and aphrodisiac	Beetroot contains a large amount of sugar up to 8% mineral and boron
38	Chenopodiaceae	*Citrus aurantifolia*	Citrus	Native of eastern Malaysia. It was introduced to the Asian mainland early in historical time was and carried by Arab traders to the Middle East	Fruits are treat coughing headache, cold and flu	Contain limonene, terpinol bisabolene and essential oil

Table 2.7 continued

39	Rutaceae	*Brassica oleracea* var. *alboglabra*	Chinese brocoli	It probably originated in the Mediterranean	Traditionally, the leaves use for breast cancer treatment	Leaves contains protein, carbohydrate, calcium and vitamin C
40	Cruciferae	*Petroselium crispum*	Parsley	It probably originated in Spain and Portugal. It was introduced into Britain in 1548 from Sardinia	The Greeks used it only as a medicine. It was used as an edible plant dates in the Roman times	The leaves contains vitamin C

(Adapted from Joyce, 1991; Ong, 2003; Vimala *et al*., 2003; Reddy *et al*., 2003)

2.11 Tissue Culture

Cell culture is the process by which prokaryotic, or eukaryotic cells are grown under controlled conditions. In practice the term "cell culture" has come to refer to the culturing of cells derived from multicellular eukaryotes, especially animal cells. The historical development and methods of cell culture are closely interrelated to those of tissue culture and organ culture. Animal cell culture became a common laboratory technique in the 1950s, but the concept of maintaining live cell lines separated from their original tissue source was discovered in the 19th century (MacLeod, *et al.* 1999; Masters, 2002). The objective of tissue culture is to maintain viable single cells (cell culture) or a functional unit of cells (Organ culture) outside of their normal multicellular organism (Martin, 1994).

In tissue culture environment, the cells are protected from external pathogens. The tissue culture has a regular supply of nutrients and oxygen and the tissue metabolic-product, potentially toxic in high concentrations and removed at regular intervals. If the tissues are part of a mammal, its environment also included a constant temperature. In tissue culture, an artificial environment is created to replace the function of the missing tissue and organ systems (Martin, 1994).

2.11.1 HeLa Cell Line

A HeLa cell is an immortal cell line used in medical research. The cell line was derived from cervical cancer cells taken from Henrietta Lacks, who died from her cancer on October 4, 1951. HeLa cells contain 10-50 copies of DNA sequences HPV 18 and are the first human tumour cells to be established as a cell line (Massad *et al*., 1996). HeLa cells contain unintact genome of HPV 18, suggesting that the virus is not presenting high replication (tandem repeats) (Pater and Pater, 1985).

Karyotype (chromosome) analysis of HeLa cells from different repositories around the world show that different strains of HeLa cells are now very different from each other, probably due to the malignant nature of the cells and differences in culture conditions in different laboratories over the decade since this cell culture was established. Although HeLa cells provided a substantial foundation for today's knowledge of cells physiology, most analysis of cell structure and function in culture is now performed with non-transformed (non malignant) cells (Jones *et al*., 1971). The polyadenylated HPV RNA species present in HeLa cells were further analyzed by fraction in formaldehyde gels and found to be similar in size to those reported.

The HeLa cell line was derived for use in cancer research. These cells proliferate abnormally rapidly, even compared to other cancer cells. HeLa cells have an active version of the enzyme telomerase during cell division, which prevents the incremental shortening of telomeres that is implicated in aging and eventual cell death. In this way, HeLa cells circumvent the Hayflick Limit, which is the limited number of cell divisions that most normal cells can later undergo before dying out in cell culture (Terry, 2006).

2.11.2 CaSki Cell Line

CaSki cells are cancer cells derived from epidermoid carcinoma of human cervix, similar to HeLa cells, but contain 600 copies of HPV-16 DNA sequences (Lappalainen *et al*., 1994). In the CaSki cell lines the HPV-16 specific RNAs are abundant with three size classes measuring 0.7, 1.2 and 2.6 kb.

2.11.3 MRC5 Cell Line

MRC 5 cells are derived from normal lung tissue of a 14 week old male fetus (Terry, 2006). The cells are capable of 42 to 46 population doublings before the onset of senescence. The MRC-5 cell line was established in a growth medium consisting of Earle's Basal Medium in Earle's balanced salt solution supplemented with 10% calf serum. Following initial cultivation, subcultures were prepared twice weekly at a 1:2 ratio. When the cells reached approximately the 7th population doubling, the majority of the cultures were harvested to be used to prepare (Terry, 2006).

2.12 Cytotoxicity

Cytotoxic screening evaluates drug-induced alterations in metabolic pathways or structural integrity which may or may not be related directly to cell death (Wilson, 2000). Some cytotoxic assay offer immediate interpretation, such as the uptake of dye by dead cells. This has been termed tests of viability, and is intended to predict survival rather than direct measurement of cytotoxicity (Wilson, 2000).

Cytotoxic screening models provided important preliminary data to select plant extracts with potential antineoplastic properties. Anticancer compounds are substances that inhibit proliferation of cancer cells. They could bind to DNA in the cancer cells, inhibit certain enzymes which are necessary for continual growth of cancer cells (enzyme inhibitors), alter the morphology of cancer cells (immunomodulators) or cause total death (cytotoxic drug) (Derelanko, 1995).

Cytotoxicity can be measured by the tetrazolium salt 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, trypan blue (TB) assay, sulforhodamine B (SRB) assay, neutral red cytotoxic assay and clonogenic assay.

2.12.1 Neutral Red Cytotoxic Assay

Viable cells can be measured with colorimetric assays utilizing a substrate whose colour is modified by living cells, but not by dead cells (Kamissarova *et al*., 2005).

Neutral red assay is a rapid quantitative colorimetric test (Borenfreund and Puerner, 1985). The neutral red (NR) cytotoxic assay procedure is a cell survival/viability chemosensitivity assay, based on the capability of viable cells to integrate and bind (uptake of cationic supervital) neutral red (NR) (3-amino-7-dimethylamino-2-methylphenazine hydrochloride, a supervital dye into viable cells (Babich, 1987).

NR is a weak cationic dye that readily penetrates cell membrane by non-ionic diffusion, accumulates intracellularly in lysosomes, where it binds electrostatically to anionic sites in the lysosomal matrix (Bulyehev *et al*., 1978). Alteration of the cell surface or sensitive lysosomal membrane lead to lysosomal fragility and other changes that gradually becomes irreversible (Komissarova *et al*., 2005). Such changes brought about by the action of xenobiotics results in a decrease uptake and binding NR, making it possible to differentiate between viable intact cells and dead/damage cells, which is the basic of this assays. The NR assay was developed and successfully used to evaluate the cytotoxic effect of metal salts in mouse BALB/c 3T3 fibroblast (Borenfreund and Puerner, 1985).

The amount of NR dye accumulated can be extracted from lysosomes and quantitated spectrophotometrically and compared to NR dye recovered from untreated control cell culture (Borenfreund and Puerner, 1985). The results are expressed as IC_{50} values which can be obtained from dose-respone curve. IC_{50} values refer to the effective dose (concentration of extracts in μg/ml) that inhibits 50% of cell growth. Extracts having an IC_{50} value equal to or less then 20

µg/ml are considered active for cytotoxicity assay against target cells (Geran *et al*., 1972; Chiang *et al*., 2003).

2.13 Immunocytochemistry

Immunocytochemistry (ICC) is a common laboratory technique which uses antibodies that target specific peptides or proteins antigens in the cell via specific epitopes. These bound antibodies can then be detected using one of several methods. ICC allows researchers to evaluate whether or not cells in a particular sample express the antigen in question. In cases where an immunopositive signal is found, ICC also allows researchers to determine which sub-cellular compartments are expressing the antigen. Immunocytochemistry differs from immunohistochemistry in that the former is performed on samples of intact cells that have had most, if not all, their surrounding extracellular matrix removed. This includes cells grown within a culture, deposited from suspension, or taken from a smear. In contrast, immunohistochemical samples are sections of tissue, where each cell is surrounded by tissue architecture and other cells normally found in the intact tissue.

There are many methods to obtain immunological detection on tissues, including those tied directly to primary antibodies or antisera. A direct method involves the use of a detectable tag (e.g., fluorescent molecule, gold particles, etc.) directly to the antibody that is then allowed to bind to the antigen (e.g., protein) in a cell.

Alternatively, there are many indirect methods. In one such method, the antigen is bound by a primary antibody which is then amplified by use of a secondary antibody which binds to the primary antibody. Next, a tertiary reagent containing an enzymatic moiety is applied and binds to the secondary antibody. When the quaternary reagent, or substrate, is applied, the enzymatic end of the tertiary reagent converts the substrate into a pigment reaction product, which produces a colour (many colours are possible; brown, black, red, etc) in the same location that the original primary antibody recognized that antigen of interest. Presence of coloured product application of chromagen indicates a positive results or presence of the antigen under study. Immunocytochemical stains are used mainly when an H&E (Hematoxylin and Eosin) diagnosis cannot be made (Babich and Borenfreund, 1987).

Some examples of substrates used (also known as chromogens) are AEC (3-Amino-9-EthylCarbazole), or DAB (3,3'-Diaminobenzidine). Use of one of these reagents after exposure to the necessary enzyme (e.g., horseradish peroxidase conjugated to an antibody reagent) produces a positive immunoreactions product. Immunocytochemical visualization of specific antigens of interest can be used when a less specific stain like H&E (Hematoxylin and Eosin) cannot be used for a diagnosis to be made or to provide additional predictive information regarding treatment.