CASPASE-DEPENDENT APOPTOTIC MECHANISM OF GALLIC ACID AND ITS DERIVATIVES ISOLATED FROM *Quercus infectoria* ETHYL ACETATE EXTRACT AGAINST CERVICAL CANCER CELLS LINES (HeLa)

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UNIVERSITI SAINS MALAYSIA

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by

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LIST OF SYMBOLS

A	Absorbance
cells/mL	Cells per millilitre
°C	Degree Celsius
g	Gram
<	Less than
L	Litre
µg/mL	Microgram per millilitre
µm	micrometer
µM	Micromolar
mM	Milimolar
mg	Milligram
mg/mL	Milligram per millilitre
mL	Millilitre
M	Molar
>	More than
nm	Nanometer
%	Percentage
±	Plus minus
Rf	Retention factor
v/v	Volume per volume
λ	wavelength

LIST OF ABBREVIATIONS

A375	Human melanoma cell line
ACE	Acetone cotyledon extract
ADP	Adenosine diphosphate
AIF	Apoptosis-inducing factor
AJCC	American Joint Committee on Cancer
AML	Acute myeloid leukemia
ANOVA	Analysis of variance
AOPI	Acrodine orang propidium iodide
Apaf-1	Apoptotic protease activating factor 1
APL	Acute promyelocytic leukemia
ATCC	American Type Culture Collection
ATP	Adenosine triphosphate
AV/PI	Annexin V/propidium iodide
Bax	Bcl-2 Associated X-protein
Bax/Bak	Bcl-2 homologous antagonist killer
BCAR4	Breast cancer anti-estrogen resistance 4
Bcl	B-cell lymphoma
Bcl-2	B-cell lymphoma 2
BCLC	The Barcelona Clinical Liver Cancer
BH3	Bcl-2 homology domain 3
Bid	BH3 interacting-domain death agonist
Bim	Bcl-2-like protein 11
C	Carbon
CAM	Complementary and alternative medicine
Caov-3	Ovarian Cancer Cell

Caov-3	Ovarian cancer cell line
CARD	Caspase recruitment domain
CC	Column chromatography
CDK	Cyclin dependent kinase
CFDA-AM	5-Carboxyfluorescein Diacetate, Acetoxymethyl Ester
$CHCl_3$	Chloroform
CI	Confidence Interval
CIN	Cervical intraepithelial neoplasia
CIN1	Cervical intraepithelial neoplasia 1
CIN2	Cervical intraepithelial neoplasia 1
CIN3	Cervical intraepithelial neoplasia 3
CINV	Chemotherapy-induced nausea vomiting
CNS	Central nervous system
CO2	Carbon Dioxide
CRC	Colorectal cancer
CT	Computed Tomography
DCM	Dichloromethane
DFS	Disease-free survival
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DPPH	1,1-diphenyl-2-picryl-hydrazyl
DR	Death-receptors
EA	Ethyl acetate
EDTA	Ethylenediaminetetraacetic acid
EGFR	Epidermal growth factor receptor
ELISA	Enzyme-linked immunosorbent assay

EMO	Moringa oleifera ethanolic extract
ER	Endoplasmic Reticulum
FADD	Fas-associated death domain
FasL	Fas Ligand
FBS	Fetal bovine serum
FDA	Food and Drug Administration
$FeCl_3$	Iron (III) chloride
FITC	Fluorescein isothiocyanate
FTIR	Fourier Transform Infrared Spectroscopy
GAE	Gallic acid equivalent
GAE-TPC	Total phenolic content was expressed as gallic acid equivalent
GCMS	Gas chromatography–mass spectrometry
H	Hidrogen
H_2O	Water
H_2O_2	Hydrogen peroxide
HaCaT	human epidermal keratinocyte
HBMC	Heteronuclear Multiple Bond Correlation
HBV	Hepatitis B virus
HCC	Hepatocellular carcinoma
HCl	Hydrochloric acid
HCT116	Colon Carcinoma
HCV	Hepatitis C virus
HDAC	Histone deacetylase
HeLa	Human cervical cancer cell lines
HER2	Human epidermal growth factor receptor 2
Hex	Hexane
HGF	Human gingival fibroblasts

HIV	Human Immunodeficiency Virus
HMQC	Heteronuclear Multiple Quantum Coherence
HPV	Human Papillomavirus
HPV16	Human papillomavirus strain 16
HPV18	Human papillomavirus strain 18
HSC-2	Human oral squamous cell carcinoma
HSG	Salivary tumour cell lines
HSIL	High-grade squamous intraepithelial lesion
HUVEC	Human Umbilical Vascular Endothelial Cell
IARC	International Agency for Research on Cancer
IC_{50}	Half maximal inhibitory concentration
ICAD	Inhibitor of caspase-activated DNase
IFN	Interferon
IR	Infrared
IUPAC	International Union of Pure and Applied Chemistry
KBr	Potassium bromide
L929	Normal fibroblast cell line
LDH	Lactate dehydrogenase
LSIL	Low-grade squamous intraepithelial lesion
MAPK	Mitogen activated protein kinase
MCF-7	Human breast adenocarcinoma cell line
MDA-MB-231	Human breast adenocarcinoma cell line
MDCK	Madin-Darby Canine Kidney
Median OS	Median overall survival
MeOH	Methanol
MgCl 2	Magnesium chloride
MMP	Matrix metalloproteinase

MRI	Magnetic Resonance Imaging
mRNA	Messenger ribonucleic acid
mTOR	Mammalian target of rapamycin
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenultetrazolium bromide
NA	Not Available
Na_2CO_3	Sodium carbonate
NaCl	Sodium chloride
NADPH	Nicotinamide Adenine Dinucleotide Phosphate
$NaHCO_3$	Sodium hydrogen carbonate
NaOH	Sodium hydroxide
NBT	Nitroblutetrazolium
NCI	National Cancer Institute
NMR	Nuclear Magnetic Resonance
NO	Nitric oxide
NOXA	Phorbol-12-myristate-13-acetate-induced protein 1
NRU	Neutral Red Uptake
O_2-	Superoxide anion
OD	Optical density
OH	Hydroxyl radical
OS	Overall survival
p53	Tumour protein
PAP	Papanicolaous
PARP	Poly ADP ribose polymerase
PBS	Phosphate buffer saline
PC 12	Pheochromocytoma Cell Line 12
PCa	Prostate Cancer cell
PD-L1	Programmed Death-Ligand 1

PD-L2	Programmed Death-Ligand 2
PET	Positron emission tomograohy
PFS	Progression Free Survival
PGE2	Prostaglandin E2
pH	Power of Hydrogen
PI	Propidium iodide
PKM 1	Pyruvate kinase isoenzyme 1
PKM 2	Pyruvate kinase isoenzyme 2
PS	Phosphatidylserine
PUMA	p53-upregulated modulator of apoptosis
QI	Quercus infectoria
QIH	Quercus infectoria galls n-hexane extract
QIM	Quercus infectoria galls methanol extract
RNA	Ribonucleic acid
ROS	Reactive oxygen species
rpm	Rotations per minute
RR	Response Rate
S.E.M	Standard error mean
SD	Stable disease
SEM	Standard error of mean
SI	Selective Index
SIL	Squamous intraepithelial lesion
SMAC	Second mitochondria-derived activator of caspase
SPSS	Statistical Package for Social Science
SRB	Sulforhodamine B
STAT3	Signal transducer and activator of transriptase 3
TA	Tannic Acid

TBMS1	Tubeimoside-1
tBOOH	Tertiary butyl hydroperoxide
TCT	Thinprep cytological test
TEM	Transmission electron microscopy
TFA	Trifluoroacetic acid
TLC	thin-layer chromatography
TNF	Tumor necrosis factor
TNF/NGF	Tumor Necrosis Factor/Nerve Growth Factor
TNM	Tumor, Nodes and Metstases
TPC	Total phenolic content
TRAF-1	Tumor necrosis factor receptor associated factor 1
TTP	Time to progression
U 87	Human glioblastoma cell
UMT	Universiti Malaysia Terengganu
UniSZA	Universiti Sultan Zainal Abidin
UPM	University Putra Malaysia
USA	The United States of America
USM	Universiti Sains Malaysia
UV	Ultraviolet
V79	c Oxidant-induced carcinogenesis cells
VEGF	Vascular endothelial growth factor
VEGFR	Vascular endothelial growth factor receptor
Vero	Normal kidney cell line
VLP	Virus-like particle
WHO	World Health Organization
WST-1	Water Soluble Tetrazolium Salts-1

MEKANISME APOPTOSIS BERGANTUNG-CASPASE ASID GALIK DAN TERBITANNYA YANG DIPENCILKAN DARIPADA EKSTRAK ETIL ASETAT *Quercus infectoria* TERHADAP TITISAN SEL KANSER SERVIKS (HeLa)

ABSTRAK

Kanser serviks merupakan salah satu kanser yang utama dalam kalangan wanita di seluruh dunia. Pada tahun 2020, kanser serviks merupakan kanser keempat tertinggi yang paling banyak didiagnosis dalam kalangan wanita Malaysia. Aruhan apoptosis menjadi salah satu mekanisme terpenting dalam menghalang proses karsinogenesis. Banyak penyelidikan terdahulu menunjukkan bahan semulajadi mampu mengaruh apoptosis dalam rawatan kanser. *Quercus infetoria* (QI) telah dilaporkan pada kajian terdahulu mempunyai aktiviti antikuman, antioksidan, antikanser dan penyembuhan luka. Walaubagaimanapun, aktiviti antiproliferatif dan mekanisme molekular terhadap sel kanser manusia masih lagi kurang jelas. Oleh itu, kajian ini dijalankan untuk menjelaskan mekanisme kematian sel yang diberi rawatan menggunakan terbitan asid galik daripada ekstrak etil asetat QI (EAQI) terhadap sel kanser serviks manusia (HeLa). Asid galik (GA) dan terbitannya iaitu metil galate (MG) dipencilkan daripada kaedah bioasai pemencilan berpandu. Kesan antiproliferatif bagi EAQI, MG dan GA dicirikan dengan kepekatan yang merencat 50% populasi sel (IC_{50}) ditentukan dengan menggunakan asai MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] pada nilai kepekatan bermula daripada 0.39 sehingga 100 µg/ml pada 72 jam tempoh rawatan terhadap titisan sel HeLa dan titisan sel normal Vero. Cisplatin digunakan sebagai kawalan positif manakala titisan sel HeLa yang tidak dirawat dan titisan sel Vero digunakan

sebagai kawalan negatif. Perubahan morfologi pada sel diukur dengan menggunakan pewarnaan acridine orange/propidium iodide (AO/PI) pada 24, 48 dan 72 jam rawatan. Pengkelasan sel hidup, sel apoptosis dan sel nekrosis ditentukan menggunakan mikroskop floresen. Penentuan eksternalisasi fosfatidilserin menggunakan asai dwi pewarnaan annexin-V Fluorescein isothiocyanate (FITC)/ propidium iodide (PI). Semua sel dirawat pada tempoh 3, 6 dan 12 jam dan dianalisa dengan 'flow cytometry'. Untuk analisa kitaran sel, kandungan 'deoxyribonucleic acid' (DNA) di dalam sel HeLa diukur pada 24, 48 dan 72 jam dengan menggunakan 'flow cytometry'. Laluan apoptosis dijelaskan berdasarkan ekspresi protein pro and anti-apoptotik (p53, Bax and Bcl-2) an diukur pada 3 jam rawatan dan caspase (caspase 8 dan 9) dengan menggunakan teknik 'flow cytometry'pada 6 jam rawatan. Hasil kajian menunjukkan EAQI, MG dan GA masing–masing mempamerkan kesan antiproliferatif terhadap sel HeLa dengan bacaan IC_{50} adalah 11.50 ± 0.5 µg/ml, 11.00 ± 0.58 µg/ml dan 10.00 ± 0.67 µg/ml. Analisa morfologi sel menunjukkan sel yang diberi rawatan EAQI, GA dan MG dengan nilai IC_{50} mempamerkan peningkatan populasi sel apoptosis jika dibandingkan dengan sel tanpa rawatan ($p<0.05$) pada 72 jam rawatan. Aruhan apoptosis disahkan dengan eksternalisasi fosfatidilserin sel pada fasa awal apoptotik yang mencirikan pergerakan sel terawat daripada kuadran sel hidup kepada kuadran awal apoptotik. Berdasarkan analisa kitaran sel, pengumpulan sel yang dirawat pada fasa sub G_0 menunjukkan pemberhentian pemecahan 'deoxyribonucleic acid' (DNA) yang membawa kepada apoptosis. Tambahan lagi, hasil kajian menunjukkan ekspresi protein p53 dan Bax (pro-apoptotik) pada sel yang terawat manakala protein Bcl-2 (anti-apoptotik) tidak diekspresikan pada 3 jam rawatan. Analisa caspase pula menunjukkan ekstrak EAQI, MG dan GA mengaruhkan apoptosis dengan mengaktifkan caspase-8 dan -9 pada 6

jam rawatan. Kesimpulannya, penemuan ini mencadangkan bahawa EAQI, MG dan GA mengaktifkan kematian sel secara intrinsik dan ekstrinsik yang menyokong aktiviti perawatan dan ciri antikanser daripada ekstrak *Quercus infectoria*.

CASPASE-DEPENDENT APOPTOTIC MECHANISM OF GALLIC ACID AND ITS DERIVATIVES ISOLATED FROM *Quercus infectoria* ETHYL ACETATE EXTRACT AGAINST CERVICAL CANCER CELLS (HeLa)

ABSTRACT

Cervical cancer is one of the most common cancers in women worldwide. In 2020, cervical cancer ranked the fourth most diagnosed cancer among Malaysian women. The induction of apoptosis is one of the essential mechanisms to prevent the process of carcinogenesis. The previous study indicated that natural products were able to induce apoptosis and showed promising advantages in cancer treatment. The *Quercus infectoria* galls (QI) have been reported to have antimicrobial, antioxidant, anticancer and wound healing activities. However, the antiproliferative activity and the underlying molecular mechanisms against human cancer cells have been poorly elucidated. Hence, the present study was undertaken to examine the cell death mechanisms of gallic acid and its derivatives isolated from *Quercus infectoria* ethyl acetate extract (EAQI) against cervical cancer cells (HeLa). Gallic acid (GA) and its derivative, methyl gallate (MG), were isolated by using a bioassay-guided isolation technique. The antiproliferative effect that characterised by inhibitory concentration at 50 % cell populations (IC_{50}) of EAQI, GA and MG were determined by using the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] assay at various concentration ranging from 0.39 to 100 μg/ml at 72 hours of treatment in HeLa cell lines and the control serving non-cancerous Vero cell lines. Cisplatin was used as a positive control, while untreated HeLa and Vero cells served as the negative control. Changes in cell morphology were measured by acridine

orange/propidium iodide (AO/PI) staining for 24, 48 and 72 h. Viable, apoptotic and necrotic cells were identified using a fluorescence microscope. Determination of phosphatidylserine (PS) externalisation was performed using annexin-V Fluorescein isothiocyanate (FITC) / propidium iodide (PI) dual staining assay. The cells were treated for 3, 6 and 12 h and analysed by flow cytometry. Cellular deoxyribonucleic acid (DNA) content was measured in HeLa cells using flow cytometry at 24, 48 and 72 h for cell cycle distribution. Apoptosis pathways were elucidated based on pro and anti-apoptotic protein expressions (p53, Bax and Bcl-2) at 3 hours of treatment and caspases activity (caspase-8 and -9) were analysed by flow cytometry technique at 6 hours of treatment. The results showed that EAQI, MG and GA exhibited the antiproliferative effect on HeLa cells with IC_{50} values of 11.50 ± 0.5 µg/ml, 11.00 ± 0.58 µg/ml and 10.00 ± 0.67 µg/ml, respectively. In the cell morphology analysis, cells treated with IC_{50} value of EAQI, MG and GA displayed an increased apoptotic cell population compared to untreated cells ($p<0.05$) at 72 hours of treatment. The induction of apoptosis was confirmed by the externalisation of phosphatidylserine on early apoptotic cells, which showed the treated cell population shifted from viable to apoptotic quadrant. Based on the cell cycle distribution, the accumulation of cells at the $subG_0$ phase in treated cells indicated the discontinuity of deoxyribonucleic acid DNA fragmentation and led to apoptosis. Furthermore, the results showed that p53 and Bax (pro-apoptotic proteins) were expressed in the treated cells, whereas Bcl-2 (anti-apoptotic protein) was not expressed at 3 hours of treatment. The caspase analysis also revealed that EAQI, MG and GA had induced apoptosis by activating caspase-8 and -9 at 6 hours of treatment. In conclusion, these findings suggested that EAQI, MG and GA significantly induced apoptotic mechanisms via the regulation of

intrinsic and extrinsic pathways, which should provide new insight into therapeutic activity and anticancer agents of QI.

CHAPTER 1

INTRODUCTION

1.1 Background of the study

Cervical cancer is one of the most prevalent cancers discovered in females worldwide. According to World Health Organization (WHO), it is estimated that approximately 604,127 women were diagnosed with cervical cancer, with around 341,831 deaths attributed to the disease in 2020. Most cases occur in developing countries where access to screening and treatment is limited, highlighting the need for research to identify effective prevention and treatment strategies that are cost-effective and feasible to implement in resource-constrained settings (WHO, 2020). In Malaysia, cervical cancer ranks as the fourth most frequently diagnosed cancer incidence for women and has more than twice the rate of the Netherlands, the United Kingdom, and Finland even after introducing screening programmes and cervical cancer vaccination (IARC, 2020) (Figure 1.2).

Cervical cancer begins in the cervix and will invade other tissues near the cervix and organs such as the lungs or liver. Signs and symptoms of cervical cancer are irregular menstruation, weight loss, heavy menstruation, abnormal menstruation, vaginal discomfort and pelvic pain (WHO, 2018). Human papillomavirus (HPV) is known as the major risk factor of cervical cancer. Chronic viral infection with high-risk HPV genotypes is the causative agent which can be observed in 99.7% of cervical cancer patients worldwide (Walboomers et al., 1999).

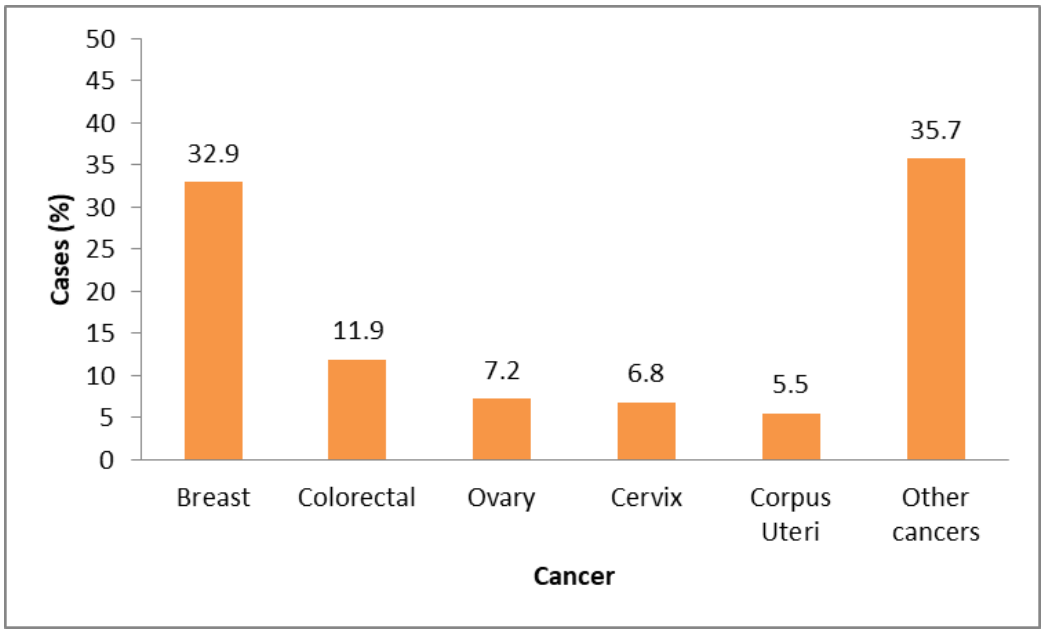

Figure 1.1 The percentage of the most common cancer for women in Malaysia Modified from IARC (2020).

Cancer treatments including chemotherapy, radiation and surgery remains the most common treatment for metastatic cancers (Smith et al., 2000). The purpose of cancer treatment is to promote the death of cancer cells by inducing apoptosis without harming the normal cells (Gerl & Vaux, 2005). Although these therapies have improved the long-term survival of cancer patients, they can cause severe and long-lasting side effects, such as the development of secondary cancer and infertility (Tajudin et al., 2012). Therefore, increased resistance to cancer treatment and adverse effects have led to the discovery and emergence of plant-derived chemotherapeutic drugs (Khalili et al., 2014). Medications derived from several plant species, such as vinblastine, vincristine, taxol, and camptothecin, have enhanced the chemotherapy of specific cancers (Mohan 2020).

1.2 Rationale of the study

In this regards, *Quercus infectoria* (QI), an oak plant that is locally known as "manjakani" has been chosen due to its wide usage and medical benefit in traditional practices worldwide, particularly among Malaysian women. QI consists of a greater amount of bioactive compounds such as gallic acids, tannins, amentoflavone, ellagic acid, β-sitosterol, methyloleanate, hexogalloyl glucose, methyl betulate and hexamethyl ether (Hwang et al., 2000).

Previous study demonstrated that, ethanol, methanol and aqueous extract of QI inhibited the growth of cancer cell lines including human cervical cancer cell line (HeLa) (Hasmah et al., 2010). The finding revealed a greater cell growth reduction in the treated HeLa with less cytotoxicity effect towards normal fibroblast cells, NIH/3T3. The inhibitory concentration (IC_{50}) in normal fibroblast cells, NIH/3T3 is greater. In addition, ethyl acetate extract of QI (EAQI) was found to exert cytotoxicity effect towards HeLa cells compared to other solvents extracts. DNA fragmentation and chromatin condensation indicated the apoptotic manifestation of EAQI in treated cells. Interestingly, EAQI also revealed the cytoselective effect towards normal cells (Wan Yusof & Abdullah, 2020).

Cytotoxicity study are generally performed because the bioactive compounds from plant-derived extracts may be targeted for use in pharmaceuticals or cosmetics industry (McGaw et al., 2014). From this finding, EAQI showed higher apoptotic activity and a better target to be studied. Therefore, the current study was designed to evaluate the cell death mechanisms of selected bioactive compounds (gallic acid derivatives) from EAQI extract in HeLa cells.

1.3 Objectives of the study

1.3.1 General objective

This study aims to evaluate the apoptotic mechanism of ethyl acetate crude extracts of QI (EAQI) and bioactive compound against cervical cancer cell lines (HeLa).

1.3.2 Specific objectives

1) To isolate, identify and elucidate the bioactive compounds from EAQI through bio-assay guided isolation.
2) To determine the antiproliferative activity of ethyl acetate crude extracts of QI (EAQI) and bioactive compounds in HeLa cells using MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] assay.
3) To assess morphological changes and cell cycle distribution in treated HeLa cells.
4) To confirm apoptotic detection of phosphatidylserine externalization in treated HeLa cells.
5) To determine the expression of tumour suppressor proteins (p53) and proteins related in mitochondrial-mediated apoptosis pathway (Bax and Bcl-2) and caspase analysis (caspase-8 and -9) in treated HeLa cells.

CHAPTER 2

LITERATURE REVIEW

2.1 Cancer

Cancer is a large group of diseases defined as abnormal cell growth beyond cell growth limits and invading other body parts and organs (Salinas-Santander et al., 2019) (Salinas-Santander et al., 2019). Cancer is also known to be a chronic disease as the normal cells are destroyed by the abnormal growth of cells in the body tissues (Amna et al., 2019). Neoplastic cells, newly growth cells transformed as they continue replicating, speciously unconscious to the regulatory influences that control normal cells (Kumar et.al. 2018)

The progressive transformation of malignant derivatives from normal cells infers the proliferation of certain genetic changes that can be inherited in the germ-line, the production of somatic mutations throughout the individual's life, or the introduction of viruses that ultimately lead to changes in the cell cycles and pathways for deoxyribonucleic acid (DNA) repair. This activates many oncogenic signaling pathways, resulting in a sequence of drastic phenotypic and biochemical cell shifts (Salinas-Santander et al., 2019).

The tumor known to be benign when it is relatively remaining localised and agreeable to local surgical removal. While the malignant is a lesion that can invade and destroy the adjacent structures and spread to distant sites to cause death. Metastasis is the spread of a tumor to the sites that are physically discontinuous with the primary tumor that indicates tumor as malignant. The malignant spread by the pathway of seeding within body cavities, hematogenous or lymphatic spread. A tumor

composed of undifferentiated cells known as anaplasia. Anaplasia, an indicator of malignancy shows the differentiation or loss of functional and structural of the normal cell (Kumar et al., 2018).

A great proportion of cancer is caused by the risk factor of cancer. Firstly, the environmental factors appear to be the dominant risk factor which is also supported by geographic variation. The most important environmental exposure linked to the cancer risk factors is diet, smoking, alcohol consumption, reproductive history and infectious agents (Kumar et al., 2018).

The diet has been implicated as predisposing influences because obesity contributes to the development of many other cancers. Furthermore, in reproductive history, there is a lifelong cumulative exposure to estrogen stimulation, particularly unopposed by progesterone, increase the risk of cancer development. Besides, infectious agents cause approximately 15% of cancer worldwide. In addition, the acquired predisposing condition is also one of the cancer risk factors where disorders related to chronic inflammation, immunodeficiency states and precursor lesions (Kumar et al., 2018). According to the American Cancer Society (2016), the most prominent risk factors that causes cancer among Asians are smoking, alcohol consumption and body weight.

2.2 Cervical cancer

2.2.1 Normal anatomy of cervix

The cervix is a part of the female reproductive system in the lower, narrow uterus region. The cervix links the body of the uterus to the vagina. The cervical mucosa is around 2 to 3 mm thick and significantly varies from the rest of the uterine endometrium in that it comprises large, branched glands. The cervix has two different parts which are covered with the two different types of cells. Endocervix, cervix closer to the body of the uterus is covered by mucus-secreting columnar epithelium, glandular cells. While the exocervix, part next to the vagina is covered with stratified squamous epithelium cells. Both cells meet at the transformation zone, where the location changes as the age increase and post-natal (Figure 1) (Cervical Cancer Action, 2009)

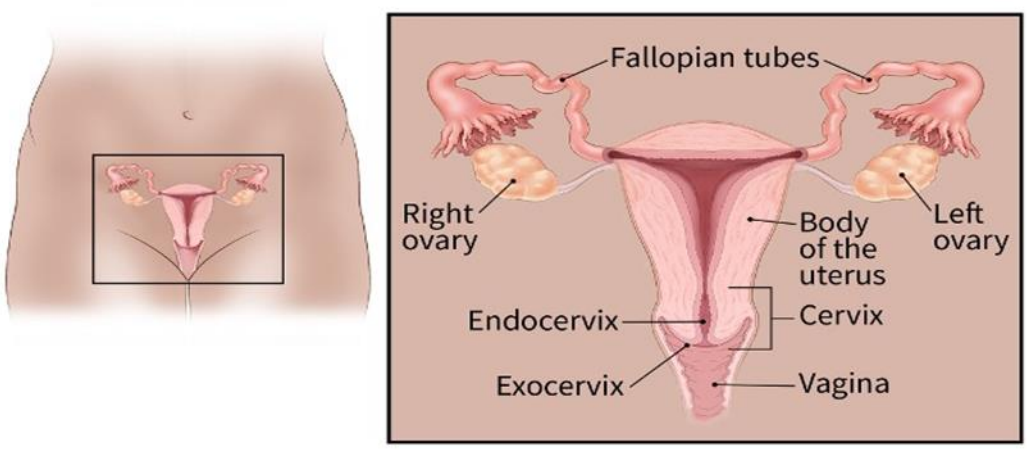

Figure 2.1 Anatomical structure of the cervix and female reproductive system. (Cervical Cancer Action, 2009)

2.2.2 Overview of Cervical Cancer

Cervical cancer occurs in the cells lining of the cervix that is located at the lower part of the uterus. Generally, cervical cancer begins in the transformation zone. Transformational zone cells do not suddenly change into cancer. The normal cells in this zone gradually develop pre-cancerous changes that turn into cancer. Pre-cancerous changes also are known as cervical intraepithelial neoplasia (CIN), the squamous intraepithelial lesion (SIL) and dysplasia (American Cancer Society, 2016a). SIL can be classified into two categories which are low-grade squamous intraepithelial lesion (LSIL) and high-grade squamous intraepithelial lesion (HSIL). LSIL is known to be the least serious pre-cancer compared to HSIL. A small percentage of tissue appeared abnormal in LSIL while HSIL shows an increase in proliferation and the maturation of epithelial arrested (Figure 2.2) (Kumar et al., 2018).

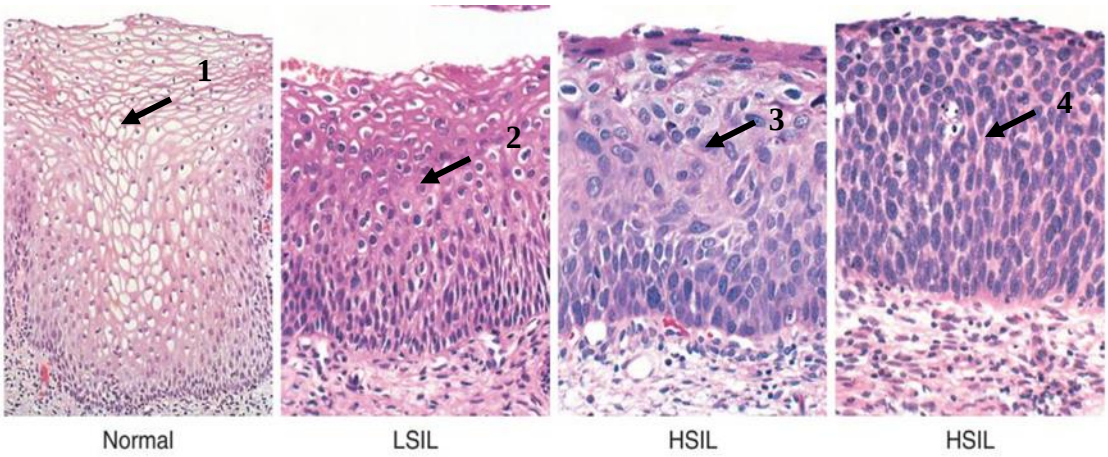

Figure 2.2 Spectrum of squamous intraepithelial lesion (SIL) with normal squamous for comparison. Arrow indicates (1) normal squamous epithelium, (2) CIN I with koilocytotic atypia, (3) CIN II with progressive atypia in all layer of epithelium and (4) carcinoma in situ with diffuse atypia and loss of maturation (Kumar et al., 2018).

2.2.3 Types and stages of cervical cancer

According to Kumar et al. (2018), the cervix's most prominent invasive carcinoma is squamous cell carcinoma (75%), accompanied by adenocarcinoma and adenosquamous carcinoma (20%) and small cell neuroendocrine carcinoma (<5%). Squamous cell carcinoma evolves as it begins in the transformational zone from the cells located in the exocervix region. Though, the adenocarcinoma develops from the mucus-producing glandular cells of the endocervix.

According to Wardak (2016) cervical cancer develops via a series of four stages (Table 2.1). Firstly, HPV transmission then proceeds to viral persistence followed by a progression of a clone of the persistently infected cell to pre-cancer and invasion.

Table 2.1 Development of cervical cancer.

Stages	**Development of cervical cancer**
1	HPV Transmission
2	Viral persistence
3	Progression of a clone of persistently infected cells to pre-cancer
4	Invasion

Source: Wardak (2016).

2.2.4 Causes and risk factors of cervical cancer

Cervical cancer dominantly caused by the DNA mutation which is a gene defect that turns on oncogenes or turn off tumor suppressor genes. HPV insists on the production of two proteins knows as E6 and E7 that turn off the tumor suppressor genes. This allows the cervical lining cells to grow at a higher rate and advance changes in additional genes, where at some cases will lead to cancer. There are more than 100 types of HPV have been identified, and among 40 of these viruses can cause genital tract infections (Villiers, 2001). This HPV is epitheliotrophic and can invade the definite type of epithelia (Doorbar, 2006). HPV is categorised into low, intermediate and high-risk subtype. The HPV high-risk subtype includes 16, 18, 31 and 45 which commonly associated with invasive cervical cancer compared to low and intermediate subtype (Munger et al., 2004).

HPV is a double-stranded circular DNA genome, comprising 7800-7900 base pairs, an unenveloped virion, and an icosahedral capsid. The genome is divided into three main regions, the non-coding upstream regulatory region and the protein-encoding early and late gene regions (Munger et al., 2004). Besides, the genome codes for 8 genes that are designed as E or L according to their expression in the early or late differentiation stage of the epithelium (Schiffman et al., 2007). The E1, E2, E5, E6, and E7 are articulated early in the differentiation, while E4 is expressed overall and L1 and L2 are expressed in the final differentiation phases. The E6 and E7 are prime HPV oncoproteins that have various cellular targets with the most prominent p53 and retinoblastoma tumor suppression protein (pRB) (Schiffman et al., 2007).

After infection occurred, the viral genome is maintained at the epithelium's basal layer. Upon the differentiation of the basal epithelial cells, the viral life cycle goes through the productive stages of genome amplification, where the virus assembly and virus release with a concomitant alteration in the patterns of the expressions from early genes to the late genes that assemble into the viral capsids (Figure 2.3) (Doorbar, 2006). During the infectious process, both of these proteins expressed at the lower levels. The E6 inhibition of p53 prevents apoptosis while the E7 inhibition of pRB repeals cell cycle arrest. At some stage in pre-cancer development, the function of the E6 and E7 is deregulated by either genetic or epigenetic changes, resulting in over-expression of full-thickness epithelial lesions (Schiffman et al., 2007).

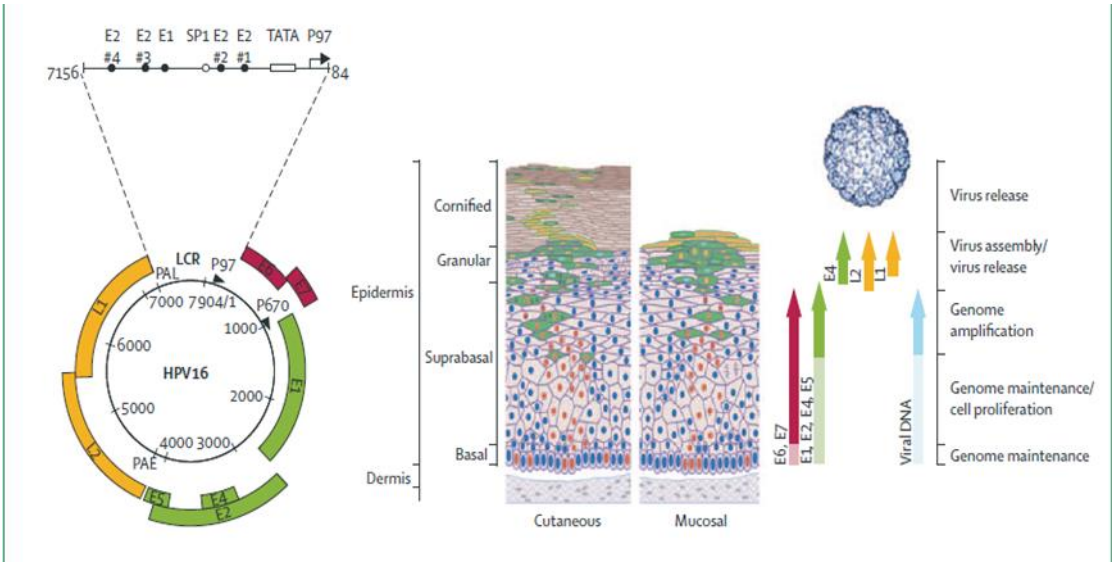

Figure 2.3 The HPV genome and the expression of the HPV genome within the epithelium (Doorbar, 2006).

Risk factors of cervical cancer include human immunodeficiency virus (HIV) infection, smoking, overweight and multiparity. Long term contraceptive pills consumption and having multiple full-term pregnancies are also risk factors of cervical cancer (American Cancer Society, 2016b). It is also known that more than 99% of cervical cancers are correlated to the infection of HPV and a certain type of HPV is carcinogenic in humans (Monsonego et al., 2004).

2.2.5 Diagnosis of cervical cancer

Cervical cancer does not exhibit any symptoms at the early stage and the pre-cancers stage. The symptoms appeared only when cancer grows and becomes larger into the nearby tissues. The symptom that commonly observed in cervical cancer is abnormal vaginal bleeding. The vaginal bleeds occur after sex and after post-menopause. The menstruation may also have occurred for a longer period or heavier than usual. Besides, the unusual discharge from the vagina is also a symptom where the discharge may be bloody and occurred between the menstruation period or at the post-menopausal. The common symptoms of cervical cancer include pain during sex and pain in the pelvic region. Besides, at the serious level of cervical cancer, signs, and symptoms such as leg swellings, haematuria, urination problem or bowel movement problem can be observed (American Cancer Society, 2016d).

2.2.6 Prevention of cervical cancer

The primary prevention of the HPV infection is through the health education programs that encourage avoidance, diligent use of condoms or both. This education could reduce the risk of cervical cancer at the population level. Furthermore, the

development of HPV L1 virus-like particle (VLP) vaccines is a possibly major improvement in the prevention of cervical cancer as the primary HPV vaccination. Screening test such as Pap test and HPV DNA test is the prevention of cervical cancer that is performed in the high-resources region (Schiffman et al., 2007).

2.2.7 Treatment of cervical cancer

2.2.8 Chemotherapy

Chemotherapy is significant cancer treatment, exclusively with its use as adjuvants to local therapy. Chemotherapy plays a role in efforts to relieve cancer-related symptoms and to improve survival in the advanced stage and metastasised tumor (Nygren, 2001). This therapy uses anti-cancer drugs that are given intravenously or orally. These drugs diffuse into the bloodstream and distributed to all body parts, and be the effective therapy to destroy cancer cells (American Cancer Society, 2016h). The addition of platinum-based chemotherapy to radiotherapy has increased survival rate compared with radiotherapy single-handedly; however, 30% to 50% unable to respond to treatment (Scatchard et al., 2012). Platinum-based chemotherapy drugs include cisplatin, carboplatin, and oxaliplatin (Oun et al., 2018). Quality of chemotherapy patient's life appeared to be the same for cisplatin and cisplatin-based combinations while the other chemotherapy found to be less effective or more toxic. The combination of other drugs with the cisplatin chemotherapy showed prolonged survival by a few months compared to cisplatin single-handedly but the side effects are higher (Scatchard et al., 2012).

Cisplatin, cisplatinum has also known as cis-diamminedichloroplatinum(II) is a metallic (platinum) coordination compound that has properties of anticancer activity

in multiple tumors. Cisplatin is also a significant component in the systemic treatment of germ cell cancer and one of the most effective medicinal drugs among many other chemotherapeutic drugs. The combination therapy of cisplatin with other cancer drugs has been applied as a novel therapeutic strategy for many human cancers regardless of drug resistance and considerable side effects by reducing the toxicity. The drug resistance found in several patients that have relapsed from cisplatin treatment. The suggested cisplatin resistance mechanisms entail changes in cellular absorption and cisplatin efflux, increased biotransformation and liver detoxification, increased DNA repair and anti-apoptotic mechanisms (Dasari & Tchounwou, 2014). In cervical cancer, cisplatin is combined with the tetraarsenic oxide to induce the apoptotic synergisms (Byun et al., 2013). Besides, it has been reported that the combination chemotherapy of cisplatin and the β-galactosyl-pyrrolidinyldiazeniumdiolate has a synergistic interaction against the human cervical cancer, glial cells of human brains and the gliosarcoma cell lines (Deng et al., 2013).

The mode of action of cisplatin has been related to its ability to crosslink with the DNA purine bases; interferes with the DNA repair mechanisms, causes disruption to DNA and ultimately induces apoptosis in cancer cells. The cisplatin initially is activated after it has entered the cells. In the cytoplasm, the water molecules display the chlorine atoms on the cisplatin (Dasari & Tchounwou, 2014). The hydrolysed product is a potent electrophile that is capable of interacting with any nucleophile, including the sulfhydryl group on protein and nitrogen donor atoms on the nucleic acids. Cisplatin bind to the N7 reactive center on the purine residues and as such can trigger damage to the DNA in cancer cells, preventing the cell division and contributing to the apoptotic cell death. However, many kinds of research implicated

DNA as a vital factor for the cytotoxicity of the cisplatin. Cisplatin also treats cervical cancer through the molecular mechanisms of cisplatin-induced oxidative stress, modulation of calcium signaling, cisplatin-induced cell apoptosis, signaling for DNA damage and modulation of gene expression (Figure 2.4) (Dasari & Tchounwou, 2014).

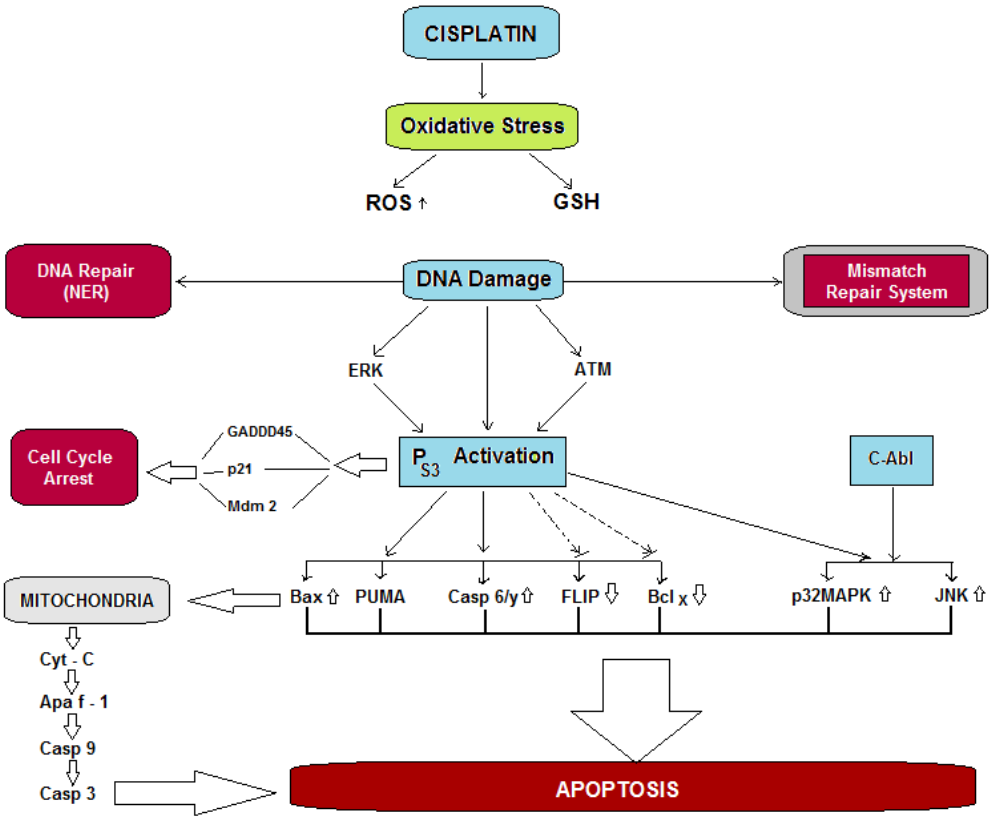

Figure 2.4 The overview of molecule mechanisms of cisplatin-induced oxidative stress in cancer treatment. Modified from Dasari & Tchounwou (2014).

Cisplatin chemotherapy accompanying with the extensive side effects. The root cause for the cisplatin cytotoxicity is the formation of the covalent adduct with purine DNA bases due to the interaction of cisplatin and DNA (Dasari & Tchounwou, 2014). The cytotoxicity of cisplatin includes nephrotoxicity. The excessive accumulation of the cisplatin in the kidney tissues, as the main route for its excretion,

contributes to cisplatin-induced nephrotoxicity. The cardiotoxicity is also proven where many cases reported cardiac diseases such as electrocardiographic changes, arrhythmias, myocarditis, cardiomyopathy, and congestive heart failures. High cisplatin dosages can also contribute to hepatotoxicity.

The reduction in the antioxidant defence system is reported due to the oxidative stress from the formation of reactive oxygen species, including the antioxidant enzymes and non-enzymatic molecules, decreased glutathione are the main alterations in the cytotoxicity of the cisplatin. Several studies have shown that combination therapy with cisplatin represents the best therapeutic approaches to overcome drug resistance and reducing the undesirable side effects (Dasari & Tchounwou, 2014). Therefore, if the platinum-based drugs are to continue to play a potential role in chemotherapy, improved control of their side effects is required, more so than creating new platinum-based drugs (Chen et al., 2013).

2.2.9 Surgery

Surgery for cervical cancer can be performed for both pre-cancer and invasive cancer (American Cancer Society, 2016f). Firstly, cryotherapy is one of the surgical methods in the pre-cancer stage. Cryonecrosis is achieved from the hypothermia produced by the evaporation of compressed refrigerant gas passing through the base of the metal probe placed into the transformation zone. The results appear to be patchy as the sub lethal damage to the tissue tends to arise at the probe's edge. But, this treatment is associated with unpleasant vasomotor symptoms (Dorsey, 1991). Besides, excisional surgery caused conisation where cone shaped-piece of tissue removed from the cervix (American Cancer Society, 2016f). These treatments ensure less cervical

damage and low blood loss but the specimen might undergo thermal damage and cause difficulty in the histological margin assessment (Dorsey, 1991).

Simple hysterectomy, trachelectomy, and pelvic exenteration is the surgical treatment for invasive cervical cancer. Simple hysterectomy performed by the removal of uterus without the structures and organs beside. This treatment can result in unusual complications and infertility. Moreover, trachelectomy leads to a high risk of miscarriage. This is because this treatment was performed by removing the cervix but not the body of the uterus and permanent purse-string is stitched (American Cancer Society, 2016f).

2.2.10 Immunotherapy

Cancer immunotherapy is a treatment that strengthens or activates the immune system to elicit the anti-tumor activity (Menderes et al., 2016). Immunotherapy has emerged as a novel alternative treatment for advanced cervical cancer (Baettig et al., 2019). The immunotherapy in cancer treatment established with the recent introduction of immune checkpoint inhibitors which release the brakes for the immune suppression. For cervical cancer, the clinical trials conducted on multiple checkpoint inhibitors. Based on the monotherapy approaches, the pembrolizumab and nivolumab appeared regular in cervical cancer, but longer observational studies required in the future. While immunotherapy and chemotherapy are not a curative as a monotherapy, accumulating evidence proposes that the combination treatment has the prospective to improve clinical results in advanced cancer stages (Menderes et al., 2016).

Currently, to achieve a greater responsibility towards cervical cancer, the immune checkpoint inhibitor studies being conducted with combination therapy. This combination therapy is based on the combination of existing therapy such as chemotherapy and radiotherapy with other molecularly targeted drugs. Thus, ongoing studies are testing the addition of immune control point inhibitors to systemic chemotherapy (paclitaxel/carboplatin, or bevacizumab cisplatin) in recurring advanced cervical cancer. For better development and establishment of this immunotherapy in cervical cancer, more studies of the immune-related genes through the polymorphism analysis and the immune of the tumor microenvironment are required (Kagabu et al., 2019).

2.2.11 Radiotherapy

Radiotherapy for cervical cancer comprises of external-beam radiotherapy, brachytherapy and the concomitant chemotherapy with cisplatin. Specifically, in the external-beam radiotherapy, basic opposing (anterior-posterior) techniques or 4-field techniques that were enable without CT based contouring or dose estimating, resulting in inadequate dosage coverage of target volumes and confines in protecting the neighbouring organs (Vordermark, 2016). Precise preparation and the implementation of the external-beam radiotherapy can improvise tumor control and lower its toxicity compared to the results published previously (Vordermark, 2016). The short-term side effect of this therapy including stomach upset, fatigue, diarrhea, changes in the skin texture, nausea and vomiting. Moreover, vaginal pain, radiation cystitis, menstrual changes, and low anaemia is also the side effects of external-beam radiotherapy (American Cancer Society, 2016g).

Intracavity brachytherapy is a type of brachytherapy commonly used in the treatment concept for chemoradiation or radial radiotherapy of cervical cancer. In a wide series of patients, it has been found that avoidance of brachytherapy leads to a significant reduction of the treatment's curative capacity (Vordermark, 2016). The short-term side effect of this therapy similar to the long-term side effect of external–beam radiotherapy. The long-term side effects of radiotherapy are vaginal stenosis, rectal bleeding, vaginal dryness, bone weakened, urinary problem and leg swellings lead to lymphedema (American Cancer Society, 2016g).

Besides, the concurrent chemoradiation therapy (CCRT) treatment technique activates the anti-tumor immunity through the abscopal effects of radiation therapy. This ensures the distant metastatic lesion shrink and disappears. Recent findings have demonstrated that tumor cells that have been damaged or killed by radiation therapy secrete new tumor antigens and activate the intracellular nuclear factors due to the DNA disruption, resulting in interferons (IFN) secretions. Those activate the antigen-presenting cells, which trigger the anti-tumor immune system (Kagabu et al., 2019). Continued individualisation in both external-beam radiotherapy and brachytherapy resulted in a more beneficial interaction between tumor management and the treatment toxicity in cervical cancer (Vordermark, 2016).

2.2.12 Targeted therapy

The development of the cancer cells relies on the transduction of oncogenic signal pathways. Molecular targeting is a new treatments are directed by modulating certain signal transduction mechanisms by obstructing the extracellular transmembrane receptors or by interfering with intracellular proteins (Markman,

2008). This therapy works by targeting the signaling cascade, inhibit the cancer cell proliferation, induce apoptosis and obstruct the metastasis. Conceptually, this therapy could contribute to more effective cancer therapy with fewer clinical side effects (Murdoch & Sager, 2008). Currently, the advances in the targeted therapy against the somatic alterations have improved cancer treatment strategies. Besides, the identification of specific clinical targets of cervical cancer may strengthen the existing approaches to fight cervical cancer (Arteaga & Baselga, 2012).

In the future, it is highly expected that the targeted therapies are enabled for the gynecological oncologist to provide customised treatments towards cervical cancer. Further studies are being conducted currently as positive results obtained from the addition of anti-angiogenic agents to chemotherapy and when given in combination with chemotherapy (Menderes et al., 2016). More researches are evaluating the importance of immune-directed therapies in cervical cancer with better knowledge of the core role of the HPV infection in the tumorigenesis of cervical cancer (Table 2.2) (Vora & Gupta, 2019).

Table 2.2 Selected studies of targeted therapy in cervical cancer.

Author/ study design/ year/ references	Drug	Patient population/n	Results/ Conclusion	Grade 3 or 4 toxicities
VEGF-targeted therapies				
Tewari et al., 2014. Randomised controlled study	Bevacizumab (chemotherapy vs chemotherapy + bevacizumab)	Metastatic, recurrent or unresectable disease as first line therapy (n=452)	Median OS: 13.3 vs 17 months HR:0.71 (97%CI 0.54 to 0.94) P=0.0035 Median PFS:5.9 vs 8.2 months HR:0.67 (95%of CI 0.54 to 0.82) P=0.002 RR:36% vs 48% P=0.008	(chemotherapy + bevacizumab arm): hypertension (11.4%), gastrointestinal perforations (10.1%), venous thrombotic events (8.3%)
Mackay et al., 2010. Single-arm, phase II	Sunitinib	Metastatic or unresectable persistent progressed on one line of therapy (n=19)	Median TTP: 3.5 months SD: 84% RR:0%	Fatigue (15.8%), diarrhoea (15.8%), hypertension (10.3%), HFS (10.3%) anaemia (23.5%)
Monk et al 2010. Phase II, a randomised controlled study, open-label	Pazopanib	The metastatic disease progressed on one or more lines of therapy (n=74)	Median OS:12.4 months TTP:4.5 months SD:43% RR:9%	Diarrhea (11%)
EGFR-targeted therapies				
Goncalves et al., 2008. Phase II single-arm study, open-label	Gefitinib	Metastatic recurrent or unresectable disease progressed on one or more line of therapy (n=30)	Median OS:3.7 months TTP:1.23 months SD:20% RR:0%	Diarrhoea (13.3%) Anorexia (6.7%)

Table 2.2 Continued

Kurtz et al., 2009. Phase II single-arm study	Cetuximab (combination with cisplatin and topotecan)	Metastatic, recurrent or unresectable disease as first-line therapy (n=19)	Median OS:7.33 months TTP:5.73 months SD:32% RR:32%	Febrile neutropenia (22%) Haemorrhage (11%) CINV (22.5%) Skin reaction (22%) Pulmonary embolisms (5%) Death due to toxicity (10.5%)
HER2 and EGFR-targeted therapy				
Monk et al., 2010. Phase II, a randomised controlled study, open-label	Lapatinib	The metastatic disease progressed on one or more lines of therapy (n=78)	Median OS: 11 months TTP:4.27 months SD:44% RR:5%	Diarrhoea (13%) Fatigue (5%) Anaemia (5%) Dyspnoea (6%)
Immune checkpoint therapy				
Frenel et al., 2016. Phase Ib multicohort open-label study	Pembrolizumab	The metastatic, recurrent or unresectable disease progressed on one or more line of therapy(n=24)	Median OS:9 months RR: 17%	Rash (9%) Proteinuria (4%) Colitis (4%) Guillain-Barre Syndrome (4%)
Therapeutic vaccines				
Basu et al., 2018. Phase II	Axalimogene filolisbac (ADXS11-001) (ADXS11-001 with cisplatin vs ADXS11-001 alone)	Metastatic, recurrent or unresectable disease progressed on one or more line of therapy(n=109)	Median OS: 8.78 vs 8.28 months Median PFS: 6.10 vs 6.08 months RR:17.1% vs 14.7%	Overall grade 3 or 4 toxicities (19.7%)
PARP Inhibitor				

Thaker et al., 2015. Limited access phase I trial	Veliparib (with paclitaxel and cisplatin)	Advanced, persistent or recurrent (n=37)	SD:41% RR:34%	NA
Antibody-drug conjugate				
Vergote et al., 2017. Multicohort phase IIA study	Tisotumab-vedotin	The metastatic, disease progressed on one or more line of therapy(n=34)	SD:18% RR:32%	Conjunctivitis (3%) Neuropathy (6%)
Immune checkpoint Inhibitor in adjuvant treatment				
Mayadev et al., 2017. Phase I study	Ipilimumab	Node-positive disease post-CTRT for sequential therapy with ipilimumab as adjuvant therapy (n=34)	1 year DFS:74%	Neutropenia (5.3%) Rash (5.3%) Lipase (5.3%)

Source: Vora & Gupta, (2019).

2.3 Apoptosis as a programmed cell death

Apoptosis is a type of cell death, a goal of cancer treatment and is characterised by cell shrinkage, blebbing of the plasma membrane, and chromatin condensation associated with DNA cleavage into ladders (Q. Liu et al., 2018). Apoptosis remains throughout the life in order to sustain tissue homeostasis, that is, a balance between cell proliferation and cell death. Therefore, this form of death differs from classical necrosis, which leads to cell membrane destruction, necrotic cell bursts, and releases its contents into the surrounding tissue, but organelles, such as the mitochondria or nucleus, remain intact during this process. Therefore, this form of death differs from classical necrosis, which leads to cell membrane destruction, necrotic cell bursts, and releases its contents into the surrounding tissue, but organelles, such as the mitochondria or nucleus, remain intact during this process (Maximov & Maximov, 2008).

In apoptosis, cell is an active participant in its own demise. This form of cell death is regulated, dependent on energy, and can affect individuals or cell clusters. Necrosis, on the other hand, is known as a toxic mechanism in which the cell is a passive victim, follows an energy-independent mode of death and typically affects a large cell area (Figure 2.5) (Jain et al., 2014).

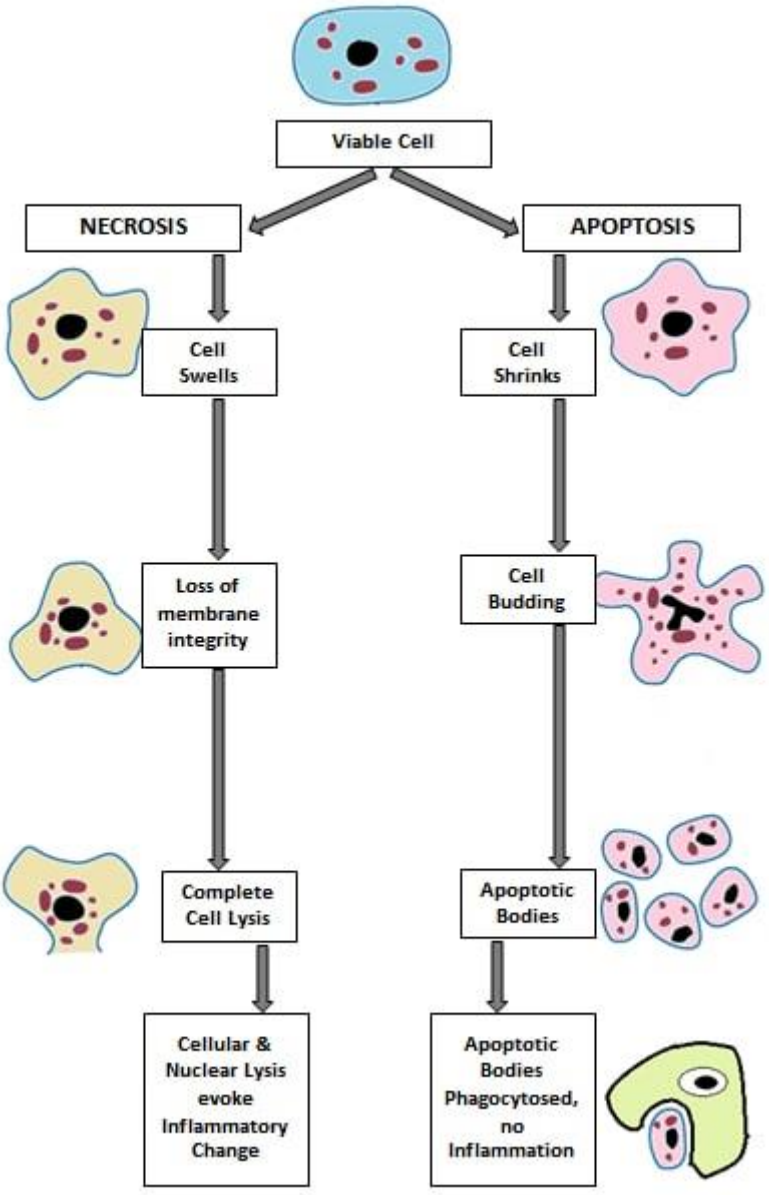

Figure 2.5 Difference between Apoptosis and Necrosis Modified from Jain et al., (2014).