ANTIOXIDANT AND ANTIPROLIFERATIVE ACTIVITIES OF SIX MALAYSIAN MEDICINAL PLANTS AND MECHANISM OF ACTION THROUGH MICRORNA IN THYROID CELL LINES

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UNIVERSITI SAINS MALAYSIA

2019

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by

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Thesis submitted in fulfillment of the requirements

for the degree of

Doctor of Philosophy

March 2019

ACKNOWLEDGMENT

I would like to express my sincerest gratitude and appreciation to my advisors, Associate Professor Dr. Khoo Boon Yin and Associate Professor Dr. Maizan Mohamed for the opportunity to have been their student for the past five years and for their guidance throughout the lab work and thesis writing. To my advisors, Professor Shaharum Shamsuddin and Professor Prabha Balaram, thank you for your ideas and comments over the course of my studies. Their dedication to research was inspiring.

To all lab mates past and present, thank you for the laughs and the memories. I would also like to thank the former and current members of INFORMM Kubang Kerian for the support and experience. I would also like to acknowledge other people who have directly and indirectly help me towards the completion of my study.

Most importantly, I would like to thank my mother, Wan Mas Wan Senik for supporting me, emotionally in completing my study. To my beloved husband, Wan Mohd Izuadi Wan Mahmud, thank you for believing in me and for being patient with me throughout this journey that made my accomplishments worth celebrating. I would like to dedicate this thesis to my daughter and son, Wan Nur Iris Wan Mohd Izuadi and Wan Mohamad Irsyad Wan Mohd Izuadi for you are the reason for me to keep on improving myself to become a better person and a better mother to you. Lastly, I would also like to thank the USM fellowship for providing the funding that has made this experience possible.

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LIST OF SYMBOLS, ABBREVIATIONS AND ACRONYMS

ANOVA	Analysis of variance
AO	Acridine orange
ATC	Anaplastic thyroid carcinoma
ATP	Adenosine triphosphate
BD	Becton Dickinson
bp	Base pair
cDNA	Complementary deoxyribonucleic acid
CO_2	Carbon dioxide
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide triphosphates
DPPH	2,2-diphenyl-1-picrylhydrazyl
DTT	Dithiothreitol
dsRNA	Double-stranded RNA
dw	Dry weight
EB	Ethidium bromide
EDTA	Ethylene diamine tetra acetic acid
FBS	Fetal bovine serum
FDA	Food and Drug Administration
FITC	Fluorescein isothiocyanate
FTC	Follicular thyroid carcinoma
GAE	Gallic acid equivalent

H_2O_2	hydrogen peroxide
IC_{50}	Half maximal inhibitory concentration
IDT	Integrated DNA Technologies, Inc.
MAPK	mitogen-activated protein kinase
mg	Miligram
mL	Mililiter
mRNA	Messenger ribonucleic acid
MTC	Medullary thyroid carcinoma
MTT	[3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide]
NCI	National Cancer Institute
nm	nanometer
O_2^-	Superoxide ion
O_2	Oxygen
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PES	Polyethersulfone
pH	Potential of hydrogen
PI	Propidium iodide
PTC	Papillary thyroid carcinoma
PTEN	Phosphatase and tensin homolog protein
QE	Quercetin equivalent
RNA	Ribonucleic acid
rpm	Revolutions per minute
RPMI	Roswell Park Memorial Institute

RT-PCR	Reverse transcriptase polymerase chain reaction
SD	Standard deviation
SEM	Standard error of the mean
TUNEL	Terminal deoxynucleotidyl transferase dUTP nick end labeling
UV	Ultraviolet
WHO	World Health Organization
w/v	Weight/volume
β-actin	Beta actin
µg	Microgram
µL	Microliter
3’UTR	3 prime end untranslated region
%	Percentage
^{o}C	Degree Celsius

AKTIVITI ANTI-OKSIDA DAN ANTI-PROLIFERATIF PADA ENAM TUMBUHAN PERUBATAN DI MALAYSIA DAN MEKANISMA TINDAKAN MELALUI MICRORNA PADA SEL-SEL TIROID

ABSTRAK

Kanser tiroid, termasuk papilari, folikular, medulari dan anaplastik, mempunyai kadar pertumbuhan berbeza dan boleh merebak jika tidak dirawat. Walaupun kebanyakan kes kanser tiroid mempunyai prognosis yang baik, risiko perulangan di kalangan pesakit adalah tinggi dan pesakit mati akibat ketumbuhan tumor yang progresif. Tumbuhan ubat-ubatan biasanya digunakan sebagai alternatif kepada rawatan kanser di banyak negara. Aktiviti anti-proliferatif oleh fitokimia telah dilaporkan dalam pelbagai jenis kanser, namun kesan tumbuhan ubat-ubatan pada kanser tiroid masih kurang diselidiki. Walaupun terdapat pelbagai laporan tentang aktiviti anti-kanser ekstrak tumbuhan pada pelbagai sel kanser, sasaran selular dan mekanisme yang terlibat masih tidak jelas. Matlamat keseluruhan kajian ini adalah untuk mengkaji kesan ekstrak daripada enam tumbuhan perubatan (*Annona muricata* L., *Angelica keiskei* Ito, *Chromolaena odorata* (L.) R.M.King & H. Rob, *Clinacanthus nutans* (Burm. f.) Lindau, *Euphorbia hirta* L., and *Leea indica* (Burm. f.) Merr.) pada sel-sel tiroid (Nthy-ori 3-1, FTC-133 dan Hth-74) dan mekanisme yang terlibat. Untuk aktiviti anti-oksida dan anti-proliferatif, kaedah *Folin-Ciocalteu*, klorida aluminium, penghapusan DPPH radikal bebas dan *MTT* digunakan. Penilaian terhadap induksi apoptosis melalui pemberhentian kitaran sel menggunakan analisis sitometrik aliran sel yang dirawat dengan *propidium iodide* dan *Annexin V-FITC/propidium iodide*, dan kaedah *TUNEL*, mengesahkan aktiviti anti-proliferatif dalam ekstrak tumbuhan.

Kaedah *RT-PCR* kuantitatif masa nyata bagi gen yang berkaitan dengan *apoptosis*, pada microRNAs dan gen target mereka juga dilakukan. Ekstrak metanol *E. hirta* mempunyai jumlah kandungan fenol dan flavonoid yang tinggi pada 307.59 ± 3.57 mg GAE/g dw dan 76.43 ± 4.34 mg QE/g dw. Ekstrak tersebut mempunyai 32% penghapusan DPPH dengan nilai IC_{50} 0.013 mg/mL. Pengurangan ketara pada ketumbuhan sel dilihat pada ekstrak metanol *A. muricata* dan *C. odorata* dengan nilai IC_{50} 20.8 dan 74.9 mg/mL dalam sel FTC-133 selepas 72 jam. Sel FTC-133 yang dirawat dengan ekstrak metanol *A. muricata* menunjukkan peningkatan ketara dalam peratusan sel pada fasa S- dan G_2/M dengan penurunan yang ketara dalam fasa G_0/G_1. Analisa sitometrik aliran sel yang dirawat dengan *Annexin V-FITC/propidium iodide* dan analisis kajian *TUNEL* mengesahkan bahawa estrak metanol *A. muricata* menyebabkan apoptosis dalam sel FTC-133. Analisa ekspresi gen-gen berkaitan dengan apoptosis menunjukkan bahawa penghapusan sel secara intrinsik disebabkan oleh peningkatan ketara dalam ekspresi gen pro-apoptosis, *BAX* dan penurunan ketara dalam ekspresi gen anti-apoptosis, *BCL2*. Di samping itu, pengurangan ketara pada ekspresi beberapa microRNAs (miR-192, miR- 197, miR-328 dan miR-346) dalam FTC-133 sel yang dirawat dengan ekstrak metanol *A. muricata* mencadangkan aktiviti anti-proliferatif yang berasaskan mekanisma modulasi microRNA. Kesimpulannya, jumlah kandungan fenolik dan flavonoid yang banyak menyumbang kepada aktiviti anti-oksida yang tinggi. Ekstrak metanol *A. muricata* menghasilkan aktiviti anti-proliferatif yang signifikan pada sel FTC-133 melalui kerencatan kitaran sel dan induksi apoptosis secara intrinsik. Kesan anti-proliferatif mungkin disebabkan oleh modulasi beberapa microRNA yang diekspres secara berlainan di dalam sel FTC-133. Potensi ekstrak metanol *A. muricata* dalam pencegahan perkembangan kanser tiroid bertumpu pada ekspresi microRNA harus diselidiki secara lebih lanjut.

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ABSTRACT

Thyroid cancers, including papillary, follicular, medullary and anaplastic carcinomas, have different growth rates and can metastasize if left untreated. Even though most thyroid cancer cases have good prognosis, the risk of recurrence among the patients is high and some patients die from progressive tumor. Medicinal plants are commonly used as alternative to cancer treatment in many countries. The anti-proliferative activity of phytochemicals has been demonstrated in various cancers, yet the effect of medicinal plants on thyroid cancer remain under-investigated. Despite various reports on the anti-cancer activities of plant extracts on a variety of cancer cell lines, the cellular targets and the underlying mechanisms remain unclear. The overall goal of the present study was to investigate the effect of crude extracts from six medicinal plants (*Annona muricata* L., *Angelica keiskei* Ito, *Chromolaena odorata* (L.) R.M.King & H. Rob, *Clinacanthus nutans* (Burm. f.) Lindau, *Euphorbia hirta* L., and *Leea indica* (Burm. f.) Merr.) on thyroid cell lines (Nthy-ori 3-1, FTC-133 and Hth-74) and the mechanism involved. For antioxidant and anti-proliferative activities of the plant extracts, methods such as Folin-Ciocalteu's, aluminum chloride, DPPH free radical scavenging assays and MTT assay were utilized. Flow cytometric analysis of treated cells stained with propidium iodide and with Annexin V-FITC/propidium iodide, and TUNEL assay further confirmed the anti-proliferative activity of the plant extract. Quantitative real-time RT-PCR of apoptotic related genes, candidate

microRNAs and their putative target genes were also performed. Methanolic extract of *E. hirta* had high amount of total phenolic and flavonoid contents at 307.59 ± 3.57 mg GAE/g dw and 76.43 ± 4.34 mg QE/g dw, respectively. The extract had 32% of DPPH scavenging with IC_{50} of 0.013 mg/mL. Significant reduction of cell viability was observed when treated with *A. muricata* and *C. odorata* methanolic extracts with IC_{50} of 20.8 and 74.9 mg/mL in FTC-133 cells at 72 hours of treatment. FTC-133 cells treated with *A. muricata* methanolic extract showed significant increase in percentage of cells at the S- and G_2/M-phase with subsequent significant decrease in G_0/G_1-phase. Flow cytometric analysis of cells treated with Annexin V-FITC/propidium iodide and analysis of TUNEL assay further confirmed that *A. muricata* methanolic extract induced apoptosis in FTC-133 cells. Gene expression analysis of apoptosis-related genes suggest that intrinsic apoptosis pathway was induced by the significant increase in expression of pro-apoptotic gene, *BAX* and significant decrease in expression of anti-apoptotic gene, *BCL2*. In addition, significant reduction in the expression of several microRNAs (miR-192, miR-197, miR-328 and miR-346) in FTC-133 cells treated with *A. muricata* methanolic extract suggest for potential underlying mechanism of the anti-proliferative property. In conclusion, large amount of phenolic and flavonoid compounds in *E. hirta* contributed to its high antioxidant activity. Treatment of *A. muricata* methanolic extract resulted in significant anti-proliferative activity in FTC-133 cells through cell cycle arrest and induction of apoptosis. The anti-proliferative activity may also be due to modulation of several microRNAs aberrantly expressed in FTC-133 cells. The potential of *A. muricata* methanolic extract in the prevention of thyroid cancer development focusing on the microRNAs regulation pathway should be further investigated.

CHAPTER 1

INTRODUCTION

1.1 Rationale of the study

With the increasing number of cancer incidence especially in developing countries, the cancer burden will likely be exasperated by the increase in growth, life expectancy, and aging of the population (Thun et al., 2009). Despite considerable effort and successes in managing cancer, it remains as one of the leading causes of death worldwide. About 7.6 million cancer deaths and 12.7 million cancer cases have occurred in 2008 worldwide and more than 50% of the cancer cases and deaths occurred in developing countries (Ferlay et al., 2010). It is projected that 26 million cases and 17 million deaths due to cancer will occur by 2030 (Thun et al., 2009).

Thyroid cancer is a disease that forms in the thyroid gland, located on the front of the neck below the thyroid cartilage. Thyroid cancers can be classified based on the histopathological characteristics including papillary, follicular, medullary and anaplastic thyroid cancers. Each cancer groups have different growth rates and can metastasized if left untreated. In certain types of thyroid cancers, the malignancies are higher in women than in men with the ratio of 3:1. In 2012, about 230,000 new cases of thyroid cancer were diagnosed in women and 70,000 in men (Ferlay et al., 2015). The incidence rates are increasing over the last few decades in most countries and if the trends remain, thyroid cancer could be in the top four of the most common cancer by 2030 in the United States (Rahib et al., 2014). However, the mortality rates have been steadily declining in most part of the world, which are likely due to the improved

strategies for the diagnosis, management and treatment of the disease (La Vecchia et al., 2015).

According to the Malaysian National Cancer Registry, between 2007 – 2011, thyroid cancer incidence was the highest among Malays for a total of 1335 cases (Azizah et al., 2016). In 2006, International Atomic Energy Agency reported that in Singapore, Malays have a higher incidence rate of thyroid cancer (males = 2.7, females = 5.0) than Chinese (males = 1.5, females = 4.3) and Indian population (males = 0.7, females = 1.1) per 100,000 population suggesting for possible genetic susceptibility in the Malays and predominantly in females. Nonetheless, thyroid cancer can occur in all age groups where in older patients, the cancer is significantly more aggressive.

In thyroid cancer, tumors can recur in 30% of survivors and may develop chemo-resistance to currently available drugs (Palme et al., 2004; Tuttle et al., 2010). The causes for thyroid cancer recurrence remain to be investigated especially at the molecular level that lead to tumor recurrence. On the other hand, the inadequacy of current chemotherapeutic agents requires the development of new, effective and affordable anti-cancer drugs. The improvements in diet richer in vegetables and flavonoids over the last few decades may have contributed to the widespread decline in the mortality of thyroid cancer (Dal Maso et al., 2009). In addition, several studies have identified a panel of mutations specific to thyroid cancer types and with deeper understanding of the genetic and epigenetic alterations, patients may benefit from personalized therapeutic approaches. Indeed, development of targeted drugs to specifically modulate molecules related to cancer is the basis for precision medicine. Phytochemicals and herbal medicine act synergistically where they attack multiple

targets at the same time in which could be great resources for the use in targeted therapies. Therefore, development of novel anti-cancer drugs based on natural products for thyroid cancers will be of immense use in planning personalized treatment modalities.

Natural products can be a major source for development of novel drugs. With more than 391 000 species in the plant kingdom, plants provide massive number of compounds with numerous chemical diversity with various pharmacological properties. When combined with high-throughput screens and the lead compounds can be optimized by combinatorial chemistry for potential drug development. The enormous diversity of plant species and the prevalence of traditional medicine practice in Malaysia provide a good platform of candidate species to be evaluated for anti-cancer drug property.

Many Malaysian plants are being used in traditional medicine to treat a myriad of diseases. Some have been suggested to be effective in treating cancer or preventing cancer recurrence, nonetheless, limited scientific evidence are available to support the local claims. Therefore, it is crucial to properly evaluate the anti-cancer properties of these plants *in vitro,* to understand the molecular basis and mechanism involve in the anti-cancer effect of the plant extracts in thyroid cancer including their involvement in apoptotic pathway and modulations of aberrantly expressed microRNAs.

1.2 Aim and scope of the study

Thus, the present study aimed to determine the antioxidant and anti-proliferative properties of six Malaysian medicinal plants – *Angelica keiskei, Annona muricata,*

Chromolaena odorata, Clinacanthus nutans, Euphorbia hirta and *Leea indica* in thyroid cell lines (Nthy-ori 3-1, FTC-133 and Hth-74) and the mechanism involved via regulation of microrna expression. The specific objectives include:

I. To evaluate the total phenolic and flavonoid contents, and free radical scavenging activity of the aqueous and methanolic extracts of *A. keiskei*, *A. muricata, C. odorata, C. nutans, E. hirta* and *L. indica* using Folin Ciocalteu's, aluminum chloride, and DPPH scavenging assay.

II. To assess the anti-proliferative activity of the aqueous and methanolic extracts and their inhibitory concentrations at 50% in thyroid cell lines (Nthy-ori 3-1, FTC-133 and Hth-74) using MTT cell proliferation assay.

III. To determine the association of anti-proliferative activity of candidate extract with cell cycle arrest using flow cytometric analysis.

IV. To investigate the efficacy of a candidate extract in inducing apoptosis in thyroid cancer cells using flow cytometric analysis of Annexin V-FITC/propidium iodide stained cells and TUNEL assay and quantitative real-time RT-PCR of apoptotic genes.

V. To investigate the potential involvement of microRNAs as an underlying mechanism of the anti-proliferative effect of candidate extract in thyroid cell line by quantifying the expression of associated microRNAs and their target mRNAs using quantitative real-time RT-PCR.

1.3 Overview of the study

Several steps were involved in completing the study. The first part of the study required preparation of the extracts and optimization of the assays involved in evaluating the antioxidant activity in the plant extracts. The second part involved the

preparation of the cells involved for the current study and evaluation of the growth condition and requirements specific to the cells. The last part of the study is the evaluation of the effect of the plant extracts on the thyroid cell lines in terms of anti-proliferative activity, establishment of apoptosis in treated cells as well as the involvement of microRNAs in the anti-proliferative activity of the candidate extract. Figure 1.1 summarizes the work involved in the present study.

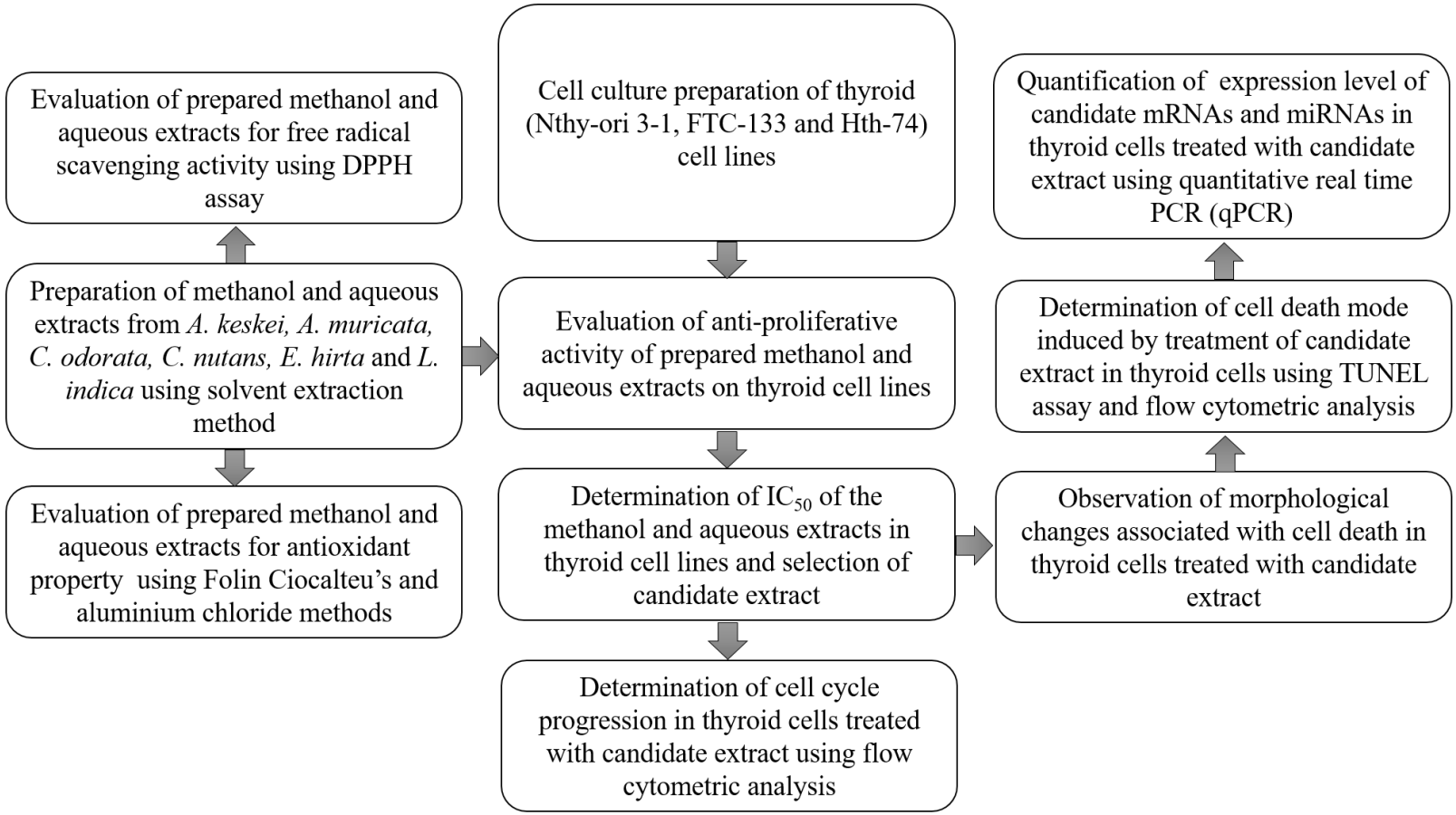


Figure 1.1 Flow chart of the study. Overview of the study design and analyses performed to determine the antioxidant and anti-proliferative activities of plant extracts in thyroid cell lines.

CHAPTER 2

LITERATURE REVIEW

2.1 Cancer

Cancer happens when abnormal cells grow uncontrollably forming tumor mass and gain the ability to invade other tissues or other part of the bodies through the blood and lymph systems. With 8.8 million of deaths recorded in 2015, cancer is one of the leading causes of death globally (World Health Organization, WHO). Breast cancer, lung and bronchus cancer, prostate cancer and thyroid cancer are several of the cancers projected to be the most common cancers in 2018 (National Cancer Institute, NCI). Based on the data collected from GLOBOCAN 2012, the number of new cancer cases is estimated at 454.8 per 100,000 population per year and the number of cancer deaths is 171.2 per 100,000 population per year (WHO). Cancer pose a major threat to public health especially in developing nations where they have inadequate resources to deal with complex and expensive treatments for cancer. According to WHO, approximately 70% of cancer deaths occur in low- and middle-income countries possibly due to diagnosis of disease at a later stage and inaccessibility or unavailability of diagnosis and treatment. In 2015, only 35% of low-income countries reported to have pathology services provided in the public sector (WHO).

Cancer causes economic, emotional and social impacts to both the patient and the society. The financial costs of cancer are high for the patient and the society where an estimated medical cost for cancer in the United States in 2014 was $87.7 billion (The Agency for Healthcare research and Quality, AHRQ). In addition, cancer

treatment often affects the patient's appearance leading to feeling of self-conscious and low self-esteem. This also could lead to clinical depression where the American Cancer Society estimates up to one in four people with cancer develop depression during or after treatment. For some cancer survivors, re-entering social and professional life can be challenging affecting patients' quality of life and work performance.

Cancer is a non-communicable disease (NCD) which is not just a burden to the person, society and economy but it is a potential societal responsibility. In 2011, United Nations met to discuss NCDs which include cancer, diabetes, cardiovascular and lung diseases, and called for new global targets and action plan to address NCDs. There are four risk factors for NCDs including the tobacco use, alcohol consumption, unhealthy dietary practices and physical inactivity. Furthermore, limited access to essential healthcare also increases the risk of NCDs. Interventions such as prevention of tobacco use and alcohol consumption may address NCDs. Additionally, WHO Global action plan focuses on achieving an 80% availability of affordable basic technologies and essential medicines in low- and middle-income countries (WHO, 2013).

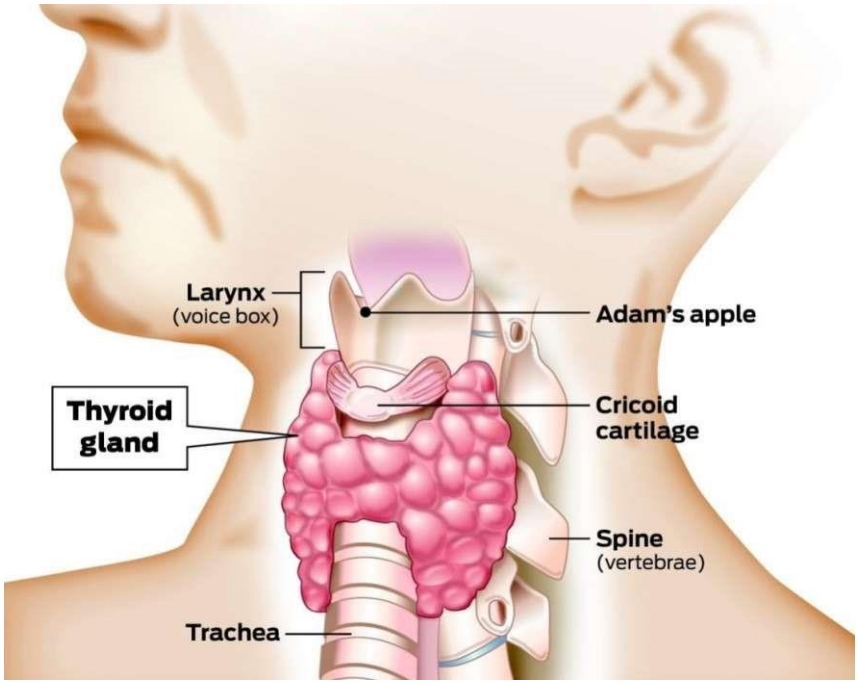
There are several categories for cancers with more than 200 diverse types of cancer. Commonly, cancers are categorized by the organ or type of cells of origin. For instance, colon cancer commonly arises in the colon and cancer originates from melanocytes of the skin is known as melanoma. These cancers are grouped into several categories including carcinoma (skin or epithelial tissues), sarcoma (connective or supportive tissues), leukemia (blood-forming tissues), lymphoma and myeloma

(immune system cells), and central nervous system cancers (brain and spinal cord tissues).

2.1.1 Thyroid cancers

Thyroid cancer is a cancer that forms in the thyroid gland, located on the front of the neck, below the thyroid cartilage (Figure 2.1 a). It is shaped like a butterfly with a right and a left lobe connected by isthmus (Figure 2.1 b). Thyroid cancers are divided into several groups based on the histopathological characteristics including papillary, follicular, medullary and anaplastic thyroid cancers (DeLellis et al., 2004). Thyroid carcinomas arise from two cell types present in the thyroid gland (Xing, 2013). Papillary, follicular and sometimes anaplastic carcinomas arise from follicular cells that make thyroid hormone that affects heart rate, body temperature, and energy level while medullary carcinoma arises from parafollicular C cells that produce calcitonin which is a hormone important for controlling the level of calcium in the blood.

(a)



(b)

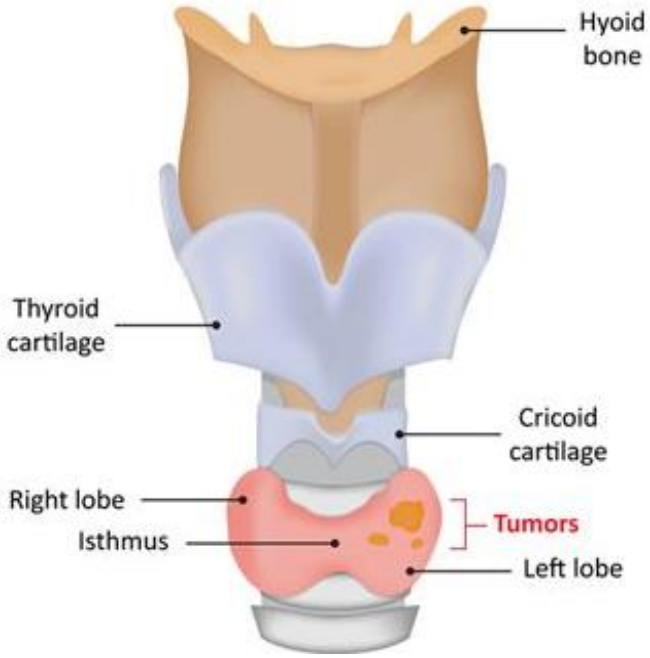


Figure 2.1 Thyroid Cancer. Labeled illustration showing the location of the (a) thyroid gland in a male figure (Colliver, 2014) and (b) tumor growth in the left lobe of the thyroid (Gleneagles Hospital Singapore, 2018).

Thyroid cancers arise in people of all ages, where its aggressiveness increases with age (Haymart, 2009). If thyroid cancers are left untreated, it can spread to other parts of the body. According to NCI, thyroid cancers represent approximately 1% of all malignancies, estimating 60,220 of new cases and 1,850 of deaths from thyroid cancer in the United States for 2013. A disturbingly steady increase in thyroid cancers worldwide has been observed (Jemal et al., 2011; Pellegriti et al., 2013). It is the fastest rising cancer in women (Durante et al., 2013). According to the Malaysian National Cancer Registry (NCR), thyroid cancer incidence rate was 2.2 for male and 6.8 for female cancer incidence per 100 000 population in 2006. Several possible factors may contribute to the increase in incidence rate of thyroid cancer including environmental factors (e.g. increased radiation exposures), better detection methods and other undiscovered factors (Davies and Welch, 2006; Nikiforov and Nikiforova, 2011). The development of thyroid cancer can be caused by exposure to ionizing radiation to the neck region at an early age. Radiation exposure significantly increases the risk for thyroid malignancies as seen in children exposed to radiation after the accident at the Chernobyl nuclear power plant and nuclear bombings in Hiroshima and Nagasaki during the World War II (Nikiforov and Nikiforova, 2011; Pellegriti et al., 2013). The alarming rise of thyroid cancer incidence is not just due to improvements in detecting thyroid cancer, other undiscovered factors may also contribute to the rise. It is suggested that obesity may increase the risk of developing thyroid cancer (Harari et al., 2012), as well as exposure to pollutants and hazardous chemicals.

With the advent of technology, thyroid cancer can be diagnosed early and the treatments work well for some types of thyroid cancer. Papillary and follicular thyroid cancers are the most curable by complete removal of the lobe that harbors the cancer.

Nonetheless, the recurrence rate among the survivors is quite high with up to 30% where the cancer may come back, sometimes decades after the initial treatment with increased incurability, morbidity and mortality (Palme et al., 2004; Tuttle et al., 2010). Therefore, better treatment and prevention methods are needed to control the disease. With the use of effective treatment and preventive method, the mortality rate can be significantly reduced.

The most common type of thyroid cancers are papillary thyroid cancer (PTC) accounting for ~80% of thyroid cancer cases (Nikiforov and Nikiforova, 2011). PTC is commonly diagnosed in patients between the ages of 30 and 50 with higher incidence rate in women. Follicular thyroid carcinoma (FTC) accounts for 10 to 15% of all thyroid cancers, are often diagnosed in patients above the age of 45 where its aggressiveness increases with age. Medullary thyroid carcinoma (MTC) makes up about 3 to 5% of all thyroid cancers where females and males are equally affected. The least common type of thyroid cancer is anaplastic thyroid carcinoma (ATC) that affects less than 5% of thyroid cancer patients. ATC is the most aggressive and invasive of all thyroid cancers and is usually diagnosed in patients older than 65 years. PTC and FTC have the highest survival rates while ATC is the least responsive to treatment. Table 2.1 summarizes the profile of thyroid cancers.

Table 2.1 Types of thyroid cancer and their profiles.

Characteristics	Papillary (PTC)	Follicular (FTC)	Anaplastic (ATC)	Medullary (MTC)
Cell type	Follicular	Follicular	Follicular	C cell
Prevalence (%)	80-85	10-15	<5	3-5
Common mutations and prevalence (%)	*BRAF* 40-45 *RAS* 10-20 *RET/PTC* 10-20 *TRK* <5	*RAS* 40-50 *PAX8/PPARγ* 30-35 *PIK3CA* <10 *PTEN* <10	*TP53* 50-80 *CTNNB1* 5-60 *RAS* 20-40 *BRAF* 20-40 *PIK3CA* 10-20 *PTEN* 5-15 *AKT1* 5-10	Familial forms: *RET* >95 Sporadic: *RET* 40-50 *RAS* 25

(Modified from Nikiforov and Nikiforova, 2011)

2.2 Cancer biology

The body is made up of cells that grow and divide to produce new cells, and eventually die when old or damage via programmed cell death, apoptosis. The cell cycle is a tightly controlled process that leads to cell division and duplication where the process detects and repairs genetic damage in the cells (Vermeulen et al., 2003). Chemical signals convey the information to cells whether to divide or stop dividing in response to growth requirements. However, sometimes this orderly process goes wrong which is a key factor in cancer. The genetic material can become damaged or changed leading to mutations affecting normal growth (Kastan and Bartek, 2004). These mutated cells escape the cellular regulation, defy death and continue multiplying forming mass of tissues called tumor. Tumor can either be benign (non-cancerous) or malignant (cancerous). Benign tumor rarely spread to other parts of the body because it lacks the invasive properties of a cancer, but it still poses negative health effects.

Malignant tumor, however, can invade surrounding tissue. The cells can break from the origin (primary site) and enter the bloodstream or lymphatic system to other sites spreading the cancer in a process termed metastasis. The more widely the cancer

metastasizes, the more difficult for it to be treated or eradicated (Kumar, 2010). The formation of cancer, termed carcinogenesis, can be divided into three distinct phases: initiation, promotion and progression (Gescher et al., 1998). The initiation of cancer begins when normal cells are exposed to endogenous or exogenous deoxyribonucleic acid (DNA) damaging agents (Greenwald, 1992; Helleday et al., 2014). Endogenous factors are those that arise within the body, often at the molecular or cellular level including age, endogenous hormones, genetic heritage and race. Exogenous agents such as carcinogenic chemicals (e.g. asbestos, tobacco), ionizing radiation (e.g. X-rays) and viruses (e.g. human papilloma virus) can cause cellular changes such as DNA alterations and gene mutations. The normal cells undergo transformation forming abnormal cells (initiated cells) that may proceed to promotion and progression process. The initiated cells acquire several biological characteristics to become malignant including loss of capacity for apoptosis, acquisition of self-sufficiency in growth signals, and acquisition to invade neighboring tissues. The promotion and progression process involve clonal expansion of the initiated cells (promotion) where the cellular damage inflicted in the initiation phase is propagated creating pre-neoplastic cells leading to formation of neoplastic cells forming malignant tumor (progression). Figure 2.2 illustrates the steps involved in the formation of cancer.

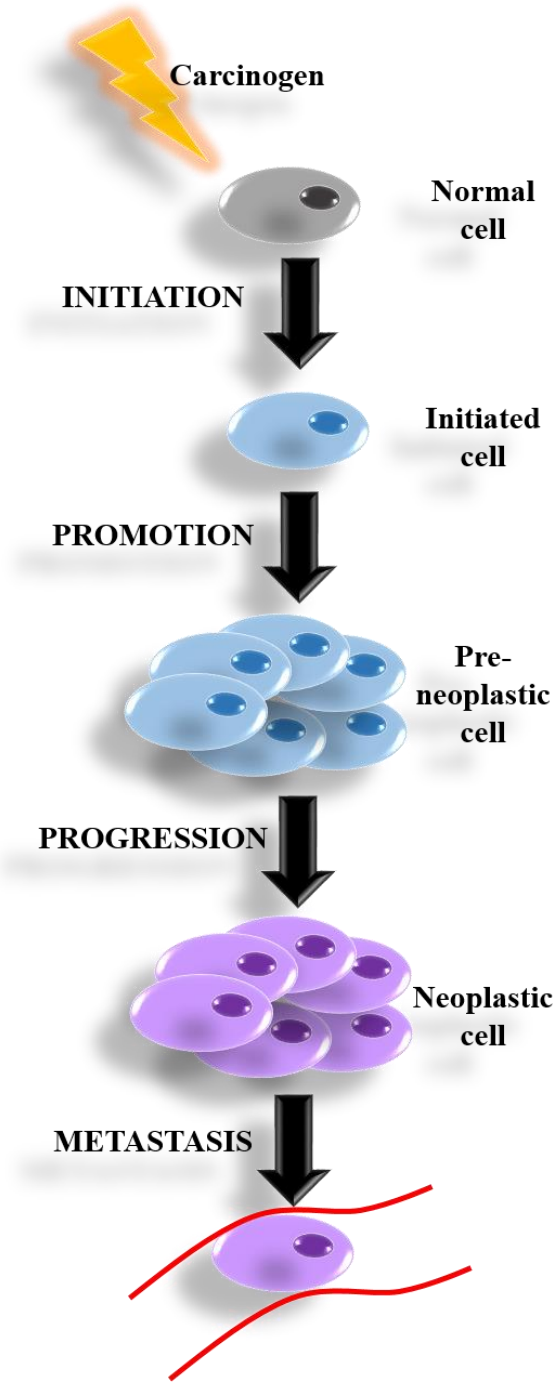


Figure 2.2 Multistage process of cancer development. Cancer involves formation of initiated cells from normal cells that have mutated due to carcinogen exposure. The initiated cells undergo clonal expansion into pre-neoplastic cells and progress to neoplastic cells after acquiring several traits making them malignant. The malignant cells may metastasize to other parts of the body via the blood stream or the lymph system. (Modified from Siddiqui et al., 2015).

2.3 Oxidative stress and cancer development

In the last three decades, growing evidence have shown that oxidative stress plays a key role in human cancer development. An imbalance between production of free radicals and oxidants and their elimination by antioxidant enzymes lead to oxidative stress. Reactive oxygen species (ROS) including oxygen, and its reduction products superoxide, hydrogen peroxide and hydroxyl radical are examples of common free radicals. Oxidative stress emerges from two main sources, internal and external sources. The sources for internal oxidative stress normally are found in the peroxisomes and mitochondrial including enzymes such as cytochrome P450 complex, xanthine oxidase and nicotinamide adenine nucleotide (NADPH) oxidase complexes, which catalyze the formation of reactive free radicals (Sosa et al., 2013). Oxidative stress from external sources could be generated from irradiation (e.g. by UV light, X-rays, gamma-rays), chemical compounds (e.g. alcohol, environmental pollutants), and exercise (Sosa et al., 2013; Valko et al., 2006).

The body generates free radicals as by-products of cells using oxygen to generate energy (Pham-Huy et al., 2008). Oxygen is a very common oxidizing agent. Oxygen-free radical is an atom or molecule with unpaired electrons that causes oxidative damage by stealing electrons from a surrounding compound or molecule (Winterbourn, 1993). At high concentrations, free radicals are capable of generating oxidative stress and damaging cells, leading to development of chronic and degenerative diseases including cardiovascular, neurodegenerative diseases and cancers (Bahoran et al., 2007; Halliwell, 2001; Valko et al., 2004). Cancer initiation and progression have been linked to oxidative stress by inducing DNA damage, introducing DNA mutations and genomic instability (Pelicano et al., 2004).

To protect cells from the negative effect of free radicals, antioxidant enzymes such as superoxide dismutase, glutathione peroxidase, and catalase reduce the free radicals by forming stable compounds (Thannickal and Fanburg 2000). For example, as shown in Figure 2.3, superoxide dismutase enzyme is responsible for disproportionation of superoxide (O_2^-) to hydrogen peroxide (H_2O_2) and oxygen (O_2) (McCord and Fridovich 1969). Then, the H_2O_2 is catalyzed into water (H_2O) by catalase or glutathione peroxidase. Apart from endogenous antioxidants, other antioxidants can also be obtained from the diet including glutathione, β–carotene and ascorbic acid (vitamin C). Studies have shown that higher consumption of Vitamin C correlates with reduced risk for pancreatic, cervical and colorectal cancers (Block 1991). Antioxidants are molecules that prevent inappropriate oxidation of other molecules where oxidizing agent steals electrons from another molecule forming free radicals (Lobo et al., 2010). It alleviates the effect of oxidative stress either by preventing ROS from being generated or by neutralizing them from the cell (Evans and Halliwell, 2001).

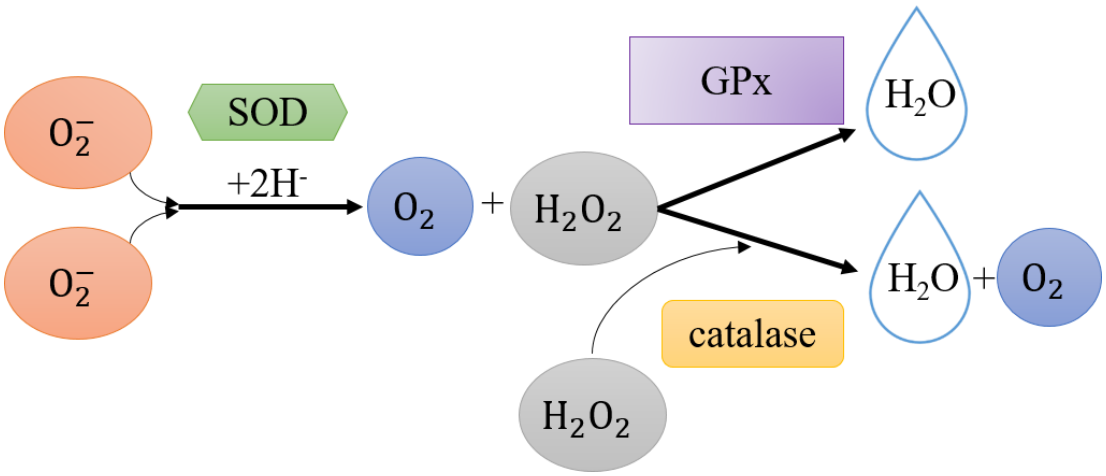


Figure 2.3 Role of superoxide dismutase in inactivation of the superoxide ion. Superoxide dismutase (SOD) catalyzes the dismutation of the superoxide ion (O_2^-) into oxygen (O_2) and hydrogen peroxide (H_2O_2). The hydrogen peroxide is then catalyzed to H_2O by catalase or glutathione peroxidase (GPx). (Modified from Peng et al., 2014)

2.4 Genetic mutations in cancer cells

Cancer cells differ from normal cells in several characteristics including decreased drug sensitivity and increased invasiveness (Hartwell and Kastan, 1994). The acquired characteristics are from a process cellular evolution where multiple genetic changes have occurred. For an initiated cell to develop into neoplastic cells, six important hallmarks of cancers are necessary during its development (Hanahan and Weinberg, 2011). The remarkable diversity of cancers is contributed by several cancer hallmarks including sustaining proliferative signaling, resisting cell death, evading growth suppressors, enabling replicative immortality, inducing angiogenesis, and activating invasion and metastasis.

The most fundamental trait of cancer cells is to proliferate uncontrollably by sustaining proliferative signaling. In normal cells, in order to maintain the homeostasis of cell number, architecture and function, the production and release of growth-promoting signals are tightly regulated (Hanahan and Weinberg, 2011). Nonetheless, cancer cells manage to deregulate these signals by producing growth factor ligands themselves thus stimulating endless growth (Sporn and Todaro, 1980), elevating levels of receptor proteins on the surface of cancer cells (Di Fiore et al., 1987), eliciting ligand-independent signaling (Su Huang et al., 1997) or by inducing adjacent normal cells to supply growth factors (Witsch et al., 2010). Somatic mutations in genes involved in MAPK (mitogen-activated protein kinase) cascades could also lead to cancer development where deregulated MAPK pathway is observed in one-third of human cancers (Dhillon et al., 2007). The MAPK pathway regulates cell proliferation regulated by external growth factors provided by surrounding cells. Additionally, cancer cells can also disrupt negative-feedback mechanisms involved in attenuating

proliferative signaling. For example, perturbations in PTEN (Phosphatase and Tensin homolog deleted from chromosome ten) signaling lead to cancer development. PTEN regulates PI3K (phosphatidylinositol 3-kinase) pathway by dephosphorylating the lipid signaling intermediate PIP_3 (phosphatidylinositol (3,4,5)-triphosphate). Loss of PTEN function leads to increased level of PIP_3 mimicking growth factor stimulation (Stambolic et al., 1998). Genetic investigations of thyroid tumors documented mutations in genes involved in the regulation of MAPK pathway commonly found in PTC and genes involved in the regulation of the PI3K pathway in FTC (Nikiforov, 2011). Table 2.1 summarizes the genetic mutations commonly found in thyroid cancers.

In addition to sustaining growth, cancer cells must also able to evade growth suppressors. The two most common tumor suppressors are p53 and pRb (retinoblastoma) proteins (Sherr and McCormick, 2002). Both proteins are involved in two main tumor suppressor pathways which control cellular responses to oncogenic stimuli. Mutations in these pathways can disrupt normal growth control or can dismantle cell cycle checkpoints. In thyroid cancer, mutations in *TP53* which encodes p53 transcription factor, are associated with overexpression of the mutant form implicated in the aggressive subtypes of thyroid cancer (Ho et al., 1996). The p53 pathway is composed of a network of genes which respond to stress stimuli to control DNA replication, chromosome segregation and cell division (Vogelstein et al., 2000). The p53 role in promoting apoptosis was first studied by Yonish-Rouach et al., (1991) who re-introduced p53 into a p53-deficient myeloid leukemia cell line.

Resisting cell death is another hallmark of cancer development. Apoptosis, a programmed cell death mechanism has been established as the natural barrier of cancer development (Evan and Littlewood, 1998). Kerr et al., (1972) suggested that a large percentage of cell death from tumors is due to apoptosis. Spontaneously regressing tumors and tumors treated with anti-cancer agents display high frequency of apoptosis (Kerr et al., 1994). In addition to loss of p53 tumor suppressor function, cancer cells circumvent apoptosis through modulation of expression of the pro- and anti-apoptotic proteins. The apoptotic pathway is divided into two major pathways; the intrinsic and extrinsic pathways (Fulda and Debatin, 2006). Stimulation of both intrinsic and extrinsic pathways activates cysteine proteases called caspases (cysteine-aspartic proteases) prior to cell death.

The intrinsic pathway involves p53-dependent pathway which is activated when signals within the cell initiate the process. The process is centered on the mitochondria where primary regulator proteins belonging to the Bcl-2 family activates the release of cytochrome c into the cytosol. The Bcl-2 family is consisted of anti- and pro-apoptotic proteins that interact with one another. The anti-apoptotic proteins include Bcl-2, Mcl-1 and Bcl-xL. On the other hand, Bax, Bak, Bad, Bid, Bik, Bim and Bcl-Xs are several of the pro-apoptotic proteins. After activation, Bax and Bak are recruited to the mitochondria and form pores (Antonsson et al., 1997) allowing for the cytochrome c to be released into the cytosol, forming apoptosome complex with Apaf-1 (apoptotic protease-activating factor 1) (Liu et al., 1996; Zou et al., 1997). The apoptosome converts inactive pro-caspase 9 to active Caspase-9 (Hu et al., 1999). Then, Caspase-9 activates other caspases including Caspase-3 and Caspase-7 leading to apoptosis.

The extrinsic pathway is initiated upon ligation of death receptor including Fas or TNF (tumor necrosis factor) receptors by death signals from outside the cell (Krammer 2000, Siegel et al., 2000). Death signals bind to the death receptors on the surface of the cell converting inactive pro-caspase 8 into active Caspase-8. Caspase-8 then activates caspase-3 initiating the caspase cascade leading to apoptosis. Figure 2.4 gives a brief overview on the factors involved in the intrinsic and extrinsic apoptosis pathways. In addition to apoptosis, cancer cells may evade cell death by modulating other cell death mechanisms including necrosis and autophagy (Choi et al., 2013, Fulda, 2013).

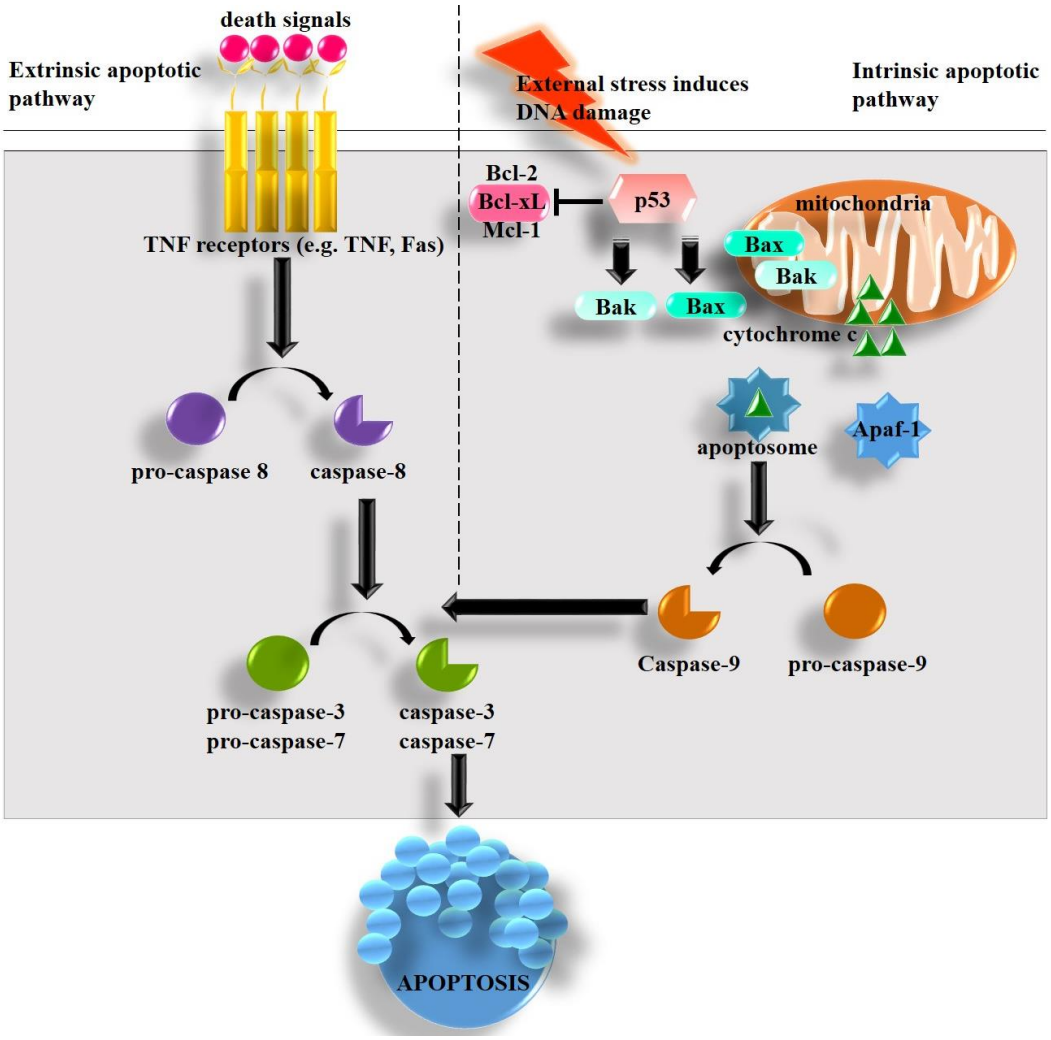


Figure 2.4 Schematic representation of the intrinsic and extrinsic apoptosis pathways. The intrinsic pathway is typically initiated by DNA damage, which activates p53. The p53 then activates Bax initiating the release of cytochrome c from the mitochondria. The released cytochrome c binds to Apaf-1 forming a complex called apoptosome. The apoptosome converts inactive pro-caspase 9 to active Caspase-9, which then activates Caspase-3. Caspase-3 begins the caspase cascade leading to apoptosis. In the extrinsic pathway, death signals from outside of the cell bind to the death receptors on the surface of the cell converting inactive pro-caspase 8 into active Caspase-8. Caspase-8 then activates caspase-3, which begins the caspase cascade leading to apoptosis. (Modified from Favaloro et al., 2012).

It is suggested that to form tumor mass, cancer cells require unlimited replication potential (Hanahan and Weinberg, 2011). Cancer cells must overcome the barriers for cell proliferation; replicative senescence and crisis. Senescence is a state of irreversible growth arrest that is triggered by multiple mechanisms including accumulation of DNA damage, epigenetic control of *INK4a/ARF* locus and telomere shortening (Collado et al., 2007). Abrogation of senescence leads to crisis, which involves cell death. Cells in crisis undergo spontaneous mitotic arrest leading to cell death. Cancer cells inhibit senescence and crisis through the upregulation of telomerase or by maintaining the telomere (Mathon and Lloyd, 2001). Additionally, inactivation of either p53 or pRb extends the lifespan of the cells despite short telomeres (Hara et al., 1991; Shay et al., 1991).

The final hallmark of cancer is the ability to induce angiogenesis where cancer cells require sustenance to support the neoplastic growth (Hanahan and Folkman, 1996). Angiogenesis is the formation of new blood vessels controlled by various signals including metabolic stress (e.g. low pH, hypoglycemia), inflammatory response and genetic mutations (Carmeliet and Jain, 2000). Without blood vessels, tumor is unable to grow or metastasize to other sites. Various proteins have been identified as angiogenic activators including angiogenin, VEGF (vascular endothelial growth factor), TGF-α (transforming growth factor α) and bFGF (basic fibroblast growth factor). Significant correlation has been shown between the VEGF expression and prognosis in several cancers including colorectal, breast, and lung cancers (Nishida et al., 2006). VEGF gene expression can be upregulated by hypoxia (i.e. reduced oxygen condition) and activation of oncogenes. Furthermore, experimental