EFFECT OF POTASSIUM KOETJAPATE, A DRIVATIVE OF KOETJAPIC ACID ISOLATED FROM *SANDORICUM KOETJAPE* MERR. ON HUMAN COLORECTAL CANCER.

by

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DEDICATION

This thesis is dedicated to

the angels of my life, my parents,

Seyedeh Pouran Hashemi

and

Seyed Sadegh Jafari,

for their sincere love, valuable

encouragement and support

throughout my life.

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TABLE OF CONTENT

CHAPTER 2 - MATERIAL AND METHODS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATION

APC gene	Adenomatous polyposis coli gene
LDH	Lactate dehydrogenase
AIF	Apoptosis inducing factor
Apaf-1	Apoptotic protease activating factor 1
BAG-1	Bcl-2 family anti-apoptotic protein
BAK	Bcl-2 homologous antagonist killer
Bax	Bcl-2 Associated-X Protein
Bcl-2	B-cell lymphoma-2
Bid	BH3 interacting-domain death agonist
Bim	Bcl-2-interacting mediator of cell death
CAM	Chick embryo chorioallantoic membrane
CC	Column chromatography
CCRF-CEM	Human lymphoblast leukemia cell line
CDDO	2-cyano-3,12-dioxoolean-1,9-dien-28-oate
CDDO-Me	2-cyano-3,12-dioxoolean-1,9-dien-28-oate methyl
CDDO-Im	2-cyano-3,12-dioxoolean-1,9-dien-28-oic imidazolide
cFLIP	Cellular FLICE-like inhibitory protein
cIAP	Cellular inhibitor of apoptosis
CL-6	Cholangiocarcinoma cells
Cox2	Cyclooxygenase-2
CRC	Colorectal carcinoma
CTL	cytotoxic T lymphocytes
Cyt c	Cytochrome c
DCC	Deleted in Colorectal Carcinoma
DCFH-DA	2′,7′-Dichlorofluorescein diacetate
DcRs	Decoy receptors

DISC	Death inducing signalling complex
DIABLO	Direct inhibitor of apoptosis-binding protein with low pI
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DPPH	2,2 Diphenyl-1-picrylhydrazyl
DSC	Differentialtial scanning calorimetry
EC	Endothelial cell nmr
ECM	Extracellular matrix
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
ERK ½	Extracellular-signal-regulated kinases
EMT	Epithelial–mesenchymal transition
EMSA	Electrophoretic mobility shift assay
FADD	Fas-associated death domains
Fas (CD95/Apo1)	First apoptotic signal/ Cluster of differentiation 95/ Apoptosis antigen 1
FasL	Fas Ligand
FBS	Foetal bovine serum
FLIP	FLICE like inhibitory protein
FRAP	Ferric reducing antioxidant power
FT-NMR	Fourier transform-nuclear magnetic resonance
GC-MS	Gas chromatography mass spectrometer
Hep-2	Human laryngeal cancer cell line
HIF-1α	Hypoxia inducible factor-1α
HREs	Hypoxia response DNA elements
HSP	Heat shock proteins
IAPs	Inhibitors of apoptosis proteins
IGF2	Insulin-like growth factor 2 receptor
IGFBP-2	Insulin like growth factor binding protein 2

IGFBP-6	Insulin like growth factor binding protein 6
IκB	Inhibitory κβ
K562	Human erythroleukemia cells
KA	Koetjapic acid
KKA	Potassium koetjape
KB	Oral carcinoma cells
LD_{50}	Lethal dose to kill 50% of animals
LEF1	Lymphoid enhancing factor-1
MAPK	Mitogen activated protein kinases
MCF-7	Breast adenocarcinoma cell line
MMP	Matixmetalloproteases
MOH	Ministry of health
MOMP	Mitochondrial outer membrane potential
MTT	(3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide
MVD	Microvessels density
NCR	National cancer registry
NF-κB	Nuclear factor-κB
NMR	Nuclear magnetic resonance
NuMA	Nuclear protein
PBS	Phosphate buffer saline
PIGF	Placental growth factor
pRb	Retinoblastoma protein
PS	Phosphatidylserine
PVP	Polyvinylpyrrolidone
Smac	Second mitochondria-derived activator of caspases
Rpm	Revolutions per minute
RPMI-1640	Roswell Park Memorial Institute-1640
ROS	Reactive oxygen species

SD	Sprague Dawley
SI	Selectivity index
SIMPs	Soluble intermembrane proteins
TCF	T-cell factor
TGF	Transforming growth factor
TGI	Tumor growth inhibition
TLC	Thin layer chromatography
TNF-R	Tumour necrosis factor cell surface death receptors
TRAIL	Tumor necrosis factor-related apoptosis inducing ligand
TRAILR1 (DR4)	Death receptors 4
TRAILR2 (DR5)	Death receptors 5
TS	Tumor spheroid
USM	Universiti Sains Malaysia
VEGF	Vascular endothelial growth factor
VEGFR	VEGF receptors
WNT	Wingless integrated
xIAP	X-linked inhibitor of apoptosis

KESAN KALIUM KOETJAPAT, SATU TERBITAN ASID KOETJAPIK YANG DIASINGKAN DARIPADA SANDORICUM KOETJAPE MERR. KE ATAS KANSER KOLOREKTAL MANUSIA.

ABSTRAK

Dalam kajian ini, usaha dilakukan untuk menambahbaik pelarutan air acid koetjapik (KA) dan mengkaji efikasi anti- kanser kolonnya, menggunakan metod-metod *'in vitro'* dan *'in vivo'*. Garam kalium KA iaitu, kalium koetjapat disediakan melalui metod separa-sintetik. Aktiviti-aktiviti antikanser kalium koetjapat dibandingkan dengan aktiviti sebatian asal iaitu, KA. Asai viabiliti sel MTT digunakan untuk mendapatkan dan membandingkan nilai IC_{50} kedua-dua sebatian. Kesan-kesan pro-apoptotik kalium koetjapat dinilaikan dengan menggunakan asai kaspas-kaspas (3/7, 8 dan 9), pewarna fluresen Hoechst 33342 dan Rhodamine 123. 'Profiler array' protiom apoptosis manusia digunakan untuk mengenalpasti sasaran protin yang bertanggungjawab bagi induksi apoptosis. Tambahan lagi, kesan antitumor *in vitro* kalium koetjapat telah dikaji menggunakan asai 'titik tergantung'. Tiga dos kalium koetjapat (25, 50, dan 100 mg/kg berat badan) telah dikaji dalam model mencit bogel 'athymic' untuk mengkaji efikasi *in vivo* anti-tumor kalium koetjapat. Dalam kajian ini pelbagai formulasi KA telah disediakan. Kajian pelarutan menunjukkan bahawa derivatif KA iaitu, kalium koetjapat, mempunyai pelarutan air lebih baik daripada dispersi pepejal KA. Kajian antikanser *in vitro* menunjukkan bahawa kalium koetjapat mempunyai aktiviti sitotoksik lebih baik daripada KA dan kompleks dispersi pepejalnya, terhadap titisan sel HCT 116. asai

pewarna fluresen menunjukkan bahawa kalium koetjapat mempunyai sifat menginduksi apoptosis. Ia menginduksi kondensasi kromatin dan menurunkan potensi membran mitokondria secara kebergantungan dos. Tambahan lagi, ia menaikkan tahap kaspas dalam sel-sel HCT 116. Keputusan protin apoptosis 'array' menunjukkan bahawa kalium koetjapat mempengaruhi aktiviti beberapa protin. Ia menurun-aturkan ekspresi beberapa protin anti-apoptotik dan regulator negatif apoptosis termasuk Bcl-2, HSP60, HSP90 dan IGF-1 dalam sel-sel HCT 116 dengan penaik-aturan protin-protin TRAILR-1 dan TRAILR-2, CD40, IGFBP-6, p27, kaspas 3 dan kaspas 8. Tambahan lagi, kalium koetjapat menunjukkan kesan antimetastatik terhadap sel HCT 116 dalam asai-asai *in vitro*. Keputusan-keputusan ini mungkin disebabkan oleh penurun-aturan laluan-laluan signal Wnt, Notch, Hypoxia, MAPK/ERC dan MAPK/JNK dalam sel-sel HCT 116, bersama dengan penaik-aturan faktor transkripsi untuk laluan-laluan putaran sel (pRb-E2F). KKA juga menghalang proses angiogenesis in vitro dengan menghalang proses-proses penembusan, penghijrahan dan pembentukan dan pembentukan tiub sel-sel endothelium . Kajian toksisiti akut menunjukkan bahawa kalium koetjapat mempunyai LD_{50} lebih daripada 2000 mg/kg dalam tikus betina SD. Keputusan kajian tumor 'spheroid' menunjukkan bahawa kalium koetjapat mempunyai kebergantungan dos potensi antitumor dan data ini berkait dengan keputusan kajian tumor *in vivo*. Kalium koetjapat menunjukkan perencatan poten pembiakan tumor (68.15%, 82.35% dan 92.76%, pada 25, 50 dan 100mg/kg, secara berurutan). Keseluruhannya, keputusan kajian ini

menunjukkan bahawa kalium koetjapat mempunyai aktiviti anti-kanser terhadap kanser kolorektal.

EFFECT OF POTASSIUM KOETJAPATE, A DRIVATIVE OF KOETJAPIC ACID ISOLATED FROM SANDORICUM KOETJAPE MERR. ON HUMAN COLORECTAL CANCER.

ABSTRACT

In the present study an attempt was made to enhance the aqueous solubility of KA and to study its anti-colon cancer efficacy using *in vitro* and *in vivo* methods. Potassium salt of KA i.e., potassium koetjapate was prepared by semi-synthetic method. Anticancer activities of potassium koetjapate were compared with the native compound i.e., KA. MTT cell viability assay was used to obtain and compare the IC_{50} values of both the compounds. Pro-apoptotic effects of potassium koetjapate were assessed using caspases (3/7, 8 and 9), Hoechst 33342 and Rhodamine 123 fluorescent staining assays. Human apoptosis proteome profiler array was used to identify the protein targets responsible for the induction of apoptosis. Furthermore, *in vitro* antitumor effects of potassium koetjapate were studied using hanging drop assay. Three doses of potassium koetjapate (25, 50, and 100 mg/kg body weight) were tested in athymic nude mice model to study the *in vivo* anti-tumorigenic efficacy of potassium koetjapate. In this study, various formulations of KA were prepared. Solubility studies revealed that resultant KA derivative i.e. potassium koetjapate had better aqueous solubility than the solid dispersions of KA. *In vitro* anticancer studies revealed that potassium koetjapate has better cytotoxic activity than KA and its solid dispersion complex towards HCT 116 cell line. Fluorescent staining assays showed

that potassium koetjapate has apoptosis-inducing nature. It induced chromatin condensation and decreased mitochondrial membrane potential in a dose-dependent manner. Furthermore, it increased the levels of caspases in HCT 116 cells. The results on apoptosis protein array show that potassium koetjapate modulated the activity of multiple proteins. It down-regulates the expression of multiple anti-apoptotic proteins and negative regulators of apoptosis including Bcl-2, HSP60, HSP90 and IGF-1 in HCT 116 cells with concomitant up-regulation of TRAILR-1 and TRAILR-2, CD40, IGFBP-6, p27, Caspase 3 and caspase 8 proteins. Furthermore, potassium koetjapate showed antimetastatic effect towards HCT 116 cells in a series of *in vitro* assays. These results are probably due to down regulation of Wnt, Notch, Hypoxia, MAPK/ERC and MAPK/JNK signalling pathways in HCT 116 cells coupled with the up-regulation of transcription factor for cell cycle (pRb-E2F) pathways. Moreover, KKA inhibited angiogenesis in *vitro* by stopping endothelial cells neovascularization, migration, tube formation and VEGF release. Acute toxicity studies reveal that potassium koetjapate has LD_{50} more than 2000 mg/kg in female SD rats. Results of spheroid tumor studies show that potassium koetjapate has dose-dependent antitumor potential and this data correlates with the outcomes of the *in vivo* tumor studies. Potassium koetjapate showed potent inhibition of tumor growth (68.15%,82.35 % and 92.76% at 25, 50 and 100mg/kg, respectively). Altogether, outcome of present study shows that potassium koetjapate has good anti-cancer activity towards colorectal cancer.

CHAPTER 1

INTRODUCTION

1.1 Cancer

A major global health issue, cancer is described as unrestrained growth of cells leading to the invasion of local tissues and tumor metastasis (1). Cancers are categorized by the type of cells from which the tumors originate including carcinoma, sarcoma, blastoma, germ cell tumor, lymphoma, leukemia and adenoma (benign tumor of glandular origin), adenocarcinoma (malignant adenoma). Cancers derived from epithelial cells that include common cancers such as most types of breast, prostate, lung and colorectal cancers are subsumed under carcinoma while sarcoma cancer types occurring in connective tissues such as fat, bone, nerve, and cartilage. Conditions such as leukemia and lymphoma start off from hematopoietic whereas germ cell tumors which are mostly existing in the testicle or the ovary result from pluripotent cells. Blastoma cancers originate from immature “precursor” cells or embryonic tissue (2). In 2008, lung, breast and colorectal cancers were reported to be the three most commonly diagnosed cancers, while in case of cancer-associated mortalities worldwide, lung, stomach, and liver cancers were found to be the most common cancer types (3) Presently more than 200 kinds of cancer have been recognized which are the second major cause of death worldwide, overtaken only by heart disease(4). In 2015, cancers of breast, colorectal, lung and prostate were estimated to be the most common causes of cancer-related mortalities (5). According a recent report, cancer is now

the leading cause of death in 21 states in the USA, cancer is currently the primary cause of death, due to exceptionally large reductions in deaths from heart diseases. Although cancer-related death rate has decreased by 23% since 1991, or more than 1.7 million deaths were prevented up to 2012, death rates are increasing for cancers of the pancreas, liver, and uterine corpus . In developing countries, there are more new cases than what is documented in the developed countries (5,600,000 vs. 7,100,000 cases respectively(6) . Cancer was found to be the third most common reason for deaths overtaken by cardiovascular and septicaemia diseases in Malaysia according to the Malaysian National Cancer Registry report in 2007. According to another report, three most commonly diagnosed cancers in Malaysia were breast, cervical and colorectal cancers followed by bone marrow, lung, lymph node and liver cancers respectively while five most commonly diagnosed cancers in the male population were lung, colorectal, nasopharynx, prostate and lymphoma respectively. Whereas in the female population, cancers of breast, colorectal, cervix, ovary and lung were at the top list (7). A range of factors may result in an increase in the risk of cancer. The causative factors involved in the progress of cancer include smoking, obesity, exposure to chemicals, oxidative stress, and radiation as external factors while inherited mutations (hyper-activation of oncogenes, and inhibition of tumor-suppressing genes), metabolic deregulations, hormone imbalance, and dysfunction of immune system are considered as the main internal factors(8) . Surgery, chemotherapy and radiotherapy are the most common treatments of cancer nowadays (9).

1.1.1 Colorectal carcinoma

Among various types of cancer, colorectal cancer (CRC), a malignant tumor of large intestine, ranks third in the world as a lethal and metastatic carcinoma while the incidence and death rates of CRC has decreased by around 3% per year in both men and women from 2003 up to the end of 2012(5). Still, CRC is a significant cause of mortality in both men and women worldwide. According to a survey, every year more than 945 000 people develop colon cancer out of which around 492 000 patients die (10). in the Malaysian Peninsula, CRC is the most prevalent type of cancer in men and the third most common cancer in women According to the National Cancer Registry Report 2003-2005. The Age-Standardized Rate (ASR) was highest among Chinese men, in whom it was more than two times that of Indian and Malay men. Chinese women also had an ASR, which was more than twice that of Indian and Malay women. Hypertension, obesity, abnormal blood lipids, and high fasting blood glucose are considered as main metabolic risk factors for colorectal cancer(11) . The growth in colorectal tumor is due to the mutational activation of oncogenes coupled with the mutational inactivation of tumor suppressor genes. Hence, it is described as a multi-step disease that converts normal epithelial cells of the colon into invasive carcinoma (Figure 1.1). An ordered series of events, recognized as the “Adenoma-Carcinoma Sequence”, causes the development of colorectal neoplasms. There must be at least four mutated genes to form malignant tumors affected by few further changes leading to benign tumorigenesis. The histopathologic changes occur due to genetic as well as environmental factors. The most important environmental factors

include pathogen invasion, toxins, generation of ROS (Reactive Oxygen Species) and stress conditions. Currently, the inherited and somatic genetic deficiencies contributing to the development of colorectal carcinogenesis have been discovered such as sustainable changes in COX2 (Cyclooxygenase-2), KRas, Ctnn-Beta (Catenin-Beta), APC (AdenomatousPolyposisColi),SMAD4 (Smaand MAD (MothersAgainstDe capentaplegic), p53, TGF-BetaR2 (Transforming Growth Factor-Beta Receptor-Type II), BAX (Bcl2 Associated-X Protein), E2F4 (E2F Transcription Factor-4) and MMR (Mismatch Repair) genes like, MSH2 (MutS Homolog-2), MSH3 (MutS Homolog-3), MSH6 (MutS Homolog-6), MLH1 (MutL Homolog-1) and MMP (Matrix Metalloproteinase)-1/2/7/9/11/12/14, loss of the 18q21 gene and microsatellites instability (12-14). At the beginning, when the normal colonic epithelia (Stage-0) is subject to unfavorable conditions such as pathogenic invasions, APC and Ctnn-Beta mutations, toxins and generation of ROS; it is changed into Dysplastic ACF (Aberrant Crypt Foci) or Dysplastic Adenoma or Dysplastic Epithelia (Stage-I) (15). An increase in microsatellite repeat sequences along with KRas and COX2 mutations play a major role in transforming Dysplastic epithelia to early adenoma phase (Stage-II). KRas mutations occur in 39% of human colorectal cancers such as KRas mutations combined with MSH2 (deletion) mutations DCC (point mutation in 70% of colon cancers) and MLH1 (substitution, deletion and hypermethylation of CpG sites), which lead to the transition from early adenoma to late adenoma (Stage-III). Lastly, transitions from late adenoma to increasingly tumor metastasis through colorectal carcinoma (Stage-IV))

comprise the less frequently targeted genes like BAX via frameshift mutations which happen in over 50% of colon adenocarcinomas along with deletion in E2F4, MSH3 and MSH6 fallowed by gene modifications of p53 and TGF-BetaR2, MMP1/MMP2/MMP7/MMP9/MMP11/MMP12 (over-expression) and SMAD4 mutations (lack of alleles on chromosomes 17 and 18 in polyploid colorectal tumors). Particularly, point mutation on p53 gene is the main reason for causing 50% of colorectal cancers whereas SMAD4 mutations (aneuploid/polyploid) are linked with metastatic carcinoma (16, 17).

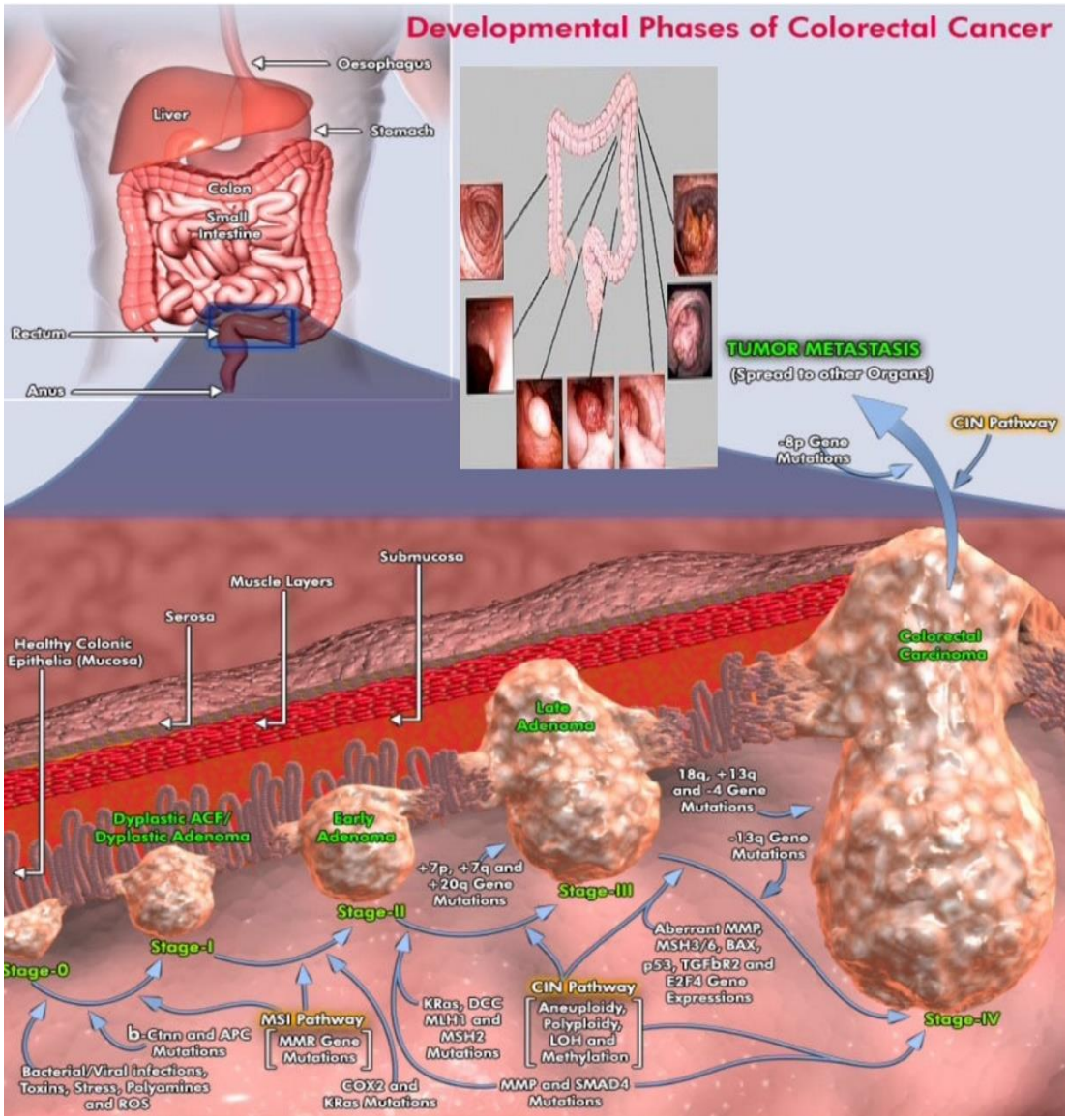

Figure 1.1: Adenoma-Carcinoma Sequence, Adapted from Qiagen (18)

1.2 Major Signaling Pathways involving in Colon Cancer:

1.2.1 Wnt/β-catenin Signaling Pathway:

The Wnt signal transduction pathway is an old pathway that is evolutionarily conserved and has a key role in the development of the embryo such as cell migration, cell polarity, along with a major role in cell to cell signaling (19, 20). Mutations in the Wnt pathway have a fundamental

role in cancer development, especially in colon cancer. The Wnts consist of a large family of nineteen glycoproteins in humans. So far, major signaling branches which are downstream of the Fz receptor have been identified such as canonical or Wnt/β-catenin dependent pathway and the non-canonical or β-catenin independent pathway. The Planar Cell Polarity and the Wnt/Ca2+ pathways are two branches of Wnt/β-catenin dependent pathway which are being actively dissected at the molecular and biochemical levels (21). Around 90% of all colon cancer cases are related to the APC tumor suppressor gene mutations, which trigger Wnt/β-catenin accumulation (22). β-catenin and TCF/LEF transcription factors combine and consequently boost the expression of c-Myc, cyclin D1 and MMP genes involved in carcinogenesis and tumor angiogenesis(23) . Therefore, a possible objective in curing different types of cancer, i.e. colon cancer, can be the down-regulation of the Wnt pathway.

1.2.2 Notch Signaling Pathway

Notch signaling pathway can promote or suppress cell proliferation, differentiation, death, and fate specification. This pathway significantly interferes in some physiological programs such as apoptosis, adhesion, migration and angiogenesis throughout adult tissue renewal leading to the development of the organism. Because of its essential role in many important processes, abnormal gain or loss of pathway components has been directly linked to multiple human diseases including cancer (24, 25). Notch can be an oncogene or a tumor suppressor gene based on factors such as the timing, cell type, signal strength, and the normal function of certain

tissues (26) . Various types of cancer including colon, melanoma, renal, breast, pancreas, and lung cancers have Notch pathway signaling (27, 28). Some studies have shown strong relations between Notch and Wnt pathways in colon cancer which strengthen the hypothesis that Notch signaling might be in a downstream of Wnt (29-31). Thus, Notch signaling pathway can be used in combination with Wnt inhibitors as a potential treatment of colorectal carcinoma.

1.2.3 P53 Signaling Pathway

The P53 is a nuclear protein, which is known as “the guardian of the genome” because of its role in the detection of genetic damage and in triggering the genetic repair mechanisms(32). It can also trigger apoptosis in case of irreparable DNA damage. When P53 is mutated, it will not be able to perform its function as frequency (>50%) of mutation in the P53 gene is the most in human cancer. This indicates that the P53 tumor suppressor gene has an a key role in cancer prevention is played by the P53 tumor suppressor gene through the cell cycle arrest mechanism which may result in inducing apoptosis (33). Mutations in the P53 suppressor gene are common in all cancers, which usually occur in the central DNA-binding core domain. Mutations mostly hamper the protein’s ability to attach to its target DNA strands, thus preventing transcriptional activation of the genes. The P53 protein controls cell death using different mechanisms that result in the regulation of genes entangled in both the extrinsic and intrinsic pathways of apoptosis either via transcriptional-independent or transcriptional-dependent mechanisms (34). Therefore, increasing the

amount of P53 could be a promising strategy in the treatment of cancers with lower side effects. Recently, small molecules such as small peptides have been used to reactivate the suppressed wild-type P53 or to return the mutant into a wild-type P53 (35).

1.2.4 Cell Cycle (pRB/ E2F) Signaling Pathway

Retinoblastoma (Rb) is a malignancy of the developing retina, which happens in children and is considered to be the most common malignancy of the eye in children (36). The retinoblastoma tumor suppressor (pRB) plays an important role in cell cycle processes and apoptosis. The pRB gene is mutated in around 50% of all human tumors . Moreover, genes encoding upstream regulators of pRB have been found to be mutated in the remaining 50% of all human tumors. About 60% of affected individuals have unilateral Rb while around 40% have bilateral Rb Heritable retinoblastoma is an autosomal dominant vulnerability to Rb RB1 was the first tumor suppressor gene cloned. Recently, it has been reported that pRB can bind with a series of transcription factors such as E2F and form dimmers that control the expression of several downstream effector genes involved in cell cycle control, mitosis, DNA repair and apoptosis(37). It appears that RB1 interacts with more than 100 cell proteins resulting in regulation of the critical G1 to S-phase transition in the cell cycle. It has also been found that the Rb/E2F complex has the main role in maintaining G1 arrest in connection with the p21/p27 family of cdk inhibitors (38).

1.2.5 NF-κB Signaling Pathway

The nuclear factor κB enhancer binding protein (NF-κB) family of transcription factors, control the expression of a large group of genes engaged in diverse cellular processes including the inflammation, immunity, migration, adhesion, cell growth, apoptosis and cell survival. Deregulated NF-κB has been linked to a variety of human diseases, particularly cancers. The oncogenic role of NF-κB seems to be mediated through its anti-apoptotic function, particularly through induction of Bcl-XL. The NF- κB family consists of five DNA binding proteins as follows: c-Rel, NF- κB 1/p50 NF- κB2/p52, RelA (p65) and RelB, which function as various homodimers and heterodimers. A highly conserved 300-aminoacid-long N-terminal Rel homology domain (RHD) which is a common domain in all five NF- κB proteins functions to dimerization, DNA binding, interaction with the inhibitors of NF- κB as well as nuclear translocation (39, 40). It was reported in several studies that NF-κB target over 200 different genes such as Myc, Rel, and Cyclin D1-4 which are engaged in cell cycle regulation, Bcl-2, Bcl-Xl, A1/Bf-1 which function in the apoptosis process, VEGF gene which has a critical role in angiogenesis process leading to the belief in the oncogenic potential of "normal" NF-kB (39, 41, 42). It was also found that NF-kB signaling system has an important role in bridging inflammation and cancer (39). Some new studies reported on the identification of cancer-associated mutations in upstream components of the IkB kinase -NF-kB (IKK-NF-kB) signaling system that can result in cell autonomous activation of NF-kB in multiple myeloma (43, 44) . Recently, Kojima and his group have reported that LPS increases

COX-2 expression in a certain colon carcinoma cell line through NF-kB which is continuously activated in colorectal carcinoma tissue samples. They also demonstrated that NF-kB is constitutively activated in colorectal carcinoma tissues by using an electrophoretic mobility shift assay (EMSA) andimmuno-histochemical staining. In addition, they found that NF-kB activation is closely related to cancer progression(45) . New recent studies suggest that activation of NF-κB appears to perform an essential role in cancer development as it was reported that NF-κB is obviously activated in 50% of CRC patients and those with colitis associated tumors and later it has been established by mouse model studies that NF-κB functions criticality in the development of Colitis-associated cancer (CAC) (40). Recently researchers tried to synthesize compounds that target this pathway such as cinnamaldehyde which was reported as an apoptosis inducer agent acting via mitochondrial pathway, and hence it has been found as a potent NF-κB pathway inhibitor. The essential roles taken by this pathway in apoptosis inhibition and tumor maintenance suggest that inhibitors of the pathway would be effective anti-cancer agents(45-47)

1.2.6 Myc/Max Signaling Pathway

Numerous studies demonstrated that a mutated version of Myc, which is constitutively expressed, leads to the unregulated expression of many genes, which result in diverse cancers. Myc has been reported to be hyper-activated in 70% of all human cancer cases including colon, breast and lung cancers while it acts as an angiogenesis switch as well (48, 49). On the other hand, Myc/Max heterodimers induce intracellular transduction

pathways which are critical for induction of apoptosis(49) . It has been found that Myc is activated via various mitogenic signals such as MAPK/ERK pathway(50). A notable tumor shrinking in transgenic mice was achieved by suppressing of the Myc/Max which resulted in targeting this pathway(51). Another study **demonstrated that c-Myc maintains** embryonal rhabdomyosarcoma (ERMS) transformed phenotype and radio-resistance by safeguarding cancer cells from radiation-induced apoptosis and DNA damage, while stimulating DNA repair induced by radiation. The findings suggest that c-Myc targeting can be an effective treatment in cancer therapy (48).

1.2.7 Hypoxia pathway

Aerobic energy metabolism processes such as oxidative phosphorylation needs oxygen in eukaryotic cell while low oxygen environments activate the hypoxia signaling pathways. Hypoxia signaling dysfunction normally happens in conditions such as tumor angiogenesis and chronic inflammation. Solid tumors frequently consist of hypoxic regions. The cells in the core of tumor which is located too far away from blood vessels become hypoxic. Being related to higher invasion risk and metastasis, intratumoral hypoxia is more robust to chemotherapy and radiation resulted in more patient mortality (52). As a transcription factor, hypoxia inducible factor (HIF) causes most of the response in hypoxia pathways. HIF-1 complex is a heterodimers protein comprising of an exclusive subunit that is tightly expressed (HIF-1α) having 3 isoforms of HIF-1α, HIF-2α, and HIF-3α and a regular constitutively-expressed beta

subunit (HIF-1β). The presence of oxygen provokes prolylhydroxylases to hydroxylate HIF, resulting in the polyubiquitination and degradation of HIF while under low oxygen conditions, prolylhydroxylase inhibition leads HIF to accumulate which in its turn activates transcription of about 100 genes encoding proteins that mediate some major biological processes such as angiogenesis, wound healing, invasion and metastasis, human metabolism, chondrocyte survival in bone growth plates, autophagy and cell death. Increased HIF-1α or HIF-2α levels are found in human colon, breast, prostate and lung carcinomas, and are linked with increased patient mortality. Some experimental data shows that hampering HIF-1 signaling blocks tumor growth in mouse models(53, 54). Research has indicated that in normal oxygen pressure, NF-κB (nuclear factor κB) directly modulates HIF-1α expression. Investigation of siRNA (small interfering RNA) as for individual NF-κB members displayed differential effects on HIF-1α mRNA levels, implying that NF-κB can control expression of normal HIF-1α VEGF-A and Ang-2 genes which are associated with extreme tumor angiogenesis and metastasis(52, 54). Another study has shown that HIF-1α and its downstream target miR-210 is provoked by hypoxia blocking Bcl-2 expression and increasing autophagy, hence this triggers resistance to radiotherapy in colon cancer cells. (55). The critical role of HIF in regulating the expression of multiple genes involved in tumor metabolism and angiogenesis makes it a potential target in cancer therapy. Hence, greater anticancer effects may be achieved by obstruction of HIF-1. Therefore, HIF inhibitors including the Klugine, Betulonic acid, phenethyl

isothiocyanate, taxol, and Acriflavine are still investigated for their anti-cancer properties (53, 56).

1.2.8 MAPK Signaling Pathways

MAPK pathway is a term used for referring to a three kinases module activated by phosphorylating one another sequentially as a reaction to different types of stimuli including neurotransmitters, cellular stress, cytokines, growth factors and cell adherencestimuli including neurotransmitters, cellular stress, cytokines, growth factors and cell adherence(57). This pathway uses one of the most generic singling designs discovered in biological signal transductions. Mitogen-activated protein kinases (MAPKs) are serine/threonine-specific protein kinases that were broadly used during evolution in many physiological processes such as gene expression, mitosis, growth control, cellular adaptation to chemical, physical stress, metabolism, motility, cell differentiation and survival, inflammation and apoptosis in all eukaryotic cells. There are 14 MAPKs in mammals, which have been divided into seven groups. Conventional MAPKs consist of the extracellular signal regulated kinases 1/2 (ERK1/2), p38 isoforms (α, β, γ, and δ), c-Jun amino (N)-terminal kinases1/2/3 (JNK1/2/3), and ERK5(58) . The MAPK/JNKs and MAPK/ERKs are involved in growth factor signaling, regulation of mitosis, migration and apoptosis while MAPK/p38 play an important role in inflammation(59). It was found that de-regulation of the above pathway resulted in various diseases such as cancer, immunological disorders, degenerative and inflammatory syndromes. As reported, MAPKs down regulate over 170

tumor suppressor genes including Tob1, JunD and Ddit3, which suppress cell growth and proliferation (60). Phosphorylation activates the MAPK pathway through a MAPKK/MKK (MAPK kinase), and that is phosphorylated by a MAPKKK/MKKK (MAPKK kinase). It was also found that MAPKs are disabled by several phosphatases including a preserved family of phosphatases named MAP kinase phosphatases (MKPs). These types of enzymes can hydrolyze the phosphate from phosphotyrosine and the phosphothreonine remains. The deletion of each phosphate group extremely reduces MAPK activity essentially giving up signaling. It has been found that some types of tyrosine phosphatases take part in inactivating MAP kinases including phosphatases such as STEP, HePTP, and PTPRR in mammals (61, 62).

Most inducers of the MAPK pathway begin signaling via activating receptors in the cell membrane, which result in activation of MAPKKK typical through a small GTPase. There is a large number of known MAPK agents including mostly of protein kinases, transcription factors, and cytoskeletal proteins. When MAPK is activated it can transfer from the cytoplasm to the nucleus, in which it controls gene transcription via impacting the structure of chromatin and transforming transcription factors activity (57, 62). It was reported that the activation of MAPK/ERK pathway provoked cell cycle arrest and apoptosis and hence could be an effective therapeutic target of different types of cancer such as osteosarcoma and pancreatic cancer therefore serious attempts were made to produce inhibitors of the ERK and JNK pathways and examine them in clinical trials (58, 63).

1.3 Apoptosis

The apoptosis process was first described in 1972 as a distinct form of cell death in term of morphology, whereas some characteristics of this phenomenon were explained earlier (64). So far apoptosis has been accepted as the most important type of genetically determined or "programmed" death of cells that is involved in cell disposal. Nevertheless, there are other types of programmed cell death which are also defined or will be discovered in the future (65, 66). Generally, organisms having multi-cells use two major methods for cell disposal: necrosis and apoptosis. Necrosis can be caused due to the breaking apart of the plasmatic membrane as a result of the formation of a swelling process. In contrast, in apoptosis, chromatin is condensed followed by fragmentation and forming apoptotic remains quickly engulfed by the macrophages hence this process does not induce any inflammatory reaction (67). Apoptosis can be activated by a variety of stimuli such as changes in the concentration of growth factors, ionizing radiations, heat shock and other cellular stress, infection by bacterial or viral particles, genetic mutations and damage of DNA(64, 67). Generally, cleavage of proteins, caspase cascade activation, changes in cellular bioenergetics and membrane potential along with expression of cell surface proteins which cause the early detection of apoptotic cells followed by DNA fragmentation are considered as the main features of apoptosis. Mitochondria perform a main role in mediating apoptosis by releasing pro-apoptotic proteins such as cytochrome c, Smac, Omi and AIF into the cytosol. Excessive apoptosis can result in the progress of acquired immune deficiency syndrome (AIDS), renal damage, neurodegenerative

disorders and cardiac ischemia. On the other hand, reduced apoptosis can result in development of cancer and autoimmune diseases(68, 69) .

1.3.1 Apoptotic Pathways

Apoptosis may be caused in mammals through three pathways based on apoptosis regulators and the location where they act: the first pathway is extrinsic pathway, started by the ligation of death receptors before the activation of caspase-8 and processing extracellular death-inducing; the second pathway is called intrinsic pathway, started by cell stress before the activation of caspase-9, and the third type of pathway is called Granzyme/Perforin pathway, which can trigger members of the caspase family by processing of caspase zymogens. Nevertheless, granzyme A functions in a caspase-independent way. Ultimately, the apoptosis pathways lead to the execution pathway ending in cytomorphological features of apoptosis suc cell h as chromatin condensation, shrinkage, cytoplasmic blebs formation prior to phagocytosing the apoptotic bodies (64). A summarized schematic image of apoptosis pathways is illustrated in Figure 1.2.

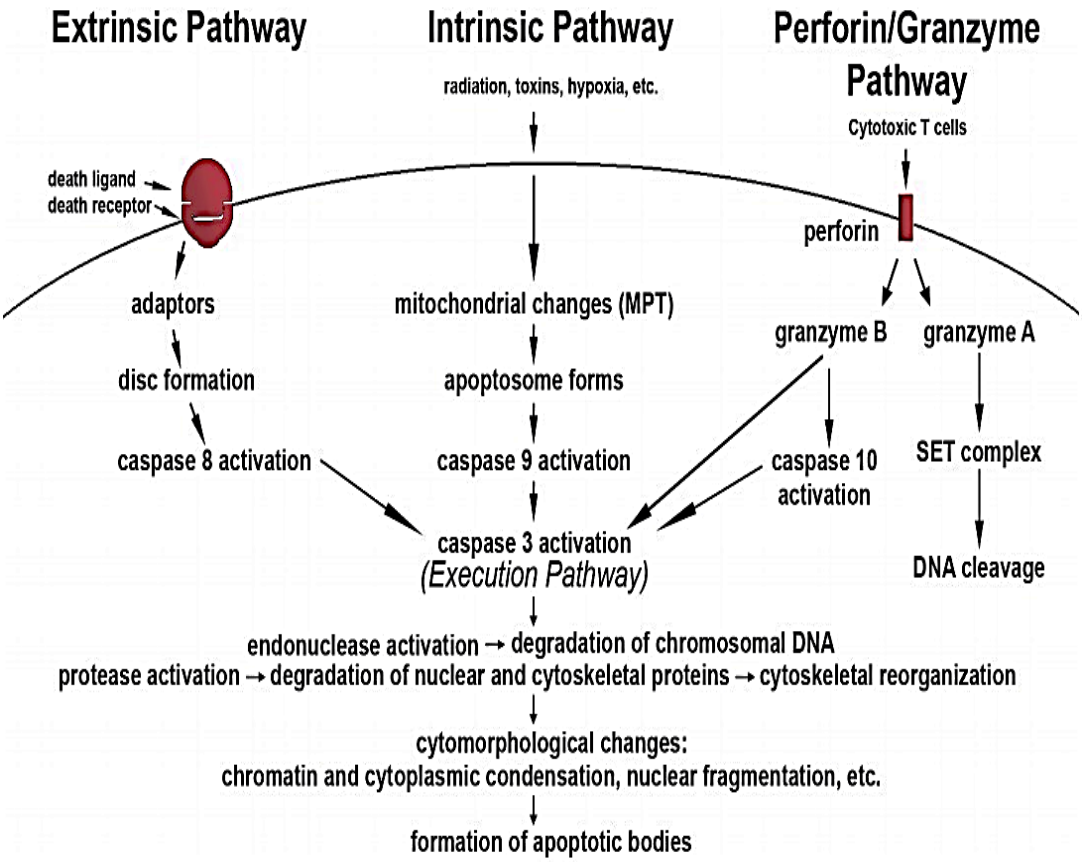

Figure 1.2: Schematic illustration of apoptosis. The three pathways of apoptosis i.e. extrinsic, intrinsic and perforin/granzyme pathways. Adopted from (64).

1.3.1(a) Extrinsic Apoptotic Pathway

The extrinsic pathway or death receptor-mediated pathway is triggered by binding of extra- cellular ligands with a family of tumor necrosis factor death receptors which are located in the cell membrane. Fas (fibroblast associated antigen) and tumour necrosis factor receptor (TNF-R) are considered as typical death receptors which are involved in this pathway. These receptors contain an extracellular cysteine-rich site in order to bind the ligand, and an intracellular site for signal conduction. FasL/FasR, TNF-alpha/TNFR1, Apo2L/DR4/DR5 and Apo3L/DR3 are considered as the most prominent ligands and their corresponding receptors

of this pathway. Binding of Fas ligand to FADD (Fas-associating protein with death domain) and the binding of TNF ligand to TNF receptor lead to the binding of the adapter protein TRADD with complex of FADD and RIP(64, 70) . Subsequently FADD binds to procaspase-8 through dimerization of the death effector domain leading to formation of death-inducing signaling complex (DISC). Next, DISC activates downstream caspases 3 or other executioner caspases resulting in destruction of cellular targets and apoptosis. Moreover, in certain cell types, BH3-only protein (Bid) is activated by caspase-8 resulting in truncated Bid (tBid). Subsequently, tBid moves to the mitochondria and activates cytochrome c release leading to activation of caspase-9 and caspase-3(67). The whole process is described in Figure 1.3.

1.3.1(b) The Intrinsic Pathway of Apoptosis

The intrinsic pathway of apoptosis is initiated via non-receptor mediated mitochondrial-stimuli that act directly on targets inside the cell by producing intracellular signals which can be either positive or negative. The lack of certain growth factors, cytokines and hormones create negative signals followed by prevention of apoptosis. On the contrary, loss of apoptotic suppression leads to activation of apoptosis via positive signals such as radiation, oxidative stress, hypoxia, viral infections, and toxins which result in Bax/Bak insertion into mitochondrial membrane. The change in mitochondrial transmembrane by Bax/Bak is followed by loss of the mitochondrial transmembrane potential and releasing of pro-apoptotic proteins such as Cytochrome c into the cytosol from the intermembrane

space(64, 71) . Moreover, BH3-only proteins, such as Bid and Bim are involved in homo-oligomerisation of Bax or Bak which induce their pro-apoptotic function. Subsequently, Cytochrome c binds to the Apaf1 and (d)ATP causing the connection of pro-caspase-9 to the complex and forming the Apoptosome. Activated caspase-9 in turn induces caspase-3 and triggers proteolytic cascade. In contrast, anti-apoptotic Bcl-2 family members, for example Bcl-2 and Bcl-XL, inhibit cytochrome c release, probably via inhibition of Bax and Bak. Furthermore, mitochondria release many other polypeptides such as AIF, endonuclease G, second mitochondrial activator of caspases (Smac/Diablo) which can promote caspase activation via suppressing the inhibitory effects of anti-apoptosis proteins (IAPs) while AIF and Endo G create DNA damage and condensation(67, 72). In case of apoptosis initiation via chemotherapeutic agents, the mitochondrial pathway is more important than the death-receptor pathway for example caspase 9–deficient cells and Apaf-1–negative thymocytes are resistant to chemotherapeutic agents, however they can be stimulated into apoptosis via Fas, TRAIL, or TNF(73).

1.3.1(c) Perforin/granzyme Pathway

Granzyme B (Gzm B) is a caspase-like serine protease that is released by cytotoxic T lymphocytes (CTL) and natural killer (NK) cells to kill virus-infected and tumor cells. Therefore, granzyme B plays a significant role in human pathologies such as anti-viral immunity and tumor immune surveillance. The serine proteases granzyme A and granzyme B are the most important component within the granules which

have been examined in recent years(64, 74). Although caspase 3 was the first substrate to be identified for Gzm B, other reports have shown that Gzm B can stimulate several members of the caspase family of cysteine proteases by proteolytic processing of their substrates(75) . Gzm B is able to cleave proteins at critical aspartate residues, thereby stimulating pro-caspase-10 which cleaves ICAD (Inhibitor of Caspase Activated DNAse). Additionally, Gzm B uses the mitochondrial pathway to improve the death signal through specific cleavage of Bid where this protein stimulates the release of mitochondrial cytochrome c into the cytosol(76, 77) . Goping and her colleagues also have shown that Gzm B can directly stimulate caspase-3 resulting in the release of pro-apoptotic proteins that suppress caspase inhibition and direct induction of the execution phase of apoptosis which suggest that both the mitochondrial pathway and direct stimulation of caspase-3 are essential for granzyme B-induced killing (75). Other research indicates that death receptors and caspases do not play any role in the apoptosis of activated T helper 2 cells induced by T cell receptors because obstructing their ligands does not have any effect on apoptosis. In contrast, adapter proteins with death domains, Fas-Fas ligand interaction, and caspases are involved in apoptosis and regulating cytotoxic T helper 1 cells whereas granzyme B does not have any impact. Also granzyme A has a major role in cytotoxic apoptosis induced by T cells and stimulation of pathways that are independent from caspases. Granzyme A causes DNA disintegration using DNAse NM23-H1. The nucleosome assemblage protein SET inhibits the NM23-H1 gene. Granzyme A protease cuts the SET complex and, therefore prevents the inhibition of NM23-H1, causing

apoptosis via degradation of DNA;; hence inactivation of this complex by granzyme A probably results in apoptosis through hindering the DNA maintenance and chromatin structure stability (64, 78).

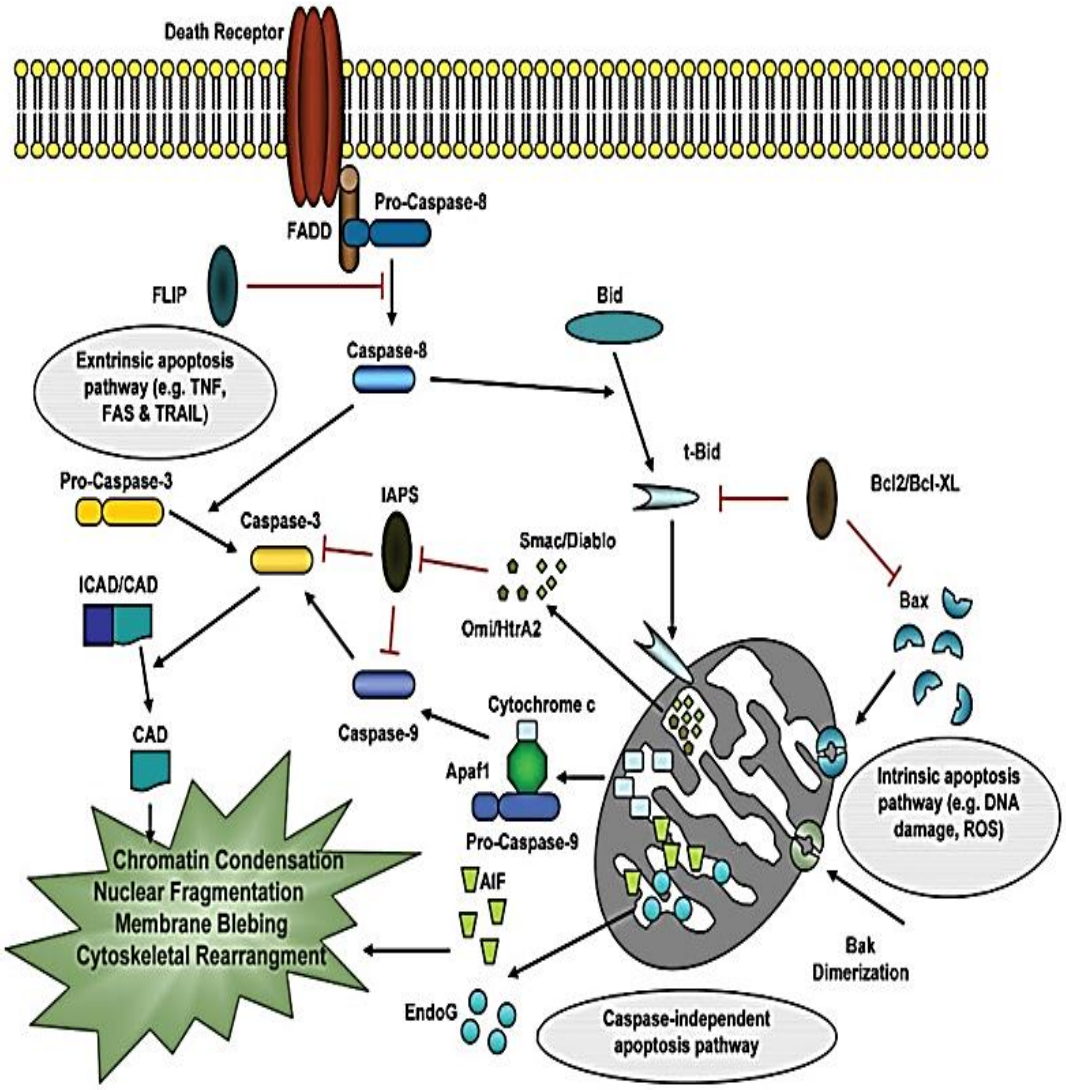

Figure 1.3: TRAIL death-receptor pathway of apoptosis.

1.3.1(d) Execution Pathway

Execution Pathway is considered the final pathway of apoptosis as the extrinsic and intrinsic pathways both terminate at the execution phase. Caspase-3, caspase-6, and caspase-7 function as effector or “executioner” caspases, cleaving various substrates such as cytokeratins, PARP, the nuclear protein NuMA and the plasma membrane cytoskeletal protein alpha fodrin resulted in degradation of nuclear material and cytoskeletal proteins that are followed by the major morphological and biochemical changes in apoptotic cells(79). Execution caspases activation in the signaling cascade is the apoptotic commitment point in which the cell kills itself (80) . Caspase-3, as the main executioner caspase, is activated by any of the initiator caspases (caspase-8, caspase-9, or caspase-10). Subsequently caspase-3 cleaves ICAD (endonuclease CAD mixed with its inhibitor) to release CAD which causes degradation of chromosomal DNA followed by chromatin condensation. Moreover, caspase-3 causes cytoskeleton rearrangement followed by disintegration of cells and formation of apoptotic bodies(64). Screening the translation yields of small complementary DNA pools recognized that gelsolin was a vital substrate for caspase-3 while in Fas-activated cells it was cleaved in vivo in a caspase-dependent manner. Caspase-3 cleaves gelsolin and its fragments which cause cleavage of actin filaments in a calcium independent way. Additionally, expression of the gelsolin cleavage yield in multiple cells resulted in cell contraction and separation from the plate before fragmentation of DNA and apoptosis induction, therefore cleaved gelsolin is considered as an important physiological effector of morphologic change

during apoptosis (81) . Endonuclease-mediated DNA fragmentation and apoptotic body formation are the final characteristic morphological features of apoptosis. The findings show that the presence of phosphotidylserine on the outer membrane leaflet, surface of apoptotic cells, permits their early uptake and disposal with no release of cellular constituents resulting in no inflammatory response (82).

1.3.2 Caspase Family

Caspases are intracellular proteases which are involved in programmed cell death, inflammation, cell proliferation, survival and differentiation. Activation of caspases is generated by a specific stimulus through a conserved mechanism. After activation, caspases perform proteolysis of downstream substrates to trigger a cascade of events that are terminated in the desired biological response(83). In fact, some caspases are vital for apoptosis but some are not required. Although aspartate at P1 position is generally necessary for all caspase substrates, different caspases demonstrate different substrate preferences. Some caspases such as caspase-8 and -10 have long pro-domains along with special motif like DED while caspase-1, -2, -4, -5, -9, - 11 and -12 use domains (CARD) which allow their responses to other proteins leading to signaling pathways(67) . Caspases were divided into two simple groups: “apoptotic” and “pro-inflammatory”, which was a helpful classification for several years. However, most apoptotic members (caspase-2, -3, -6, -7, -8, -9, and -10) are attributed to at least one non-apoptotic role. Likewise, “non-apoptotic” candidates such as caspase-1, -4, and -5 are expected to