# ANTI-TUMOR STUDY OF SCOPOLETIN AND RUBBING-MERCAPTO-NITRILE FROM *NICOTIANA GLAUCA*

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# ANTI-TUMOR STUDY OF SCOPOLETIN AND RUBBING-MERCAPTO-NITRILE FROM *NICOTIANA GLAUCA*

**By**

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*This thesis is dedicated to*

*my people in Syria who have long been suffering from the war*

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## LIST OF ABBREVIATION

| | |
|---|---|
| ANOVA | Analysis of variance |
| APC | Adenomatous polypsis coli |
| ATCC | American Type Culture Collection |
| ATP | Adenosine triphosphate |
| Bcl-1 and 2 | B-cell lymphoma 1 and 2 |
| bFGF | basic Fibroblast growth factor |
| BT20 | Breast cancer cell line |
| CCD-18Co | Normal human colonic fibroblast |
| c-myc | Myelocytomatosis oncogene cellular homolog |
| CO2 | Carbon dioxide |
| Conc | Concentration |
| 3D | Three-dimensional |
| DMEM | Dulbecco's modified eagle medium |
| DMSO | Dimethyl sulphoxide |
| DNA | Deoxyribonucleic acid |
| EC50 | Concentration that provides 50% inhibition |

| | |
|---|---|
| ECM | Endothelial cell medium |
| e.g. | For example |
| etc. | Et cetera, it means “and other things” |
| FTIR | Fourier transform infrared spectrometry |
| GC-MS | Gas chromatography mass spectrometry |
| 1 H-NMR | Hydrogen Nuclear Magnetic Resonance |
| HCT 116 | Human colorectal carcinoma cell line |
| Hep G2 | Human hepatocellular carcinoma cell line |
| HIFBS | Heat-inactivated fetal bovine serum |
| HUVECs | Human umbilical vein endothelial cell line |
| HIFs | Hypoxia inducible factors |
| i.e. | That means |
| IC50 | Half-maximal inhibitory concentration |
| IL | Interleukin |
| IR | Infrared |
| LD50 | Lethal dose of 50% of the tested animals |
| MCF 7 | Human hormone sensitive and invasive breast cancer cell line |

| | |
|---|---|
| MDA-MB-231 | Human hormone resistant breast cancer cell line |
| MDA-MB-468 | Breast cancer cell line |
| MEM | Minimum essential medium |
| MIC | Minimal inhibitory concentration |
| MTT | 3-(4,5-Dimethylthiazol-2-yl)-2,5diphenyl tetrazolium bromide |
| mRNA | Messenger Ribonucleic acid |
| N. glauca | Nicotiana glauca |
| NO | Nitric oxide |
| p53 | Tumour suppressor protein 53 |
| PBS | Phosphate buffer saline |
| PC-3 | Human prostate cancer cell line |
| PDGF | Platelet-derived growth factor |
| PE | Plating efficiency |
| ROS | Reactive oxygen species |
| RMN | Rubbing-Mercapto-Nitrile |
| RPMI-1640 | Roswell Park Memorial Institute medium |
| RT | Retention time |

| | |
|---|---|
| SD | Standard deviation |
| SF | Surviving fraction |
| SPSS | Statistical Package for the Social Sciences |
| TGF-β | Transforming growth factor-β |
| TNF | Tumour necrosis factor |
| US | Ultrasound |
| USA | United States of America |
| USM | Universiti Sains Malaysia |
| UV | Ultraviolet |
| VEGF | Vascular endothelial growth factor |
| VEGFRs | Vascular endothelial growth factor receptors |
| WHO | World health organization |

## LIST OF UNITS

| | |
|---|---|
| Cm | Centimetre |
| g | Gram |
| h | Hour |
| kg | Kilogram |
| mg | Milligram |
| ml | Millilitre |
| mm | Millimetre |
| mm3 | Cubic millimetre |
| min | Minute |
| U | Unit |
| Mg | Microgram |
| Ml | Micro litter |
| µM | Micro Molar |
| µm | Micron |

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**KAJIAN ANTI-TUMOR SCOPOLETIN DAN RUBBING-MERCAPTO-NITRILE DARI *NICOTIANA GLAUCA***

## ABSTRAK

*Nicotiana glauca* (Solanaceae) telah digunakan secara tradisional untuk merawat jangkitan dan kanser. Tumbuhan ini dilaporkan kaya dengan komponen–komponen yang baik untuk kesihatan. Dalam kajian ini, dua komponen anti-tumor daripada ekstrak n-heksana *N. glauca* telah berjaya diasingkan, iaitu scopoletin dan satu komponen baru, Rubbing-Mercapto-Nitrile (RMN). Pengasingan scopoletin berpandukan aktiviti anti-angiogenesis telah dijalankan menggunakan ekstrak daripada batang *N. glauca*. Aktiviti anti-angiogenesis scopoletin dikaji menggunakan model–model angiogenesis secara *ex vivo* dan *in vivo*. Keberkesanan anti-tumorigenik scopoletin dikaji menggunakan xenograf tumor kolorektal manusia pada mencit nude atimik. Scopoletin menyebabkan perencatan signifikan dalam percambahan salur mikro pada eksplan aortik tikus dengan nilai kepekatan perencatan 50% adalah 34 µg/ml. Scopoletin (100 dan 200 mg/kg) merencat dengan berkesan vaskularisasi plak matrigel yang diimplan pada mencit nude, masing–masing sebanyak 59.72% dan 89.4%. Dalam model xenograf tumor, scopolectin menunjukkan potensi perencatan pertumbuhan tumor (34.2% dan 94.7%, masing–masing pada kepekatan 100 dan 200 mg/kg). Kajian histologi tumor menunjukkan penurunan drastik dalam proses vaskularisasi. Secara *in silico*, scopoletin menunjukkan afiniti ligan yang kuat dan daya ikatan yang tinggi terhadap faktor–faktor angiogenik berikut: protin kinase (ERK1), faktor pertumbuhan endothelial vascular-A (VEGF-A), dan faktor pertumbuhan fibroblast-2 (FGF-2). Seterusnya, kompaun baru yang dikenali sebagai Rubbing-Mercapto-Nitrile (RMN) juga telah diasingkan dari *N. glauca* dan dikaji dengan lebih lanjut. RMN

mempunyai kesan sitotoksik yang poten dan terpilih terhadap sel–sel kanser kolorektal HCT-116. Kompaun ini meransang proses apoptosis di dalam tisu neoplastik dengan nilai indeks terpilih yang tinggi (SI = 4.3). Keadaan ini menyebabkan berlakunya kondensasi nuklear, pereputan kromatin, dan kerosakan pada membran mitokondria. RMN juga menyebabkan penurunan pengawalaturan laluan isyarat bagi TGF dan HIF dan kenaikan pengawalaturan pada laluan–laluan isyarat WNT, NOTCH, NF-κB, ERK, P53, dan JNK secara *in vitro*. Secara keseluruhannya, dua komponen anti-kanser telah diasingkan dan dikenalpasti daripada *N. glauca* dan aktiviti anti-tumor scopoletin telah disahkan secara *ex vivo* dan *in vivo* dan aktiviti RMN telah dipastikan secara *in vitro* beserta dengan target molekular.

**ANTI-TUMOR STUDY OF SCOPOLETIN AND RUBBING-MERCAPTO-NITRILE FROM *NICOTIANA GLAUCA***

## ABSTRACT

*Nicotiana glauca* (Solanaceae) has been traditionally used as a folk remedy to treat infections and cancer. It has been reported as a rich source of beneficial phytochemicals. In the present study, two anti-tumor compounds derived from n-hexane extract of *N. glauca* namely scopoletin and, a novel compound, Rubbing-Mercapto-Nitrile (RMN) have been isolated. Antiangiogenesis-guided isolation of scopoletin was conducted using an extract from the stem of *N. glauca*. The anti-angiogenic activity of scopoletin was investigated using *ex vivo* and *in vivo* angiogenesis models. The antitumorigenic efficacy of scopoletin was studied in human colorectal tumor xenografts using athymic nude mice. Scopoletin caused significant suppression of microvessel sprouting in rat aortic explants with $IC_{50}$ of 34 µg/ml. Scopoletin (100 and 200 mg/kg) strongly inhibited (59.72% and 89.4%, respectively) vascularization in matrigel plugs implanted in nude mice. In a tumor xenograft model, scopoletin showed potent inhibition of tumor growth (34.2% and 94.7% at 100 and 200 mg/kg, respectively). Tumor histology reveals a drastic decline of vascularization. *In silico* studies suggest that scopoletin strongly inhibit protein kinase (ERK1), vascular endothelial growth factor A (VEGF-A), and fibroblast growth factor 2 (FGF-2). A novel anti-cancer compound Rubbing-Mercapto-Nitrile (RMN) was also isolated from *N. glauca* and characterized. RMN has potent but selective cytotoxic activity towards HCT-116 colorectal cancer cells. The compound stimulates strong apoptosis in the neoplastic tissue with a high selective index (SI = 4.3). It causes nuclear condensation, chromatin degradation, and damage to the mitochondrial membrane. RMN also causes

down-regulation of the TGF and HIF signaling pathways, and up-regulation of the WNT, NOTCH, NF-κB, ERK, P53, and JNK signaling pathways in vitro. Overall, in this study, two anticancer compounds were isolated and identified from *N. glauca* and the antitumor activity of Scopoletin was verified *ex vivo* and *in vivo* and the RMN activity was confirmed *in vitro* with their molecular targets identified.

# CHAPTER ONE: INTRODUCTION

## 1.1.Overview of cancer

Over 200 types of cancer that harm the human body have been recognized. Cancer is the second leading cause of death worldwide, surpassed only by heart disease. It is reported in all ages; and one in three people may contract this disease in his or her lifetime. Unfortunately, this ratio is expected to increase to 55% by 2020 (Cerveira & Bizarro, 2012). Cancer is characterized by excessive and uncontrolled cellular growth followed by local tissue invasion and tumor metastasis (Chabner, 2006). A tumor may be comprised of either normal cells (benign, hyperplasia) or abnormal cells (dysplasia, malignant), probably due to genetic mutation. Tumorigenesis occurs when there is dysregulation in more than six cellular pathways, leading to prolonged angiogenesis, irresponsiveness to anti-growth signals, evasion of programmed cell death, continuous growth independently of proliferation signals, lack of apoptosis, and metastasis. A variety of factors may lead to an increase in the risk of cancer. Some may be genetically linked, resulting in irregular control of cell-cycle checkpoints, hyper-activation of oncogenes, and inhibition of tumor-suppressing genes. Environmental factors comprise absence of physical activity, oxidative stress, radiation, smoking, and obesity (Hanahan & Weinberg, 2000; Johnson et al., 1996)

The main methods of cancer treatment currently applied are: surgery, chemotherapy and radiotherapy. Traditionally, cancer is diagnosed using available medical imaging techniques and biochemical screening tests (Shewach & Kuchta, 2009).

## 1.2. The incidence and mortality of cancer

Cancer has become one of the most common health problems in the world. In 2012, more than 14 million new cases were reported and about 8.2% resulted in death (Ferlay et al., 2014). The highest incidence of cancer among men is that of prostate cancer, which accounts for 25% of all newly diagnosed cancer cases in men. On the other hand, breast cancer represents 27% of all newly diagnosed cancer cases in women and is followed by lung and colon cancers respectively (A. Jemal et al., 2009). In the developed countries, including North America, Japan, the European countries, Russia, Australia and New Zealand, the number of new cases is less than that reported in developing countries of lower resources (5,600,000 vs. 7,100,000 cases respectively) (Boffetta et al., 2014). In addition , the cancer survival rate tends to be lower in the developing countries; and that is most likely because of lacking educational facilities and financial resources, limited access to health services, and the kind of dietary intake (Jemal et al., 2011). The World Health Organization (WHO) has estimated that the number of people diagnosed with cancer will rise in 2030 by 21.4 million.

Based on the latest Health Facts released by the Malaysian Ministry of Health (MoH) in 2013, cancer is one of the top-ten causes of hospitalization and one of the top-five causes of death in Malaysia. The incidence of cancer increased from the annual 32,000 new cases in 2008 to about 37,000 in 2012. Mortality due to cancer stood at 20,100 deaths in 2008, and has increased to 21,700 deaths in 2012.

### 1.3.The differences between normal and cancer cells

What makes a cancer cell different is its abnormal behavior and inability to react properly under a whole set of conditions (Iyer et al., 2009). Cancer cells have many distinct characteristics compared with the normal ones, some of which are well understood, whereas others are still under research. These differences pave the way for researchers to discover new treatments which target cancer cells and spare the normal ones (Polyak & Weinberg, 2009). First, normal cells are controlled by the tumor suppressor genes, which control the growth of cells by sending death signals when the cells are damaged or too old; and any mutation in these genes may result in an uncontrolled cancer cell growth. Second, a normal cell responds to signals from the neighboring cells to stop its growth when such is needed. In contrast, malignant cells ignore these signals and move outward from the focus of the origin of the neoplasm (Abercrombie & Ambrose, 1962). Third, cancer is an age-related disease in organisms with renewable tissues; and it is widely acceptable that age-related degenerative diseases generally trigger a loss of cell function. As a result, a cancer cell does not perform the function compared with a normal cell. For example, in leukemia, the cancerous white cells are not functional and cannot treat an infection despite the very high count of white blood cells. And that also applies to cancerous thyroid cells, which cannot produce thyroid hormones like the normal ones (Campisi & Yaswen, 2009). Fourth, cancer cells are not limited by oxidative metabolism as they produce most of their energy in the absence of oxygen, unlike normal cells, which require oxygen to complete the krebs cycle (Moreno-Sanchez et al., 2007). Fifth, normal cell populations are mortal, which distinguishes them from cancerous cells which have an immortality property i.e., they don't have a life span (Hayflick, 2000). Researchers addressed the causes and mechanisms underlying this phenomenon and they concluded that the limitations in the growth cycles of

normal cells are attributable to the length of their telomeres (long strands at the end of the DNA). As a cell divides multiple times, the telomeres get shorter and shorter till the cell dies. However, in the event of cancer, the cells renew their telomeres so that they can continue to divide endlessly–basically becoming immortal (Hayflick, 2000). Sixth, cancer cells are "undifferentiated" i.e., they don't have the ability to differentiate into specialized mature cells, whereas normal cells are "well-differentiated" (Edge & Compton, 2010). The term 'differentiation' is also used to provide information for cancer assessment. It is an indicator that provides information on the speed of growth and the spreading of a tumor. Hence, the 'degree of differentiation' is associated with a tumor's developmental process, whereby it gives information on cancer progression and aggressiveness. The closer to normal (differentiated) a cancer cell appears, the lower the grade is, and the less aggressive the tumor is considered (Jögi et al., 2012). Cancer cells are characterized by genomic instability, which is proportional to the number of chromosomal abnormalities resulting from mutations in the DNA-repair genes. The greater this number is, the higher the stage of cancer should be (Negrini et al., 2010). Other contributing factors are the decrease and increase in the oligonucleotide repeats present in microsatellite sequences (Leach et al., 1993), and the increase in the number of base-pair mutations (Al-Tassan et al., 2002; Fishel et al., 1993). On the other hand, normal cells have intact, normal DNA; and a normal number of chromosomes (Negrini et al., 2010). Finally, cancer cells have the ability to spread from a primary site (the origin of the cancer) to a distant organ in a process called "Metastasis", which consists of many distinct sequential steps (Table 1.1) (Figure 1.2) (Judah Folkman, 2002b) summarized as follow: A cancer cell is shed from the primary tumor into the body's circulatory or lymphatic systems, a phenomenon known as intravasation. The cells must then survive in the circulation till they are arrested in an

organ. This process is referred to as extravasation, in which the cells go from the circulation into a surrounding tissue where they would maintain their growth rate and undergo pre-angiogenic micrometastasis (Moreno-Sanchez et al., 2007). Breast and prostate cancers often metastasize to the bones (Woodhouse, Chuaqui, & Liotta, 1997) (Woodhouse et al., 1997). On the other hand, normal breast and prostate cells cannot undergo this process (Chambers et al., 2002).

Table 1.1: The differences between normal and cancer cells

| Proper (-) Improper (+) | *Normal* | *cancer* |
|---|---|---|
| Response to stop signals | - | + |
| Response to death signals | - | + |
| Response to aging signals | - | + |
| Growth | - | + |
| Differentiation | - | + |
| Recruitment of food | - | + |
| Movement | - | + |
| Immortality | - | + |
| Genomic instability | - | + |

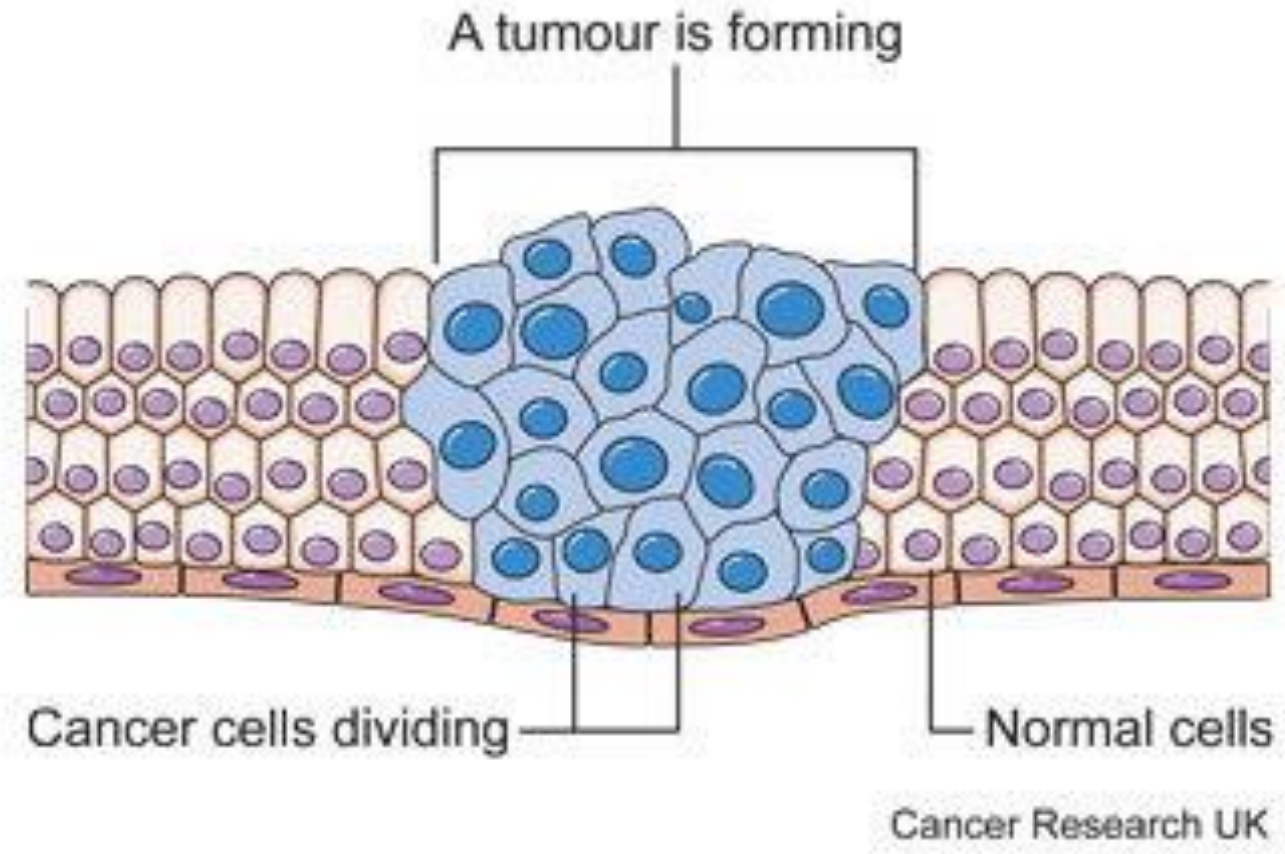


Figure 1.1: Forming tumor among normal cells

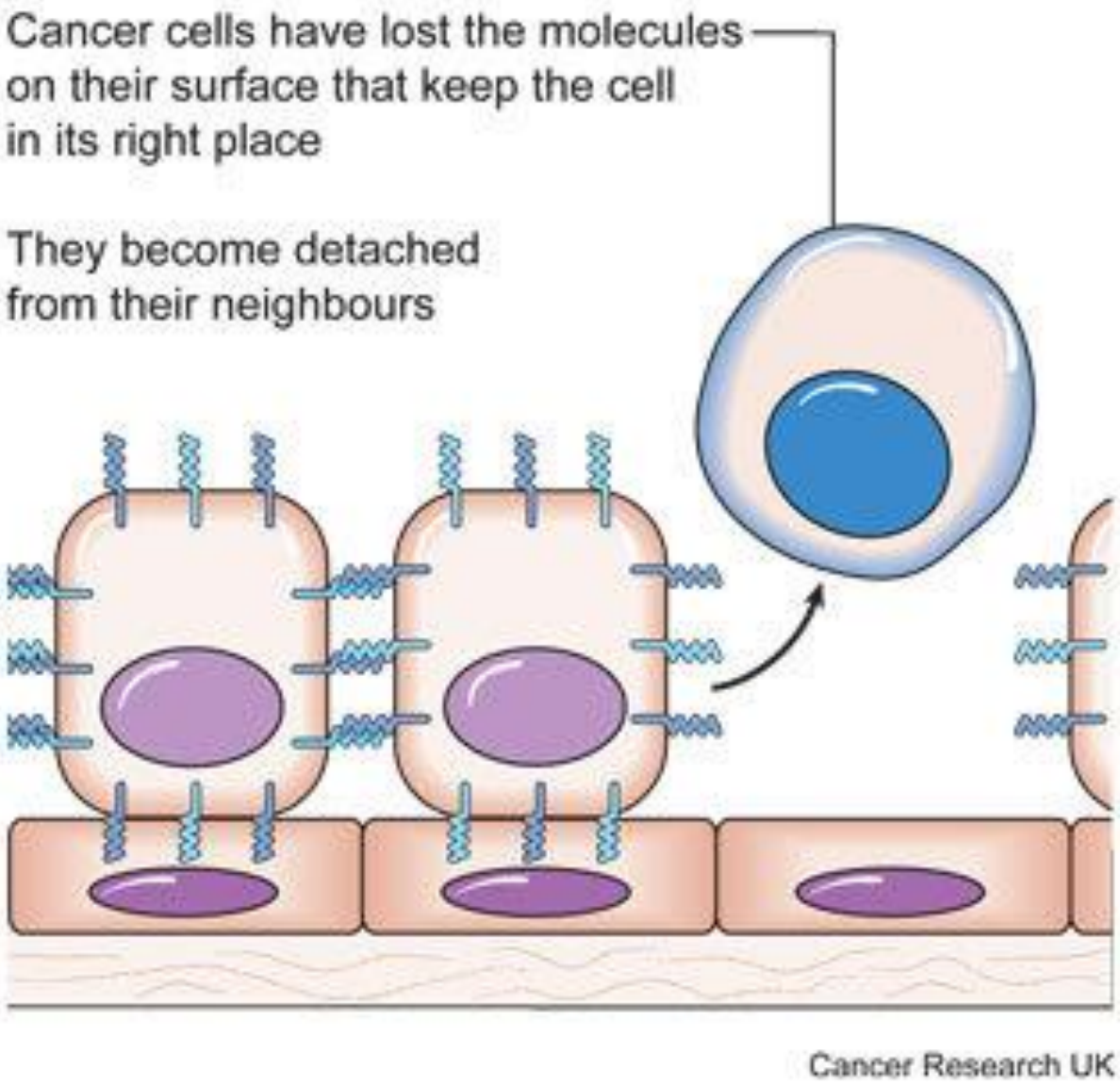


Figure 1.2: Metastasis of a cancer cell

## 1.4.The molecular biology of cancer

Cancer, in essence, is a heterogeneous disease caused by the accumulation of many somatic mutations. Each single gene defect "causes" cancer, because each mutation drives a wave of cellular proliferation, tumor size exaggeration, disorganization and malignancy (B. Vogelstein & Kinzler, 1993). The majority of these mutations are acquired and "not inherited". They casually arise as consequences to damage at the genome level. Such damage could result from an endogenous process, like a mistake occurring during DNA replication; an attack by free radicals produced during metabolism; or the instability of certain DNA bases. It might also involve exogenous agents, such as UV and ionizing radiations, or chemical carcinogens (Bertram, 2000). Tumors show different behaviors in different tissues. For example ,cancer cells of the pancreas are more aggressive than those in the prostate (Pedraza-Fariña, 2006). The mechanism through which cells transform to cancer is incompletely understood. However, it is assumed that it implicates many factors which disturb the balance between cell proliferation and cell death, leading to abnormal cell differentiation and proliferation. A cancer is believed to take place through the activation of oncogenes, or the downregultion of the tumor-suppressor genes; which are linked to signal transduction pathways (Cerveira & Bizarro, 2012). Oncogene activation from Proto-oncogenes could be the result of gene amplification or chromosomal translocations. It promotes cell growth and proliferation analogous to a car which still moves forward even after the driver has removed his foot off the pedal. Tumor-suppressor genes work in the opposite direction as they attenuate cell growth processes. Inactivation of these genes could be the result of missense mutations, mutations resulting in truncated proteins, or epigenetic silencing. It is analogous to having dysfunctional brakes in an automobile preventing the car from stopping even when the driver tries to engage them (Vogelstein & Kinzler, 2004).

Five major pathways must be activated/inactivated in the process of cell genesis for a normal cell to transform into a cancer cell (Bertram, 2000), contributing to the development of independence from growth stimulatory signals; development of a refractory state to growth inhibitory signals; development of resistance to programmed cell death, apoptosis; development of an infinite proliferative capacity i.e., overcoming cellular senescence; and development of an angiogenic potential i.e., the capacity to form new blood vessels and capillaries.

Table 1.2 Cancer predisposition genes (Vogelstein & Kinzler, 2004)

| Gene synonym(s) | Syndrome | Hereditary pattern | Pathway | Major heredity tumor types |
|---|---|---|---|---|
| **Tumor-suppressor genes** | | | | |
| APC | FAP | Dominant | APC | Colon, thyroidal, stomach, intestinal |
| AXIN2 | Attenuated polyposis | Dominant | APC | Colon |
| CDH1 (E-cadherin) | Familial gastric carcinoma | Dominant | APC | Stomach |
| GPC3 | Simpson-Golabi-Behmel syndrome | X-linked | APC | Embryonal |
| CYLD | Familial cylindromatosis | Dominant | APOP | Pilotrichomas |
| EXT1,2 | Hereditary multiple exostoses | Dominant | GLI | Bone |
| PTCH | Gorlin syndrome | Dominant | GLI | Skin, medulloblastoma |
| SUFU | Medulloblasto-ma predisposition | Dominant | GLI | Skin, medulloblastoma |
| FH | Hereditary leiomyomatosis | Dominant | HIF1 | Leiomyomas |
| SDHB, C, D | Familial paraganglioma | Dominant | HIF1 | Paragangliomas, pheochromocytoma |

| | | | | |
|---|---|---|---|---|
| | | | | s |
| VHL | Von Hippel–Lindau syndrome | Dominant | HIF1 | Kidney |
| TP53 (p53) | Li-Fraumeni syndrome | Dominant | p53 | Breast, sarcoma, adrenal, brain |
| WT1 | Familial Wilms tumor | Dominant | p53 | Wilms’ |
| STK11 (LKB1) | Peutz-Jeghers syndrome | Dominant | PI3K | Intestinal, ovarian, pancreatic |
| PTEN | Cowden syndrome | Dominant | PI3K | Hamartoma, glioma, uterine |
| TSC1, TSC2 | Tuberous sclerosis | Dominant | PI3K | Hamartoma, kidney |
| CDKN2A (p16INK4A, p14ARF) | Familial malignant melanoma | Dominant | RB | Melanoma, pancreas |
| CDK4 | Familial malignant melanoma | Dominant | RB | Melanoma |
| RB1 | Hereditary retinoblastoma | Dominant | RB | Eye |
| NF1 | Neurofibromatosis type 1 | Dominant | RTK | Neurofiber |
| BMPR1A | Juvenile polyposis | Dominant | SMAD | Gastrointestinal |
| SMAD4 (DPC4) | Juvenile polyposis | Dominant | SMAD | Gastrointestinal |

**Oncogenes**

| | | | | |
|---|---|---|---|---|
| KIT | Familial gastrointestinal stromal tumor | Dominant | RTK | Gastrointestinal stromal tumor |
| MET | Hereditary papillary renal cell carcinoma | Dominant | RTK | Kidney |
| PDGFRA | Familial gastrointestinal stromal tumor | Dominant | RTK | Gastrointestinal stromal tumor |
| RET | Multiple endocrine | Dominant | RTK | Thyroidal, parathyroida, adrenal |

## 1.5.Apoptosis in cancer

Apoptosis, programmed cell death, is a physiological process of cellular suicide that is integral to tissue homeostasis and is more feasibly observable during embryonic development, whereas at which stage more than 10 billion cells are made in the human body every day to balance those dying of apoptosis (Abud, 2004). It is characterized by a number of cellular morphological changes, such as cell shrinkage, membrane blebbing, organelle relocalization, nuclear fragmentation, chromatin condensation, and the production of the 'apoptotic bodies', which are membrane-enclosed particles containing intracellular material (Kerr et al., 1972). Another pathway of cell death is "necrosis", which lacks the features of apoptosis and autophagy. It could be differentiated from apoptosis by certain changes in cellular mechanisms and morphological characteristics, including dysfunctional organelles, collapsing mitochondria, ATP depletion, cellular swelling, and extreme cellular disintegration, all of which end up forming an inflammation in the cells. This is in contrast with apoptosis, which is not accompanied by any inflammation (Gerl & Vaux, 2005; Kerr et al., 1972).

Triggering cells by apoptotic inducers, such as viral infections, bacterial toxins, free radicals, death ligands (like Fas), glucocorticoids, chemotherapeutics, radiation therapy, heat shock, growth factor withdrawal and/or irreparable DNA damage; makes many distinctive changes in the cells (Elmore, 2007). One of the distinguished families of proteins is called caspases. It plays a significant role in the initial stages of apoptosis. It breaks down the main components required for normal cellular functions, like the proteins which shape the cytoskeleton and the DNA repair enzymes. In addition, caspases induce the degradation enzymes, like DNases, leading to DNA cleavage in the nucleus (Song & Steller, 1999). The apoptotic process promotes different morphological changes that are

specific to the apoptotic cells. Mainly, cells shrink due to the cleavage of actin and laminin in the cytoskeleton, and the nuclei take on a "horse-shoe" shape because of the breakdown of their chromatin contents. As shrinking continues, the cells package themselves to become easy victims for removal by macrophages. Changes in the apoptotic cells' membranes are responsible for triggering the macrophagal response (Ghatage et al., 2012)

Table 1.3 the differences between apoptosis and necrosis

| Apoptosis | **Necrosis** |
|---|---|
| **Physiologic, regulated** | Pathologic, unregulated |
| **Cell shrinkage** | Cell swelling |
| **Chromatin condensation** | Irregular chromatin |
| **Preservation of Intracellular organelles** | Clumping, dysfunction and destruction of organelles |
| **Membrane blebbing** | Disruption of cellular membranes |
| **apoptotic bodies** | Non-specific and random degradation of DNA ('DNA smears') |
| **Organized chromatin** | |
| **Digestion to small fragments ('DNA ladders')** | |

### 1.5.1. Caspase family regulates apoptosis:

Caspases, which are cysteine-dependent, aspartate-specific proteases, are a family of enzymes that is vital for the initiation and execution of apoptosis (Earnshaw, Martins, & Kaufmann, 1999). They remain in their inactive proenzyme forms in most healthy cells; however, caspases' action is irreversible once activated by death signals; because the protease cascade starts the initiation process, whereas the single-chain procaspases are driven into a breakdown mechanism at specific aspartic acid residues, whereby the inhibitory N-terminal pro-domain is removed to form the active proteases. Apoptotic caspases are divided into two types: the first one comprises the initiators, or "the upstream caspases". They usually come with long pro-domains (>90 amino acid); and start the cascade such as caspase-2,-8,-9,-10. The second one comprises the effectors, or "the downstream caspases". They come with short pro-domains, containing 20-30 residues, and play a major role in the cleavage processes that disassemble the cell. An example is caspase-3,-6,-7 (Thornberry & Lazebnik, 1998). Hence, the activation of an effector caspase, like caspase-3 or caspase-7, is achieved by an initiator caspase, such as caspase-9. However, this process requires many components, such as apoptosome, which is a protein complex responsible for the activation of caspase-9. The latter then activates caspase-3, leading to changes in morphology and biology in the apoptotic cell (Ghatage et al., 2012).

### 1.5.2. Mechanism of apoptosis

The mechanisms of apoptosis are highly complex and advanced processes. They make an energy-dependent cascade of molecular events. There are two main pathways of apoptosis: the intrinsic or the mitochondria-initiated apoptosis pathway, and the extrinsic or

death-receptor initiated apoptosis pathway (Igney & Krammer, 2002). However, there is an additional pathway, referred to as the perforin/granzyme pathway, which could induce programmed cell death via either granzyme B or granzyme A. All of the intrinsic, extrinsic, and granzyme B pathways lead to the execution pathway, which starts with the cleavage of caspase-3, leading to the morphological changes associated with apoptosis. On the other hand, the granzyme A pathway activates a parallel caspase-independent cell death pathway via single-strand DNA damage (Martinvalet et al., 2005).

The intrinsic pathway acts directly on targets within the cell. It may be induced by many triggers, like DNA damage, hyperthermia, radiation, free radicals, hypoxia and/or a viral infection (Abud, 2004), all of which could cause changes in the inner mitochondrial membrane and lead to the release of pro-apoptotic proteins, such as cytochrome C, Smac/DIABLO, AIF, Endo G and HtrA2/Omi, from the intermembrane space into the cytosol. Consequently, activation of caspase-3 starts and the execution pathway begins. The Bcl2 families play a primary role in mediating and controlling this process. The Bcl2 family has 4 homologous (BH) domains, which might either be anti-apoptotic or pro-apoptotic. The pro-apoptotic proteins in the cell include Cl-10, Bax, Bak, Bid, Bad, Bim, Bik, and Blk, which may be localized at different parts of the cell. The anti-apoptotic members of the Bcl2 family are Bcl2 and $Bcl_{xl}$. Bcl2 are localized completely within the intracellular membranes and in the cytosol (Ghatage et al., 2012). These protein are key in determining whether the cell must perform apoptosis or not as they regulate the release of cytochrome C from the mitochondria in a process labeled as mitochondrial outer membrane permeabilization (MOMP).This is accomplished by an interaction between pro-apoptotic Bcl2 and membrane pores and channels which might be accompanied by a loss of mitochondrial membrane potential (Saelens et al., 2004). When the pro-apoptotic Bcl2 units

are released from the inter-membrane spaces into the cytosol, the main caspase-dependent group, which comprises cytochrome c, Smac/DIABLO and HtrA2/Omi, works to induce the caspase cascade. It binds to and activates Apaf-1 and procaspse-9, leading to the formation of the apoptosomes, which result in the activation of caspase-9 and the effectors, caspase-3 and caspase-7; and this marks the end of the apoptotic pathway (Fulda & Debatin, 2004).

The extrinsic Pathway is a rapid mechanism that results in apoptosis. It is a caspase-dependent process which requires transmembrane receptor-mediated interactions, like the reactions between the ligands corresponding to the death receptors, including FasL/FasR, TNF-α/TNFR1, Apo3L/DR3, Apo2L/DR4 and Apo2L/DR5 (Ashkenazi & Dixit, 1998). After a ligand and a death receptor bind, the receptor changes its conformation (Abud, 2004) allowing them to interact with specific intracellular adaptor proteins, leading to changes in the "death domain"; which is made up of an extracellular domain and a cytoplasmic domain, and plays an essential role in transmitting the death signal from the surface of the cell to intracellular signaling pathways (Ashkenazi & Dixit, 1998). For example, TRADD, a TNFR-associated death domain, recruits many apoptotic proteins to the receptor, at which point, the protein complex formed is often called the Death-Inducing Signaling Complex (DISC). DISC results in the autocatalytic activation of caspase-8, and the initiation of apoptosis. Alternatively, FADD, a FAS-associated death domain, could form a link with TRADD, which would lead to the cleavage of pro-caspase 8, and the induction of apoptosis (Kischkel et al., 1995; Wajant, 2002).

The perforin/granzyme pathway is an extrinsic pathway used by cytotoxic T lymphocytes (CTLs) to kill specific cells, such as tumor and virus-infected cells (Brunner et

al., 2003). Its mechanism is based on the release of perforin, proteases granzyme A and granzyme B. The perforin units form transmembrane pore proteins in the attacked cells' membranes (Trapani & Smyth, 2002). Thus, perforin proteins enable the entry of granzyme A and granzyme B, which are considered the main components inside the granules. Granzyme B units activate pro-caspase-10 and caspase-3, which results in the induction of apoptosis (Sakahira et al., 1998). On the other hand, granzyme A, also released by T cells, induces apoptosis via a caspase-independent mechanism as it indirectly activates a tumor suppressor gene product, an effect which is called DNA nicking. The expression of this product, known as DNAse NM23-H1, is diminished in cancer cells (Fan et al., 2003). The activation of NM23-H1 occurs by cleavage of the proteins which inhibits NM23-H1 inside of the SET complex. Hence, the activation process promotes the release of NM23-H1, causing DNA fragmentation (Lieberman & Fan, 2003)

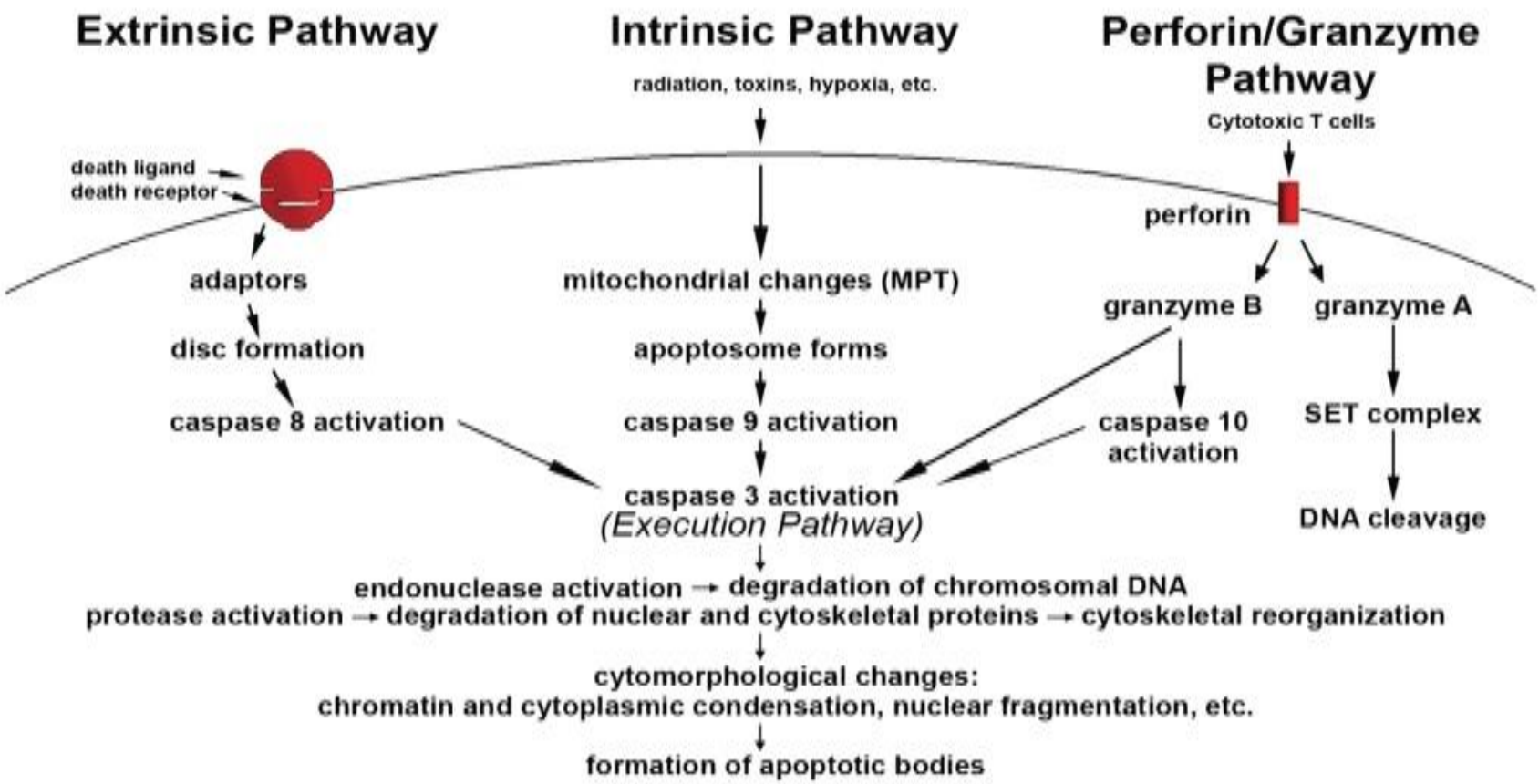


Figure 1.3: Pathways of apoptosis

## 1.6.Colorectal carcinoma

Colorectal carcinoma (CRC) is the term used to describe tumor formation in the large intestine, colon and/or the rectal area. It has the third highest incidence rate among all cancer types globally, preceded only by lung and breast cancers; and it is considered to be one of the leading causes of death among patients of both sexes. The risk rises after the age of 50. However, it could be managed if detected early. (Gustin & Brenner, 2002).

Specialists have divided colorectal cancer into four main stages in terms of the morbidity of the disease: Dukes' A, B, C and D. This pathological staging system has been in use for more than 50 years. The first stage, Dukes' A, refers to a condition whereby contiguous lesions contiguous exist only in the innermost lining bowel wall of the colon or the rectum, without reaching the muscularis. Dukes' B refers to cases in which the cancer penetrates the muscularis. Dukes' C describes conditions whereby the cancer has spread to the lymph nodes around the bowel area. Dukes' D stage is the most dangerous and death-leading phase as cancer would have spread to other organs in the body, such as the lungs and/or the spleen (Dukes, 1932).

Colon cancer is a multi-step disease. It occurs as a consequence of a series of pathological changes that transform normal epithelial cells of the colon into invasive carcinoma. Several studies have shown that the multi-step process of colon cancer is accompanied by certain mutations affecting the adenomatous polypsis coli (APC) gene and cyclooxygenase-2 (COX-2), as well as K-ras mutations (caused by Kirsten-rat sarcoma virus), loss of the 18q21 gene, microsatellite instability, mutations in the transforming growth factor-β II receptor (TGFβR2), and stabilization and translocation of β-catenin (Kanwar et al., 2010; Kobayashi et al., 2000)

## 1.7.Major signaling pathways in colon carcinogenesis

### 1.7.1. Wnt /β-catenin signaling pathway

Wnt (Wingless and INT-1) – which refers to a large family of secretory glycoproteins – signaling pathway has a significant role in embryogenesis, cell-to-cell signaling, and adult tissue homeostasis (Clevers, 2006). Wnt/β-catenin, which is considered the most important Wnt pathway, regulates the transcription of cofactor β-catenin, and, therefore, enhances the expression of its respective gene (Logan & Nusse, 2004). Abnormal Wnt/β-catenin signaling is a primary factor in the development of tumorigenesis, degenerative disorders, osteoporosis and aging (Moon et al., 2004). It is well known that over 90% of colon cancer cases result from the accumulation of Wnt and β-catenin due to mutations of the APC tumor suppressor gene, or oncogenic mutations of β-catenin (Giles et al., 2003; Luu et al., 2004). An increase in the levels of β-catenin, a key component of the Wnt signaling pathway, has been observed in many types of human cancer, such as prostate cancer, colon cancer, melanoma, and breast cancer (Chien et al., 2009; Luu et al., 2004). The Wnt /β-catenin complexes promote the expression of many oncogenes, like the c-Myc, cyclin D1 and MMP genes, which are essential for tumor angiogenesis and cancer development (Dihlmann & Doeberitz, 2005). Thus, compounds which target the Wnt signaling pathway and downregulate it could make for a potential treatment of cancer (Luu et al., 2004; Tetsu & McCormick, 1999).

### 1.7.2. Notch Signaling Pathway

Notch signaling plays a fundamental role in the development of normal cells and tissues, like those of the cardiovascular system, central nervous system, and endocrine system. Its role is also implicated in bone development and tissue renewal (Allenspach et

al., 2007). In addition, Notch is considered as a conserved cell signaling mechanism. It is associated with different effects on cellular processes, such as cell fate determination, differentiation, cell proliferation, programmed cell death (apoptosis), adhesion, epithelial-mesenchymal transition, migration, and angiogenesis. Regulation of these processes can become faulty in Notch-mediated pathological situations (Artavanis-Tsakonas, Rand, & Lake, 1999; Bolós, Grego-Bessa, & de la Pompa, 2007). In mammals, there are four Notch proteins (Notch 1–4) and one Notch membrane-bound type 1 receptor with one transmembrane domain (Baron, 2003; Bolós et al., 2007). Preclinical and clinical studies have attributed a pro-oncogenic function to Notch signaling in many solid tumors (Rizzo et al., 2008). An increase in the rate of Notch pathway signaling is associated with many kinds of cancers, such as melanoma and lung, breast, pancreas, renal and colon cancers (Collins et al., 2004; Strizzi et al., 2009).

An *in vivo* study has demonstrated strong synergies between Notch and Wnt signals in colon cancer that result in the formation of intestinal adenomas and, more significantly, colorectal tumor (Fre et al., 2009; Hayward et al., 2008). Since Notch pathway is complicated, a better understanding of its interactions with other pathways is required for future therapeutic applications (Bolós et al., 2007; Rizzo et al., 2008).

### 1.7.3. P53 signaling pathway

The p53 tumor suppressor gene was identified in 1979. It performs an essential role in the activation of apoptosis and cell arrest pathways. It has been labeled as "the guardian of the genome" because of its role in preventing genome mutation (Fridman & Lowe, 2003; Read & Strachan, 1999) Activation of p53 by DNA damage, aberrant oncogene expression or hypoxia causes an induction in cell-cycle checkpoints, DNA repair, cellular senescence,

and apoptosis. Hence, disruption of this pathway or a mutation in p53 may end in checkpoint defects, genomic instability, inappropriate survival, cancer, neurodegeneration, ischemia, cholestasis and/or atherosclerosis (Amaral et al., 2010). Many factors make p53 gene mutation very prevalent in human malignancies. First of all, a single base change in the coding sequence results in a dysfunctioning P53. Secondly, a single abnormal p53 allele or allele loss can alter the phenotype of the p53 proteins. Thirdly, since p53 plays a role in multiple pathways, defects in its coding gene make the cell's defenses completely diminished against carcinogenesis (Bellamy, 1997).

It is well-established that any mutation in the p53 tumor suppressor gene causes its function to be lost, leading to profound proliferation of cancer cells and chemoresistance (Beroud & Soussi, 2003; Hussain & Harris, 1998; Wallace-Brodeur & Lowe, 1999). On the other hand, hyper-activation of p53 product has been associated with many different diseases, like arthritis, multiple sclerosis, and neuropathies (Mattson et al., 2001; Wosik et al., 2003). Moreover, *in vivo* studies have suggested that acute p53 activation is the mechanism behind the side effects of cancer chemotherapy (Komarova & Gudkov, 2001; Tyner et al., 2002).

### 1.7.4. TGF-β Signaling Pathway

TGFβ and its receptors are extensively expressed in all tissues; and TGFβ signaling has a dual role in human diseases (Massagué, 1998). In cancer, TGFβ acts as both a tumor suppressor and an oncogene which promotes tumor metastasis (Katz et al., 2013). Its tumor suppressive effects, which are observed in the early stages of cancer and in normal cells, include an inhibition of cell proliferation, an induction of apoptosis, and an inhibition of cell immortalization. In contrast, the tumor promoting effects of TGFβ include invasion,

migration, cell adhesion, tumor metastasis and an induction of Epithelial-Mesenchymal Transition (EMT), which has been observed in invasive and aggressive tumors (Massagué, 1998; Xu et al., 2009) . Furthermore, as a tumor progresses and increases in size, it secretes a huge amount of autocrine TGFβ, which gets released into the surrounding area. The increased levels of TGFβ affect the tumor and the vicinity, and promote immunosuppression and angiogenesis, inhibit cell adhesion, and promote the metastatic processes (Gorsch et al., 1992). Using antibodies to target the TGF-β proteins or receptors has shown a promising anticancer effect. However, the dual role of TGFβ translates into potential side effects for any novel compounds targeting this pathway (Massagué, 2008).

### 1.7.5. Cell cycle (pRB/ E2F) signaling pathway

The main element in this pathway is the retinoblastoma tumor suppressor protein (pRB), whose role is to induce apoptosis and inhibit cell cycle progression (Robert A Weinberg, 1995). pRB stops premature G1/S transitions by physical interaction with several transcription factors, namely E2F, which is vital for the activation of the S-phase genes (Bartek et al., 1996). It is believed that mutations in the Rb gene are present in the vast majority of human cancer conditions (Hunter, 2002). For example, such mutations have been detected in cases of osteosarcoma, small cell lung carcinoma, breast carcinoma and other cancer types (Nevins, 2001). In addition, a downregulated $E_2F$ activity has been reported in human tumor cases, and has been attributed to many mechanisms, such as a functional loss of pRB; amplification of cyclin D, a cyclin-dependent kinase inhibitor that inhibits the phosphorylation of pRB, which promotes the phosphorylation of pRB; a loss of p16; and an expression of E7, the human papillomavirus (HPV) oncoprotein, which disrupts the pRB–E2F complexes (Sherr & McCormick, 2002).