Chapter 1

1. Introduction

1.1 Breast Cancer

Breast cancer is the most potent nemesis of women's health worldwide, every year as per WHO 2.1 million new cases are registered[1]. In the last three decades, researchers around the globe made immense progress in understanding and treating such a complex disease like breast cancer. Cancer which originates from breast tissue is known as breast cancer, these most commonly originate from the lobular or inner lining of the milk duct [2]. In 2018 around 627,000 women lost their life because of breast cancer, which comprises of 15% of the total death in women related to all cancer types (WHO)[1]. In recent times the rate of breast cancer has significantly increased in south-eastern Asia, including India. The number of breast cancer patients have succeeded the patient count of cervical cancer some of the major cities, such as Mumbai, Thiruvananthapuram, Bangalore and Delhi[3].

Cancer cells are generally very similar in the genetic makeup to the cells of the organism where they develop that is why many times, they are not detected by the host immune system[4]. Erratic growth and uncontrolled proliferation of breast tissue gives rise to breast cancer[5]. Breast is made up of glandular tissues and stromal tissues. Milk producing glands or lobules are a part of glandular tissues and fibrous connective tissues and fatty tissues are included in the stromal or supportive tissues of the breast[2]. Different types of tumors can form is different area of breast, mostly tumors are of benign nature and occur due to the changes in the breast. Non-cancerous condition such as fibrocystic, where women develops cyst or fibriosis, where formation of scar tissue occurs etc[6]. Breast cancer can originate from cells lining the ducts(ductal carcinoma) or cells lining the lobules (lobular carcinoma) or few can even originate from other cells[7].

1.2 Types of breast cancer

According to the site of breast cancer

1.2.1 **Invasive breast carcinoma-** In this type of cancer uncontrolled growth of cells invades into the fatty and connective tissues of the breast, through the lobular (invasive lobular carcinoma) and ductal (invasive ductal carcinoma) wall of the breast. It can be non-metastatic (spreading) to the lymph nodes or other organs, even if it is invasive in nature[8].

1.2.1.1 **Medullary carcinoma-** This is another invasive form of breast cancer which form a distinct boundary between tumor and normal mammary tissue[9].

1.2.1.2 **Mucinous carcinoma-** It is also known as colloid carcinoma; mucinous carcinoma is one of the rarest forms of invasive breast carcinoma. It is caused by mucus producing tumor cells[10].

1.2.1.3 **Tubular carcinoma-** Tubular carcinoma is a unique type of invasive breast carcinoma as it's easy to prognosis in comparison to the common type of breast cancers. Only 2% of the entire breast cancer cases consist of tubular carcinoma[11].

1.2.1.4 **Inflammatory breast cancer-** Inflammatory breast cancer is very rare (consist of only 1% of total cases) and extremely invasive in nature. It is commonly identified by swollen breast (red and warm) with dimples and by presence of distinct ridges form via blocking of lymph vessels around the breast skin[12].

1.2.1.5 **Paget's disease of the breast-** It is another rare form of invasive breast carcinoma. Paget's disease of the breast starts from the milk producing glands and extends up to the nipples. It consists of 1–3% of all the breast cancers cases and can affect both men as well as women[13,14].

1.2.1.6 **Phylloides tumor-** This form of breast cancer can either form benign or malignant tumor. Phylloides tumor mostly form in the connective tissues of breast and they can be simply removed by surgical procedures[15,16].

1.2.2 **Non- Invasive breast carcinoma-** This is a non-invasive type of carcinoma, where the cells are confined to the ductal cells and do not invade the peripheral fatty and connective tissue. The most common non-invasive breast cancer is Ductal Carcinoma In Situ (DCIS) and the less uncommon form is Lobular Carcinoma In Situ (LCIS). LCIS is even considered as a marker for increased risk of breast cancer[8].

According to the histological type, grade, immunohistochemical (IHC) evaluation

1.2.3 **Luminal A-** It is the most common subtype of breast carcinoma, accounting for 50-60% of all breast cancer cases. It is identified as ER-positive and/or PR-positive tumors with a negative HER2 and low Ki67 (proliferating cell nuclear antigen) index. Patients with luminal-A subtype of breast cancer have a better prognosis rate and even the rate of relapse is low in comparison to other breast cancer types[17].

1.2.4 **Luminal B-** It comprises of 15-20% of all breast cancer cases and it is more aggressive form of breast carcinoma in comparison to luminal-A. It is identified as HER2-positive (ER-positive; HER2-positive; any Ki67; any PR) and HER2-negative (ER-positive; HER2-negative; Ki67% high; PR low)luminal B subtypes[18].

1.2.5 **Her2neu positive-** It comprises of 15%- 20% of newly diagnosed all breast cancer cases[19]. This subtype is identified by a high expression of HER2 (> 10%), negativity for ER (< 1%) and PR (< 20%), and high expression of Ki-67 (> 20%)[20].

1.2.6 **Basal-Like-** Basal-like breast cancer (BLBC) is another form of aggressive molecular subtype characterised by cluster of genes expressed by epithelial cells in the basal or outer layer of the adult mammary gland. BLBC is prevalent in young woman and it often relapses rapidly, making it a clinical challenge[21]. It lacks expression of estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2)[22](TNBC).

1.3 Stages of breast cancer

After diagnosis of cancer, different stages are assigned to it as per its advancement. Method of treatment and prognosis are determined as per the stage of the cancer[2].

Stage	Description
Non-invasive Carcinoma	
0	When tumor is confined in milk producing glands or milk duct and it has not invaded the surrounding tissues.
Invasive Carcinoma	
I	Tumor diameter is less than 3/4 inch and it is confined in the breast.

IIA	The tumor is ¾ inch or less in diameter and it has spread to one to three lymph nodes in the armpit, microscopic amounts have spread to lymph nodes near the breastbone on the same side as the tumor, or both or the tumor is larger than ¾ inch but smaller than 2 inches (5 centimeters) in diameter but has not spread beyond the breast.
IIB	The tumor is larger than ¾ inch but smaller than 2 inches in diameter, and it has spread to one to three lymph nodes in the armpit, microscopic amounts have spread to lymph nodes near the breastbone on the same side as the tumor, or both or The tumor is larger than 2 inches in diameter but has not spread beyond the breast.
IIIA	The tumor is 2 inches or less in diameter and has spread to four to nine lymph nodes in the armpit or has enlarged at least one lymph node near the breastbone on the same side as the tumor or The tumor is larger than 2 inches in diameter and has spread to up to nine lymph nodes in the armpit or to lymph nodes near the breastbone.
IIIB	The tumor has spread to the chest wall or skin or has caused breast inflammation (inflammatory breast cancer).
IIIC	The tumor can be any size plus at least one of the following: It has spread to 10 or more lymph nodes in the armpit. It has spread to lymph nodes under or above the collar bone. It has spread to lymph nodes in the armpit and has enlarged at least one lymph node near the breastbone on the same side as the tumor. It has spread to four or more lymph nodes in the armpit, and microscopic amounts have spread to lymph nodes near the breastbone on the same side as the tumor.
Metastatic cancer	
IV	The tumor, regardless of size, has spread to distant organs or tissues, such as the lungs or bones, or to lymph nodes distant from the breast.

1.4 Present scenario of breast cancer

In the present era a heterogenous disease like breast cancer is difficult to combat as it comprises of different sub-types such as estrogen receptor (ER) or progesterone receptor (PR) positive which includes 60-70% of all breast cancer cases, human epidermal growth factor receptor 2 (HER2) protein amplified or modified consist of 15-30% and 10-15% cases comprise of lack of ER and PR expression and HER2 upregulation regarded as Triple Negative Breast Cancer (TNBC)[23]. TNBC epidemiological data have shown it to be most common in premenopausal women, under the age of 40 years[24]. Survival rate of TNBC

patients are lesser in comparison to the other sub-type patient group, mortality rate of patients within first five years of diagnosis is 40%[25]. TNBC is very invasive in nature, distant metastasis is found in nearly 46% of patients. It often metastasis to brain and visceral organs and metastasis is usually observed after 3 years of diagnosis[26]. Non-TNBC patients have an average time of relapse as 35–67 months but in case of TNBC patients the average time of relapse is 19–40 months. After reoccurrence, the mortality rate in TNBC patients are as high as 75%[27,28]. In India the TNBC patient count is as high as of 31% of the all breast cancer type[29]. Due to the chemotherapy resistant nature of TNBC, targeted gene therapy based on studies identifying genes related to the disease is one of the frontlines for treatment of TNBC.

1.5 Tumor heterogenicity

Intra-tumor heterogeneity revelled co-existence of genetically and epigenetically diverse cancer cells residing in throughout the landscape of tumor or in the entire process of tumor progression[30]. Reason behind these variations ranges from genetic alteration, epigenetic modifications, different conditions of microenvironment, plasticity of genes[30],[31]. Intra-tumor heterogeneity is at the core of tumor development and even the by-product of the tumor evolution process. This diversity in tumor population creates a barrier in clinical management of the cancer patients and success of the therapeutic approaches. Understanding of the heterogenicity of the tumor cells is very critical for clinical developments, so that the therapeutic approaches can be based on the identified resistant subpopulation and the differential regulation of cellular phenotypes[32]. Recent study in breast cancer indicated that resistance to chemotherapy and progression of tumor are result of generation of a subpopulation with different genetic makeup in comparison to the original population[33]. Conditional fluctuation in the protein expression levels have shown produce hindrance against chemotherapeutic drugs. Cancer stem cells are a perfect illustration of the complexity of the tumoral heterogenicity, these cells are particularly resistant to targeted therapies and chemotherapy[34].

1.6 Immune surveillance

Immune system is the defence mechanism of an organism, which is a collection of structures and processes within the body and those main function is to protect the organism against any foreign invasions which causes diseases or any kind of health hazards.

Different kind of stimuli gives rise to different kind of immune response. Immune system can even discriminate between different stimuli,choosing between those who will lead to active immune response and those who will lead to tolerance[35].

Immune surveillance is a phenomenon where the immune system which is protecting the body not only recognizes the invading pathogens but also its own body cells which are getting converted into cancerous cells and destroys them. When the immune system specifically identifies the bodies own cells as cancerous cells on the basis of their expression of tumor-specific antigens, this process is known as tumor immune surveillance[35,36].

The concept of tumor immune surveillance was first proposed by Burnet and Thomas in 1957.They postulated that immune surveillance eliminates precancerous or cancerous cells. This data was supported by many experiments conducted between mid-1970 and 1990s by different research groups and by 2003 many evidences proposed that immune system can recognize and eliminate the tumor cells[37]. However, even in presence of active immune system the spontaneous growth of tumors can't be stopped.Therefore a more refined concept of tumor immune surveillance was proposed, known as tumor immunoediting comprising of more information about the role of immune system in tumor development[35].

The idea of tumor immunoediting initiates with immune surveillance and goes up to immune escape, so the three major steps of tumor immunoediting are: Elimination, Equilibrium and Escapes[38]. Thus, some tumor cells are eradicated by the immune cells but certain cells show either reduced immunogenicity or an increased capacity to inhibit protective anti-tumor immune response[39].One of the main mechanism of eradicating cancerous cells is apoptosis. Apoptosis is an energy-dependent process of cell suicide in response to a variety of environmental stimuli and is characterized by a number of distinct morphological features and biochemical processes including cell shrinkage and partial detachment from substratum, plasma membrane bleeding, chromatin condensation and intra-nucleosomal cleavage and, ultimately, cell fragmentation into apoptotic bodies which are phagocytosed without provoking an inflammatory response[38,40] . Apoptotic pathways are of two types: the intrinsic or mitochondrial death pathway and the extrinsic cell death pathway initiated by the tumor necrosis factor (TNF) family members [41,42].

1.7 Apoptosis

Apoptosis is an energy-dependent process of cell suicide in response to a variety of environmental stimuli and is characterized by a number of distinct morphological features and biochemical processes including cell shrinkage and partial detachment from substratum, plasma membrane blebbing, chromatin condensation and intra-nucleosomal cleavage and, ultimately, cell fragmentation into apoptotic bodies which are phagocytosed without provoking an inflammatory response[41–43]. Apoptosis is genetically controlled and plays an important role in both embryonic development and tissue homeostasis in adults, where this process is crucial for the formation and maintenance of body form[44]. Apoptosis also plays a protective role, eliminating cells which might prove harmful if they were to survive, e.g. removing cells harboring mutations following irradiation or chemical insult which could lead to the emergence of cancer, and as a protection against virus infection[44,45].

Apoptotic pathways are two types: the intrinsic or mitochondrial death pathway and the extrinsic cell death pathway initiated by the tumor necrosis factor (TNF) family members (Kumar S, 2007; Tsujimoto, 2003). Mitochondrion-mediated apoptosis is usually initiated by many apoptotic signals, which imbalance the major apoptosis regulators, such as Bcl-2, Bax, and Bid[46,47].

In one of the pathways the pro-apoptotic protein Bax accumulates on mitochondria after being activated and triggers an increase in the permeability of the outer mitochondrial membrane[48]. There after the mitochondria release cytochrome c and other key molecules that usually facilitate apoptosome formation to activate caspase 9. This, in turn, activates downstream apoptotic pathways such as caspase 3 and poly (ADP-ribose) polymerase (PARP)[48,49]. The mitochondria also release apoptosis-inducing factor and endonuclease G to initiate the caspase-independent apoptosis[50]. In the extrinsic cell death pathway activation of caspase 8 is achieved through binding to the adaptor protein Fas-associated protein with death domain (FADD), which in turn activates caspase 3 to facilitate cell death[51].

1.8 Complement induces apoptosis

TRAIL mediated modulation of our immune system has been one of the key features of TRAIL signalling. Components of complement system provides a platform for cross-talk between complement system and systemic and regulatory functions[52]. Complement system

as a definitive role in innate immune system, adaptive immune system, organ development, homeostasis, regulation of the coagulation system, synaptic maturation, angiogenesis, mobilization of hematopoietic stem-progenitor cells, tissue regeneration, lipid metabolism and even in cancer regulation. Complement system can activated by classical pathway (antigen-antibody complex formation system), lectin pathway and/or alternative pathway[52–54]. Initiation of the classical pathway is regulated by C1q, C1r, C1s, C2, C4, CFH; lectin pathway starts with MBL, MASP2, C2, C4; and the alternative pathway involves C3, CFB, CFP, CFH, CFI, CD59. All the three pathways convert inactive C5 into the active C5 convertase, which finally generates the MAC (membrane attack complex) resulting in cell lysis[52]. Several studies have shown the complement system to be activated in response to tumor antigens[52,54]. In our study we do find an interesting link between complement system and TRAIL, which has not been reported before.

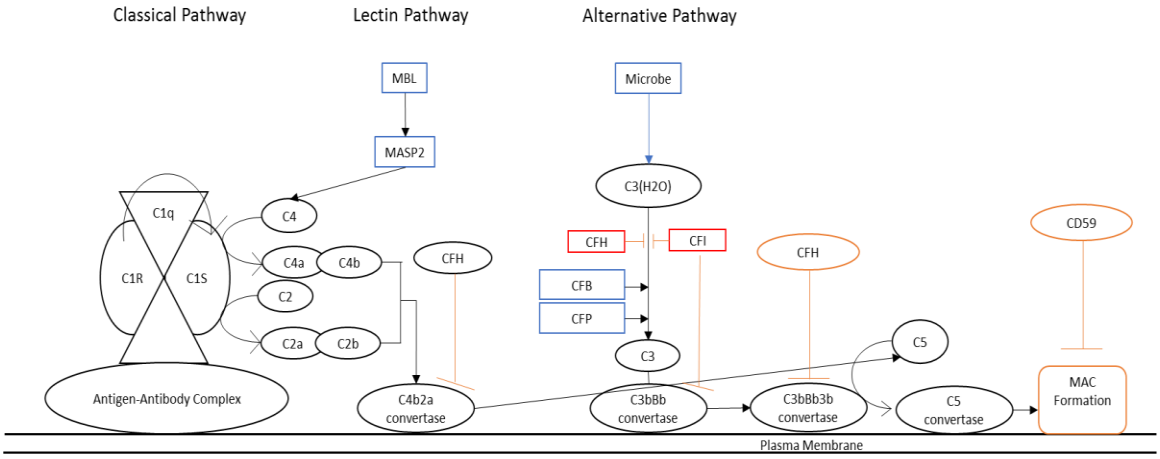

Figure 1.1. *Complement Pathway: Schematic representation of the complement pathway genes. Two of the pathways of complement cascade are the classical pathway and the alternative pathway. Classical pathway initiates with binding of C1q with antigen-antibody complex, C1r present on C1q cleaves and activates C1s which in turns cleaves C2 and C4 into C2a and C4b, and form the C3 convertase. In case of alternative pathway hydrolysed plasma C3 binds with CFB with the help of CFD and CFP to further cleave more plasma C3 to generate active C3b, which on the surface binds to Bb (cleaved form of CFB) to form the convertase. CFH gives hindrance to the C3 convertases of classical pathway and C3bBb3b convertases. CFI inhibits C3 convertases of alterative pathway. C3 convertase binds to additional C3b to generate C5 convertase, which cleaves C5 into C5a and C5b. Further C5b facilitates the formation of MAC. Blue represents activator genes and red represents inhibitor genes in both the pathways.*

1.9 Molecules inducing apoptosis

Apoptotic pathways are of two types: the intrinsic or mitochondrial death pathway and the extrinsic cell death pathway initiated by the tumor necrosis factor (TNF) family members[46],[47]. One of the members of tumor necrosis factor (TNF) family named TNF-related apoptosis-inducing ligand (TRAIL/Apo-2L) can induce apoptosis in transformed cells or the carcinogenic cells mostly without harming the primary cells. TRAIL can activate both the arms of apoptosis, therefore indicating a major role in elimination of tumor cells in tumor immune surveillance[55],[56]. In a tumor microenvironment (TME), TRAIL is expressed by different immune cells such as NK cells, monocytes, macrophages, and cytotoxic T cells[57],[58]. These cells under normal physiological conditions do not express TRAIL. TRAIL expression occurs on exposure of these immune cells to proinflammatory cytokines such as TNFA, IFNA, IFNB, IFNG and IL2 in the tumor microenvironment[57].

1.10 TRAIL Pathway

Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) (systematic name, TNFSF10; formally Apo-2L or TL2)[59] is a Type II transmembrane protein, which was first recognized because of its homology with extracellular domain of CD95 L, TNF and LTa[60] and it usually binds to one of the four receptors, which are express only in humans, it includes TRAIL-R1/DR4, TRAIL-R2/KILLER/DR5, TRAILR3/DcR1/TRID , and TRAIL-R4/DcR2/TRUNDD[61]. Among these four receptors only DR4 and DR5 has functional death domain and they are membrane anchored proteins with transmembrane and cytoplasmic domain. While DcR2 contains truncated, non-fuctional death domain(DD), on the other hand DcR1 does not have any DD.DcR1 is a glycosylphosphatidylinositol-anchored receptor without any transmembrane and cytoplasmic domain. TRAIL even binds another receptor called OPG (Osteoprotegerin), which work in the similar manner asDcR1 and DcR2 and is a secreted protein without any membrane anchor. Four of the TRAIL receptors are mapped to chromosome cluster 8p22-p21. OPG, which is least related to TRAIL was mapped to the same chromosome , to 8q24.

TRAIL is mapped to3p26 loci[59]. TRAIL trimer binds to its receptors and activates receptor trimerization resulting in initiation of apoptosis. Activated DD of the receptors recruits the adaptor protein Fas-associated protein with death domain (FADD), which in turns recruits pro-caspase8/10 leading to the formation of death-inducing signaling complex (DISC). The death effector domain(DED) of both FADD and caspase8 and caspase10 activates DISC. The enzymatically activated caspase8 is the initiation caspase in DISC. The initial activation of caspase8 is done by proximity-induced conformational changes at DISC and the ultimate activation is done by auto-catalytic cleavage and formation of homodimers. The activated homodimer of caspase8, after getting released from the DISC, recruits the downstream apoptosis molecules[62]. TRAIL not only recruits FADD through DISC, but also activates a pro-survival transcription factor NF-κB. TRAIL binds to DR4, DR5 and DcR2 and through triggering TNFR1 associated death-domain (TRADD) activates NF-κB by recruitment of DD containing protein RIP and TNF receptor-associated factor-2 (TRAF2). A dominant TRADD can counter the activation of NF-κB through TRAIL receptors[63]. In type-I cells the strength of caspase8/10 activity is strong enough to recruit the downstream apoptosis molecules but in type II cells, the strength of caspase8/10 is insufficient for activation of downstream effectors and therefore to amplify the extrinsic signals caspase8/10 must cleave the pro-apoptotic Bcl-2 family member Bid to truncated Bid(tBid), which ultimately links the extrincsic and intrinsic pathways [64],[65],[66],[67].tBid translocates to the mitochondria and activates Bax and Bak. Bax accumulates on mitochondria and triggers an increase in the permeability of the outer membrane, thereby releasing apoptotic factors such as cytochrome c, second mitochondria-derived activator of caspase/ direct inhibitor of apoptosis(IAP) binding protein with low pI(Smac/DIABLO). Then apoptosome complex is formed as cytochrome c binds to apoptotic protease-activating factor 1 (Apaf-1) in presence of ATP/dATP. In proximity induced manner apoptosome enhances the auto-catalytic activation of caspase9, then in turn activates caspase3 or caspase7. The active sites of caspase3, caspase7 and caspase 9 are blocked by IAP proteins like c- IAP1, c-IAP2 and X-linked IAP (XIAP). The activity of caspases are restored by the release of Smac/DIABLO from mitochondria which binds to IAPs and make them inactive[68],[69].

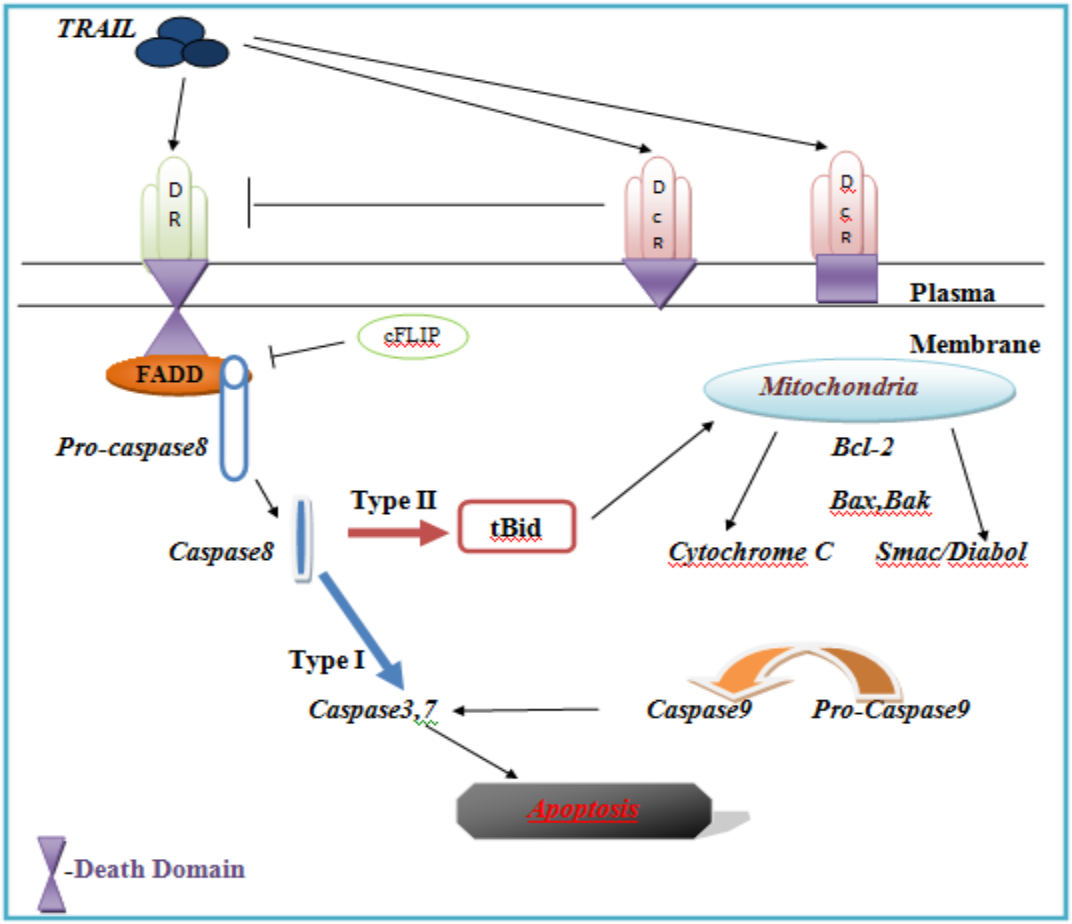

Figure 1.2. *Apoptotic pathway: Schematic representation of apoptotic pathways induced by TRAIL. TRAIL induces extrinsic pathway via FADD and caspase-8, and intrinsic pathway via mitochondria. Both pathways intersect at caspase-7 and 3 which are the final executioners of apoptotic cascade.*

1.11. TRAIL resistance

Resistance towards TRAIL- induced apoptosis can be acquired at any stage of signaling pathway, starting from the death receptor(DR) to the expression of apoptotic inhibitors. Mutation occurred at the death receptors causing loss of function or by blocking the receptors from binding to the TRAIL can confer resistance to the apoptotic pathways. Dysfunctioning of protein Fas-associated death domain (FADD) and absence of different caspases can even produce resistance to cell death. Recruitment of cFLIP to disc, overexpression of Bcl-2 and Bcl-XL, dysfunctioning of Bax, accumulation apoptotic inhibitors and reduced release of second mitochondria-derived activator of caspases (Smac/Diablo) from the mitochondria to the cytosol are some of the main ways of TRAIL resistance. Resistance is even offered by Inhibitors of apoptosis (IAP) proteins, Six1

expression and O-glycosylation of receptors. Mutation in DR4 or DR5 or internalization of the DRs can cause resistance towards TRAIL-induced apoptosis. Lastly TRAIL not only activates apoptosis but also pro-survival factor NF-κB activation of which may counteract apoptosis.

1.11.1. Resistance through Caspase-8

Downregulation or loss of caspase-8 expression can lead to TRAIL resistance[30,70].In one experiment caspase-8-deficient Jurkat cells were shown to be completely resistant to TRAIL, whereas the corresponding wild-type cells remained sensitive to TRAIL. In another study, the specific caspase-8 inhibitor ZIETD-FMK could inhibit TRAIL-induced apoptosis in most TRAIL-sensitive cell lines[71]. Downregulation or absence of caspase-8 expression is shown to be related with resistance to TRAIL-induced apoptosis in many cancers like cancer cells, including Ewing's tumor, neuroblastoma, malignant brain tumors, melanoma, and small-cell lung cancer. In a very few cases, loss of caspase-8 expression resulted from a gene deletion, as demonstrated by Southern blot analysis. In most cases, however, absence of caspase-8 expression resulted from gene silencing by DNA methylation in its promoter region[70,72,73]. Treatment of such cells with the demethylation agent 5-aza-20-deoxycytidine reversed the hypermethylation of the caspase-8 promoter, thereby restoring expression of caspase-8 and sensitivity to TRAIL-induced apoptosis[70,73].

1.11.2. Resistance through cFLIP

To avoid excessive apoptosis induction by TRAIL, several mechanisms to countervail TRAIL-induced apoptosis have evolved in normal cells and are frequently exacerbated in tumor cells to escape TRAIL-mediatedapoptosis. Splice variants, cFLIP-long (cFLIPL), cFLIP-short (cFLIPS) and cFLIP-Raji (cFLIPR), are expressed on the protein level. All three of these cFLIP variants contain two N-terminal DEDs that are highly homologous to the two DEDs of caspases-8 and -10. cFLIPL contains a long C-terminal domain, which closely resembles the caspase portion of caspase-8 but lacks catalytic activity[74]. cFLIPL contains two DEDs and a caspase-like domain, but it cannot activate caspase cascades because that domain lacks a cysteine residue essential for catalytic activity. cFLIPS also

contains two DEDs, but it lacks almost the entire caspase-like domain. Both cFLIPL and cFLIPS can be recruited into the DISC, where they bind to either FADD or caspase-8 through DED–DED interactions, resulting in inhibition of caspase-8 activation and inhibition of subsequent apoptosis[75]. Changes in the cFLIP/caspase-8 ratio have also been reported to correlate with TRAIL resistance in several different tumors, including melanoma, hepatocellular carcinoma, Burkitt's lymphoma, and B-cell chronic lymphocytic leukemia[63].

1.11.3. Resistance offered by Bcl-2 Family

There are three different classes of Bcl-2 family members: (i) pro-apoptotic effectors like Bax, Bak and possibly Bok, (ii) anti-apoptotic factors like Bcl-2, Bcl-xL and Mcl-1 and (iii) pro-apoptotic inducers, like Bid, Bim, Puma,Noxa, which belongs to BH3 protein family[76]. As per a study in one of the colon cancer cell line for the TRAIL-induced apoptosis by Bax, even the expression of Bak is required[77]. Another experiment showed that when apoptosis is induced by staurosporin, UV, VP-16 in wild type jurkat cells, with the same treatment apoptosis is nearly absent in Bak deficient jurkat cells[63] . In pancreatic and adenocarcinoma cell lines TRAIL resistance is conferred by high expression of Bcl-XL expression. Even in neuroblastoma, glioblastoma and breastcancer cell lines, the over-expression of Bcl-2 gives rise to TRAIL-resistance[64]. The third class of Bcl-2 family is activated by posttranslational modification, transcriptional induction or by caspase-8 mediated proteolysis of Bid in presence of apoptotic stimulus[78].

1.11.4. Resistance due to Inhibitors of apoptosis (IAP) proteins

IAPs usually confers resistance to TRAIL-induced apoptosis by inhibiting activities of caspases(caspase-3,7) through binding to their BIR(Baculovirus IAP Repeat) domain or by directly interfering with the active site of apopsomal caspase-9[63,79]. The cellular IAPs(cIAPs) even degrades the caspase- 3 and 7 by ubiquitin mediated proteasomal degradation ultimately repressing the apoptotic pathway[39,80].A mitochondrial protein known as Smac/Diablo released during the apoptosis cascade, functions as an inhibitors of IAPs making the path open for apoptotic proteins[30]. Smac/Diablo takes place of caspases in the BIR region of IAPs, releasing the caspases for apoptotic pathways. Some

of the melanoma cell lines have developed pathways for inhibiting the release of Smac/Diablo[63].

1.11.5. Resistance due to Six1 expression

Six1, a homeobox transcription factor that is not expressed in most normal adult tissues but is often re-expressed in tumors causes marked resistance to TRAIL-induced apoptosis(94)[81]. Six1 expression is correlated with metastasis in breast cancer[82]. High Six1 expression in tumors occurs in more than 60% of women with metastatic ovarian cancer[81]. Six1 is additionally associated with worsened survival in hepatocellular carcinoma[83,84]. In case of ovarian cancer over expression of Six1 is correlated with higher expression of DcR2.This may be done by binding of Six1 to the promoter region of DcR2, as a consensus for Six1 is found upstream to the translation initiation site in the mRNA[84].

1.11.6. Resistance offered by O-glycosylation of receptors

Loss of peptidyl O-glycosyltransferase GALNT14correlated with reduced sensitivity to TRAIL because O-glycosylation of DR4 or DR5 promotes the ligand stimulated clustering of receptors leading to more efficient recruitment and activation of caspase-8[85].

1.11.7. Resistance through TRAIL receptors

As it is already known that the apoptotic signalling begins with the TRAIL binding to its receptor and till date only five receptors of TRAIL has been found as mentioned earlier. The two main pro-apoptotic receptors are DR4 and DR5 and any genetic mutation caused in these two receptors can give rise to TRAIL resistance[85]. As in case of ovarian cancer and bladder cancer polymorphism of DR4 is observed. A conversion of arginine for leucine because of the change of base from A to G at nucleotide 1322 of codon 441 in the DD region of DR4 is found in both the cancer cell lines. This polymorphic form of DR4 is less effective in triggering the apoptotic signals than its counter wild type version. In case of lung cancer, head and neck squamous cell cancer cells, two missense nucleotides occur at two different positions on DR4. First occurring at the 209 codon converting the

arginine base for threonine by altering the C base by G and the later occurs at the 141 codon by substituting G by A, giving a outcome of histidine instead of argennine. As these two mutations occur in or near the ligand-binding domain of DR4, it usually causes abnormal death receptor trimerization or TRAIL binding. Usually a mutation is also found in DR4 in case of metastasizing breast cancer cell line.

Another study found that in case of breast cancer and non-small cell lung cancer, a mutation found in the intracellular domain of DR5 receptor binds to the FADD molecule. A mutation in DR5 had also been found in colon cancer which induced resistance to certain anti-cancer agents like 5-fluoruracil. In head and neck cancer, non-small-cell lung cancer, breast cancer, non-Hodgkin's lymphoma, and hepatocellular carcinoma two kinds of mutation were found in DR5, one of which was a 2-bp insertion in the DD of DR5 that resulted in a premature stop codon and a truncated DR5[86].

Higher mutation frequency and higher loss of heterozygosity (LOH) frequency of 8p21-22 loci of DR4and DR5 genes are characteristics of metastatic Invasive Ductal Carcinomas (IDCs) of breast cancer. Three mutations of DR4 and four mutations of DR5 are found in different stages of metastatic breast cancer One mutation of DR4 and two mutations of DR5 are found in primary tumor and in metastatic focus and the remaining four kinds of mutations are present only in metastatic lesions, indicating that they might help in metastasis of breast tumor. The death domain of DR4 contains three mutations. Among the three mutations, two are hemizygous mutations (Ala420Val and Pro376Leu) allelic deletion. It rises a possibility of binding of these hemizygously mutated DR4 with the normal DR4 and producing a resistance in binding of TRAIL to its receptors. DR5 might be biallelically inactivated by the four mutations as they show LOH at intragenic markers and/or the microsatellite markers. Two mutations in DR5 Q416R and G426R shows lesser apoptosis than the wild types[87]. Other than the three mutations in DR4, a polymorphism in death domain (Lys441Arg) may even help in the TRAIL resistance. This polymorphism is found in 20% of the normal population[88].

1.11.8. Endocytosis of death receptors

In one study MDA-MB-231 cells, DR4-GFP-WT was expressed on the cell surface but was found exclusively in the cytoplasm of BT474 cells. Additionally, treatment with

chlorpromazine, an inhibitor of clathrinmediated endocytosis[89,90], increased the cell surface receptor expression. Knockdown of adaptor protein 2 (AP2) or clathrin significantly increased DR4 and DR5 cell surface expression without changing their total protein levels in BT474 cells. The resultant cells became highly susceptible to TRAIL-induced apoptosis. Taken together, these results suggest that DR4 and DR5 may undergo constitutive endocytosis through clathrin-dependent pathway in TRAIL-resistant cells and experimental inhibition of endocytosis restores DR4- and DR5-mediated signaling in response to TRAIL[90,91].

A recent study identified two nuclear localization signals in DR protein which mediates its nuclear localization through the nuclear import pathway by importin beta1 in HeLa and HepG2 cells[91] . The over expression of DRs in cytoplasm may reflect receptor-ligand internalization, a rapid process occurring after ligand binding. Numerous studies have shown that TNFR1 and Fas undergo rapid internalization in response to ligation[92,93]. TRAIL receptors, DR4 and DR5, were also shown to follow a similar mode of action[79,90,94]. In a tumor setting, this might be triggered by soluble cytokines (TNFα, FasL or TRAIL) in the tumor microenvironment[95]. The signaling events may involve clathrin-dependent endocytosis or other uncharacterized mechanisms[90,96]. However, studies cannot exclude the possibility that intracellular localization of DRs may be newly synthesized molecules within the endoplasmic reticulum or Golgi that have yet to be processed and inserted properly into the plasma membranes. In any event, the trapped DR in cytoplasm inevitably reduces its accessibility to incoming ligands, thereby making the cells resistant to the targeted therapies[96,97].

1.11.9. Resistance through NF-κB

NF-κB which is a prosurvival protein involved in cell proliferation, cell survival and oncogenesis[98] is even activated by DR4, DR5 and DcR2. NF-κB activation is achieved in a TRADD/TRAF2/RIP dependent manner[99]. Interaction of TRADD transcription factor with TRAF2 and RIP activates NIK, a member of mitogen-activated protein kinase family[100][103]. Activated NIK recruits another two downstream kinases, IKKα and IKKβ[101]. Activated IKKα and IKKβ forms a heterodimer which phosphorylates IKBs. Phosphorytaled IKBs gets degraded, thereby active NF-κB is released[99] [102]. Some of the studies have shown that FADD interacts with TRADD molecule for activation of NF-

κB pathway. TRADD recruits FADD for activation of caspases for apoptosis pathway and it even recruits TRAF2 and RIP for activation of NF-κB. Therefore TRADD which is present upstream of FADD has an important role in activation of NF-κB[102]. In contrast another study shows that a unidentified adaptor molecule is recruited by DR4, DR5 and DcR2 other than FADD or TRADD which activates the TRAF2 molecule[99] [102]. Thereby the original pathway for activation of NF-κB by TRAIL induced death receptors are yet not well understood but by activating NF-κB TRAIL produces hindrance in the process of cell death.

1.11.10. Role of EMT in TRAIL resistance

It has been already reported that EMT plays an important role in TRAIL resistances, role of migration and invasion have shown to be critical factors regulating TRAIL resistance in TNBC cell lines[103]. One of the essential epithelial transmembrane proteins regulating EMT is CDH1[104], a homophilic calcium-dependent cell adhesion molecule[105]. Loss of tumor suppresser CDH1 plays a crucial role in dysfunction of cell-cell adhesion. Therefore, the loss of cellular integrity will lead to local metastasis and invasion of the tumor cells[104]. Reduced cell-cell adhesion and increased cell-EMC adhesion is one of the definitive markers of tumor cells. In gastric cancer high density of collagen helps in integrin-mediated cell–ECM interactions and loss of membranous E-cadherin[106]. Homotypic interaction of E-cadherin regulated actin and membrane dynamics of extracellular matrix during early stage of cell-cell adhesion[107]. Earlier it has been reported that loss of CDH1 causes aberrant cell cycle entry of post-mitotic neurons, thereby leading to apoptosis[108]. Proteolysis of CDH1 leads to accumulation of cyclin B1 that in turn promotes apoptosis[109]. Studies have shown ability of TRAIL to form death receptor complex relies on EC1 extracellular domain of E-cadherin. E-cadherin/α-catenin mediated linkage of actin cytoskeleton plays a vital role in activation of DISC[110]. Embodiment of DR4 and DR5 in CDH1 adhesion structures, utilizes the structural ability of actomyosin contractility for effective assembly of DISC components[111].

1.11.11. Resistance against TRAIL in tumor microenvironment

The increased knowledge of the diversity of the TRAIL signalling pathway, revealed more about the intricate mechanism by which TRAIL induces apoptosis or pro-survival pathway on basis of cell type or various specific mutations[112]. TRAIL pathway can be modulated by both cellular and non-cellular components of the tumor microenvironment (TME), it can just be a simple inhibition of delivery of TRAIL expressing leucocytes to the site of active tumor development[113]. One study have shown TRAIL downregulation in dendritic cells (DCs) in murine lymphoma, they have stated that r whole tumor lysate or tumor cells are capable of total impairment of TRAIL expression on DCs which facilitates tumor invasion and these process id completely irreversible even in presence of DC stimulator IL-5[114]. Other immune cells such as macrophages and neutrophils are capable of expressing TRAIL and they do move towards DR expressing cells, they can even produce soluble TRAIL and protect themselves via overexpression of DcRs[115]. Another study has stated that tumor invasion and infiltration can be achieved via MMP9 pathway, activated by IL-35. IL-35 can be also responsible for downregulation of TRAIL expression in neutrophils via STAT3 pathway activation[116].

Certain cancer-related stroma cells are known for their contribution towards TRAIL resistance, such as cancer-associated fibroblasts (CAFs) shows enhanced expression of decoy receptors specially around TRAIL resistant tumor cells, providing a protumerogic environment to the cancer cells[117]. In myofibroblasts TRAIL pathways is hindered via secretion platelet derived growth factor BB and via activation of prosurvival hedgehog pathway in cholangiocarcinoma[118]. It has been already reported that resistant cells also have an ability to convert anti-tumor immune cells into tumor related stroma cells. DR4 and DR5 signaling pathway in TRAIL resistant NSCLC, pancreatic, and colorectal cells are used for protumerogic functions such as secretion of CCL2 and activation of polarization of myeloid cells into M2-like cells[119].

1.12. TRAIL therapeutic approaches

To tackle different resistance mechanism adapted by different cancer types against TRAIL induced apoptosis, various therapeutic approaches are adopted by various groups in multiple studies. The most common approach is combination therapy to enhances the effect of TRAIL on the targeted tumor type, others being targeting multiple pathways at one which reduces the chances of multiple simultaneous mutations.

It has been shown that combining TRAIL with DNA damage causing therapeutic agents like radiation therapy and chemo therapy resulted in positive outcomes, one recent study have shown relation between p53 status and TRAIL sensitivity[120]. As p53 plays a central role in regulating some proapoptotic genes such as BAX, DR4, DR5[121]. Thereby, DNA damage causing agents activates the p53, which in turn help in upregulating apoptosis via TRAIL pathway. Other chemo therapeutic agents such as gemcitabine and cisplatin have been used in combination with TRAIL for killing different tumor types[120]. Similarly, inhibition of known negative regulator MDM2 of p53 can lead to accumulation of p53 in breast, lung and colon cell lines, which can further lead to sensitization of these cells towards TRAIL treatment[122].

Another approach of enhancing TRAIL-mediated killing is via targeting multiple pathways at the same time, as tumor becomes TRAIL resistant even via upregulating survival pathways accompanied by antiapoptotic pathways[112]. Multiple antibody-based inhibitors showed promising combinatory effect with chemotherapy in comparison to the small molecule inhibitors[123]. Thereby, combining TRAIL with these antibodies with common target genes can be a promising way to overcome TRAIL resistance in cancer cells. In one study TRAIL was combined with nanobodies (VHH domain fragment) or the single-chain variable fragment (scFv) for targeting EGFR, the product ENb2–TRAIL was far smaller than the entire antibody and it targeted proliferation and apoptosis pathway simultaneously[124]. When ENb2–TRAIL was added directly or delivered via stem cells, it showed enhanced cell death and it was even competent to overcome resistance against TRAIL or ENb[125]. Next TRAIL was infused with the three type 1 repeat (3TSR) domain of the antiangiogenic protein thrombospondin 1 (TSP) to overcome the resistance conferred against TRAIL induced apoptosis. This compound also elicited the apoptosis pathway when applied systematically or via stem cell delivery. Angiogenesis is mainly targeted by 3TSR in not only tumerogenic cells but also in the tumor-associated endothelial cells, when treated in combination with TRAIL it can increased the life expectancy of tumor bearing mouse[126].

Many natural compounds were tested via high through put screening to check their synergic effects with TRAIL. 16480 compounds were screen for their activity in renal adenocarcinoma, out of these 18 compounds showed promising results when treated in combination with TRAIL and 12 out these activated apoptosis pathways via caspase-8 dependent mechanism[127]. 55 FDA- or few approved antineoplastic drugs were tested on a

small scale on TRAIL-resistant prostate and pancreatic cancer cells. The promising candidate sensitized cells toward TRAIL treatment via know mechanisms such as promoting DR4 or DR5 lipid raft distribution, inhibiting XIAPs and causing DNA damage[128].

Lastly, the newest developed way of treating cancer is cancer immunotherapy, where the immune cells are weaponised directly or indirectly for eradication of cancer. Certain lymphocyte-mediated tumor cell death mechanisms are regulated via TRAIL signalling pathway such as NK cell and T-cell response, thus using TRAIL in synergy could result in favourable outcomes[106]. Different approaches addressing cell-based therapies were tested, among them certain immune checkpoint blockers were used for activation of tumor antigen specific, MHC-restricted T-cells. This includes fusion of TRAIL with the a scFv fragment of anti-PD-L1, these molecules shown multiple favorable therapeutic results by activating suppressed DCs or macrophages into active proapoptotic response cells and also helps in production of IFN-γ, thereby sensitizing cells towards TRAIL induced apoptosis by suppression of c-FLIP[129]. Fragment of antibody which can stimulate CD3 and CD7 were fused with TRAIL and it was observed that they can bind with the DR5 receptor present on the surface of cancer cells activating both proapoptotic signalling pathway and tumoricidal T-cells. This treatment has shown positive outcomes in a wide range of cancer cells and it is believed that it has potential to be a pan-carcinoma therapeutic agent. Even a very minimal expression of DR5 on the cancer cell surface is sufficient for activation of T-cell via CD3[130]. Similar TRAIL combination with scFv antibody fragment was even used for specific C-type lectin-like molecule-1 (CLL1) to DCs, activate granulocytes and monocytes[131]. In another study ex-vivo engineered Natural killer cells were used to secret glycosylate TRAIL in peritoneal tumors for reduction of peritoneal colorectal carcinomatosis. Another approach was used for developing different multivalent TRAIL for better results. Among several variants TRAIL timer dimerization was achieved using a fusion form of TRAIL with the Fc portion of the human IgG1. This particular recombinant protein is composed of six different binding sits that can induce superior clustering and this protein has entered the first phase of clinical trial[132].

1.13. Present status

Even though multiple novel approaches are developed in battle against overcoming the resistance to TRAIL induced apoptosis in several cancer types, only few of periclinal studies are considered for clinical trials. Previous clinical trial with TRAIL therapeutics have failed to show better combinatory effect with present chemotherapy agents. But butter developed synergic compounds and antibodies against TRAIL receptors (DR4/DR5) have shown promising results in some of the clinical trials.

Its shown that Recombinant Human TRAIL (rhTRAIL) is more capable of inducing apoptosis via both of its death receptors in comparison to the antagonistic antibodies used against DR4 or DR5[74]. rhTRAIL Dulanermin has been a candidate for Phase Ib/II randomized clinical trials in combination with certain sensitizing compounds like rituximab, good tolerance has been reported but failed to show any significant effect in OS(overall survival)[133]. Several DR4/ DR5 antagonistic antibodies are considered for clinical trials such as mapatumumab against DR4 and tigatuzumab, conatumumab and lexatumumab against DR5[133–135]. scFv-TRAIL has shown very promising preclinical effects, therefore cilinal trials are urgently needed for this[136]. Another clinical trial is investigating a multivalent TRAIL death receptor agonist ABBV-621 antibody, which consist of six-TRAIL binding sites fused with the Fc domain of human IgG1, it showed antitumor activity in the clinical trial[137]. Even though previous TRAIL related clinal trials have shown disappointing results but with the new developed periclinal approaches it is possible to find better solution in overcoming TRAIL resistance in tumor cells.

1.14. Aim of the study

Until this day chemotherapy is the main stream method applied to treat breast cancer patients around the world, even though other therapies are considered for the treatment of the patients such as targeted hormonal receptor therapy, but the effectiveness of these methods are not as much as they were hoped for treating the growing cancer. In last two decades TRAIL has been extensively studied and even consider for clinical trials. TRAIL because of its unique character is considered as an alternative of the chemotherapy, but setbacks have been reported in the clinical trial because of which TRAIL is still not avail for treating breast cancer patients. In our study we aim to understand the detailed

mechanism of TRAIL induced cell death in breast cancer cell line. So that we can identify the key regulators of TRAIL pathway which can be utilised for using TRAIL in main stream treatment for breast cancer patients in future.

1.15. Objectives

- Study the expression pattern of pro-apoptotic, anti-apoptotic and proliferation pathway genes on treatment with recombinant human TRAIL (rhTRAIL)
- Study the molecular mechanism of apoptosis resistance on prolonged TRAIL treatment.
- Study the effect of TRAIL on theophylline mediated cell death and/or mitotic arrest.