2
Review of Literature

A nascent polypeptide chain achieves its native structure with the help of folding machinery present inside the cells that includes molecular chaperones and other associated proteins. However, many of these proteins are not able to fold properly and are needed to be cleared from the cell by the cellular protein quality control system that includes mainly ubiquitin proteasome system (UPS) and autophagy. These two systems either work independently or in collaboration with each other [Goldberg, 2003a; Korolchuk *et al.*, 2010]. Molecular chaperones are the supervisors of newly synthesized polypeptides in the crowded cellular *milieu*. The guidance of chaperones assists immature polypeptides to acquire a functional macromolecular structure and move towards appropriate cellular localization to perform pre-defined functions [Ellis and Hemmingsen, 1989; Hartl, 1996]. Two well-studied chaperones are heat shock proteins, Hsp70 and Hsp90. Heat shock proteins function as stress-induced proteins to monitor changes in the molecular environment of the cell [Nollen and Morimoto, 2002]. The chaperones not only assist new proteins in achieving the functional activity by proper folding, but also play crucial roles in removal of aberrant proteins, either by UPS or by autophagy pathway [Hartl *et al.*, 2011].

UPS involves ubiquitin-dependent degradation of various misfolded proteins [Ciechanover, 1994; Hochstrasser, 1996]. It consists of three enzymes E1 ubiquitin activating enzyme, E2 ubiquitin conjugating enzymes and E3 ubiquitin ligases. Other than these enzymes a small 76 amino acid long ubiquitin protein, and 26S proteasome, the proteolytic machinery of the cell with different types of protease activities, are other major components of the UPS [Hershko and Ciechanover, 1998]. Ubiquitination of a particular protein, with the help of successive activities of E1, E2 and E3 enzymes generates a degradation signal onto a protein, for its translocation to the 26S proteasome interior, where the catalytic sites of proteasome cleave polypeptide into smaller oligopeptides. The specificity in the functioning of UPS comes from E3 ubiquitin ligase enzymes, which recognize misfolded proteins and continue their elimination through catalytic activities of 26S proteasome [Pickart, 2001].

E3 ubiquitin ligases are the specialized class of approximately 1000 different proteins [Nakayama and Nakayama, 2006], which maintain the turnover of cellular proteins under the normal basal conditions by tagging them with a small protein, ubiquitin, and direct them towards the 26S proteasome, for their degradation. E1 ubiquitin activating and E2 ubiquitin conjugating enzymes assist them in ubiquitination mechanism [Hershko and Ciechanover, 1992]. At certain instances, they along with molecular chaperones, utilize lysosomal degradation machinery of the cell, by orchestrating a process called autophagy, to remove the bulk of the cellular inclusion bodies.

These E3 ubiquitin ligases have been classified in different ways depending upon their structures and functions. Based on structural similarity, i.e., the presence of specialized domains, these proteins can broadly be classified into really interesting new gene (RING), homologous to E6-AP carboxyl terminus (HECT), U-box and plant homeodomain (PHD) domain containing E3 ubiquitin ligases [Metzger *et al.*, 2012]. Apparently, they could also be

separated by their functional similarities. Quality control (QC) E3 ubiquitin ligases keep on monitoring and identifying any unwanted intracellular modifications in three-dimensional structures of the proteins, under various kinds of biotic and abiotic stress conditions; and by delivering them to cellular proteolytic systems they facilitate the degradation of these toxic inclusions formed inside the cells [McClellan *et al.*, 2005].

2.1 PREVIEW OF Gp78: WHAT IS HISTORY AND STRUCTURAL PROPERTIES AND HOW IT IS DIFFERENTIALLY DISTRIBUTED WITHIN VARIOUS SUBCELLULAR LOCATIONS?

Gp78 was initially reported as an intracellular intermediate of synthesis of viral glycoprotein Gp80, in rabies virus infected baby hamster kidney (BHK-21) cells [Madore and England, 1977]. Few, years later, a group of scientists reported a membrane bound glycoprotein with molecular mass of 78 kDa. They found that alterations in shape of metastatic cells lead to increased O-glycosylation of this glycoprotein Gp78, which enables it to participate in establishing interaction between cell and its external environment [Nabi and Raz, 1987]. Further studies by the same group found that structural and functional characteristics, like surface localization and involvement in mediating cellular motility of melanoma cells, of Gp78 are similar to AMFR [Nabi *et al.*, 1990]. Experimental studies confirmed that Gp78 is the same surface bound molecule, which binds AMF and functions as its receptor to further mediate cell motility and metastasis of cancer cells [Watanabe *et al.*, 1991b]. Later, protein-protein binding assay analysis of AMF and cell surface glycoprotein also established AMF as a natural ligand of AMFR or Gp78 in B16-F1 melanoma cells [Silletti and Raz, 1993; Watanabe *et al.*, 1991a]. Internalization of Type 1 membrane receptor, Gp78 and its ligand during metastasis is found to be associated with regulatory functions over cell kinesis [Watanabe *et al.*, 1991b]. Another study also confirmed that AMF and its binding with its receptor cause signal transduction to stimulate cell motility, same as chemotactic stimulation do in neutrophil mobility [Nabi *et al.*, 1992]. After observing these significant roles of Gp78 in metastasis, a group of researchers tried to reduce expression of this receptor to control tumor cell mobility [Lotan *et al.*, 1992]. It was found that the surface of carcinoma cells shows an increase in the population of AMFR, as compared to normal cells; however, in both type of cells, structure and copy number of the gene remains same [Silletti and Raz, 1993]. In later years, several studies elaborated active involvement of AMFR in the maintenance of metastasis in various types of cancer cells [Nakamori *et al.*, 1994; Otto *et al.*, 1994; Silletti *et al.*, 1995].

Human AMFR gene is located on chromosome 16; whereas in the mouse it is present on chromosome 8, with both the mRNA transcripts encode a protein of 643 amino acids in length [Chen *et al.*, 2006; Shimizu *et al.*, 1999]. The N-terminus of the protein forms five transmembrane domains [Ponting, 2000], while C-terminus cytoplasmic tail contains most of the functional domains of the protein, e.g., RING finger domain, which is essentially required for Gp78 E3 ubiquitin ligase activity, spans from 340 to 382 amino acids [Song *et al.*, 2005]. RING finger motif comprises two histidines at fourth and fifth positions of the motif, termed as RING-H2 finger domain [Fang *et al.*, 2003].

The presence of the C-terminal RING finger motif also gives another designation to this molecule as a RING finger protein 45 (RNF45) [Fairbank *et al.*, 2009; St-Pierre *et al.*, 2012]. Another important domain necessary for interaction with Ubc, E2 ubiquitin conjugating enzymes, is a coupling of ubiquitin conjugation to the ER degradation (CUE) domain, which is located towards C-terminus of the RING finger [Ponting, 2000; Song *et al.*, 2005]. The similarities observed by analysis of domains and functions of Gp78 with yeast E3 ubiquitin ligase Hrd1p and its cofactor Cue1p, suggest an evolutionary relationship between these two proteins [Chen *et al.*, 2012]. The hydrophobic segment of the cytosolic domain of Gp78 is one between the two oligomerization sites (OS) and form hetero-oligomer with its E2 conjugating enzyme [Li *et al.*, 2009]. Further structural analysis of C-terminus of AMFR protein identified another region,

called Ube2G2 binding region (G2BR), which comes into play for binding of AMFR withUbe2G2, an E2-conjugating enzyme [Chen *et al.*, 2006]. The interaction of Ube2G2: G2BR domain brings conformational alterations in E2 ubiquitin conjugating enzyme and increases the affinity of Ube2G2 for AMFR/Gp78 [Das *et al.*, 2009]. The study of Gp78 and its binding with E2 conjugating enzyme Ube2G2 confirms the two oligomerization sites, among which hydrophobic site is present towards the cytosolic domain of Gp78. The hetero-oligomer formed by Gp78 and Ube2G2 enables other Ube2G2 molecules to come closer, which provide easy transfer of ubiquitin molecules to nearby E2s that lead to active site-linked polyubiquitin chains [Li *et al.*, 2009]. Valosin-containing protein (VCP)-interacting motif (VIM) is the last domain, which is present on distant C-terminus of the protein and is crucially required for interaction of AMFR with AAA ATPase p97 enzyme, and its cofactor Ufd1-Np14, during ERAD [Ballar *et al.*, 2006]. Interestingly, a subsequent study demonstrated that Ufd1 (ubiquitin fusion degradation 1) might bind to AMFR without VCP [Cao *et al.*, 2007]. Figure 2.1 represents the structural overview of AMFR mRNA and protein with descriptive arrangements of its functional domains.

Fluorescence microscopic analysis revealed that apart from its presence over the cell surface, AMFR is also distributed throughout the cytoplasm in vesicular and tubular structures [Benlimame *et al.*, 1998]. Perinuclear and peripheral distribution of AMFR tubular structures was also observed through electron microscopy, which gets disrupted by the disruption of microtubular organization of the cells [Benlimame *et al.*, 1995]. Moloney sarcoma virus (mos)-transformed MDCK (MSVMDCK) cells have shown concentrated AMFR tubules at the pericentriolar region of microtubules, further confirming AMFR with cell motility-related functions [Nabi *et al.*, 1997]. Ilimaquinone, a drug known for its Golgi-vesiculation abilities, may also disrupt AMFR tubules; and the morphological similarities of fragmented tubules with smooth ER suggest that these tubules could be the subdomains of smooth ER [Wang *et al.*, 1997]. Electron microscopic studies revealed that a small fraction of AMFR along with its ligand AMF could also be localized in cell-surface caveolae, which are recycled between surface and ER by internalization and trafficking to ER membranes via clathrin-independent endocytic pathways [Benlimame *et al.*, 1998]. However, in later years, it has been found that AMFR may also be endocytosed through MVBs and are recycled even after microtubule disruption and inhibition of endocytosis, showing a possible mechanism of AMFR internalization in a clathrin-dependent manner [Le *et al.*, 2000].

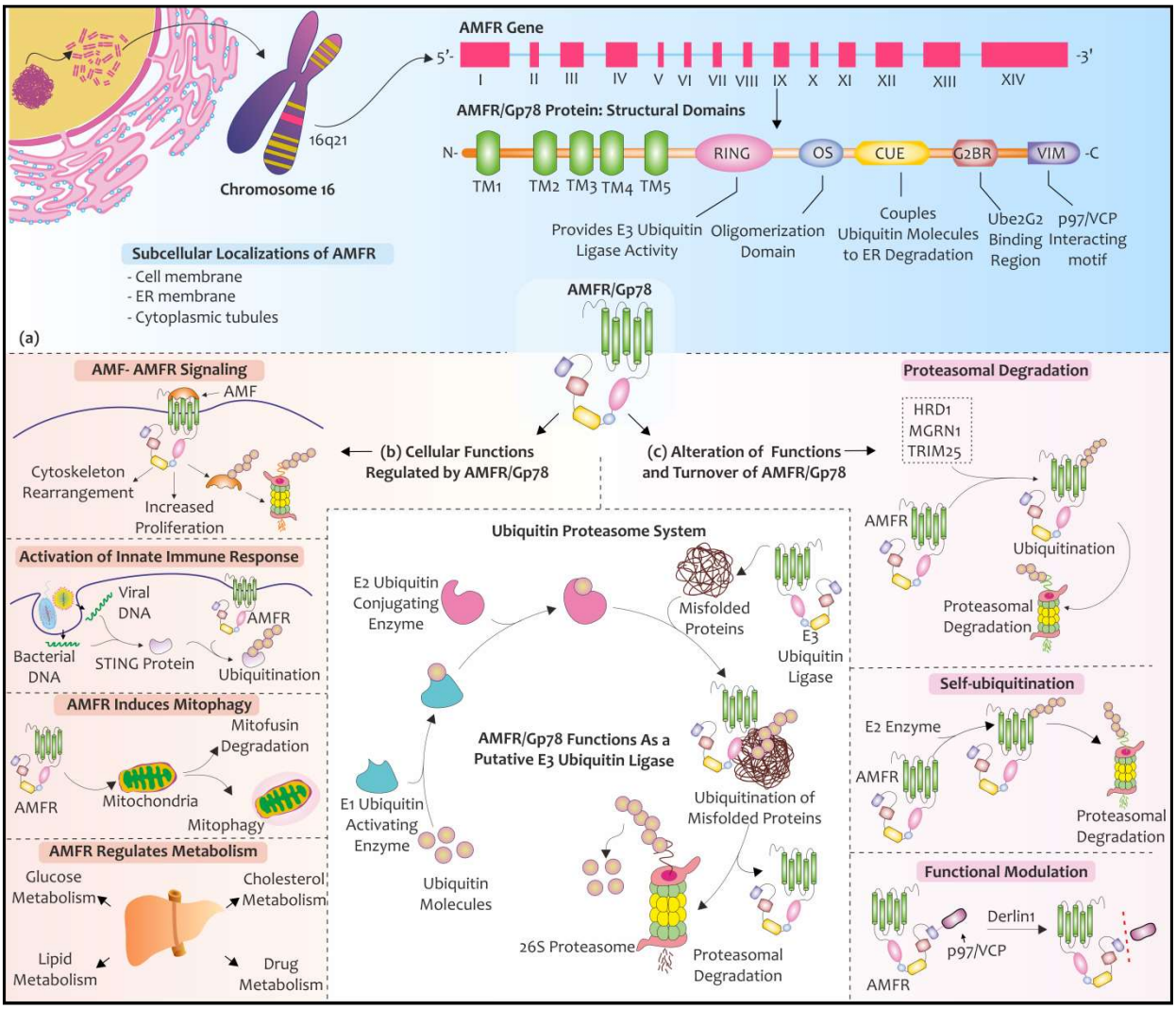

Figure 2.1 : *Overview of structure, functions and regulations of autocrine motility factor receptor (AMFR)/Glycoprotein 78 (Gp78):* Membrane-bound receptor, as well as really interesting new gene (RING) E3 ubiquitin ligase AMFR/Gp78, is encoded by AMFR gene, which is located at chromosome number 16. AMFR/Gp78 is known to participate in various cellular pathways and is itself regulated in multiple ways. (a) The mRNA coding for AMFR/Gp78 consists of fourteen exons [Tsai *et al.*, 2012], which form a mature protein with five transmembrane domains at N-terminus, followed by other functional domains, viz., RING, an oligomerization domain, Coupling of ubiquitin conjugation to the ER degradation (CUE), G2BR and Valosin-interacting motif (VIM). (b) Gp78 is involved in different cellular pathways, such as signaling, innate immune response, metabolism and induction of mitophagy. (c) The turnover of Gp78 is regulated either by E3 ubiquitin ligase activities of Hrd1, mahogunin ring finger 1 (MGRN1) and tripartite motif-containing protein 25 (TRIM25); or it could ubiquitinate itself in the presence of E2 conjugating enzymes. AMFR is functionally modulated by endoplasmic reticulum associated degradation (ERAD) E3 ubiquitin ligase Derlin 1. In the centre, it has been shown a general overview of ubiquitin proteasome system (UPS). Published as Joshi and Mishra et al., 2017.

2.2 IS Gp78 A PROMISING E3 UBIQUITIN LIGASE? OPTIMUM REGULATION OF DIFFERENT CELLULAR FUNCTIONS UNDER CRUCIAL CONDITIONS

The most studied and well-known function, for which AMFR is known, is its involvement in the motility and metastasis of different types of cancer cells [Liotta *et al.*, 1986; Nabi *et al.*, 1990; Watanabe *et al.*, 1991b]. The ligand-receptor binding of AMF-AMFR regulates multiple signaling processes and hence affects cell growth, motility and the programmed cell death apoptosis [Yanagawa *et al.*, 2004]. Overexpression of Gp78 is not only involved in progression or mobility of cancer cells, but it has also been found that in NIH3T3 cells, it

induces transformation; whereas, in nude mice, enhanced expression of this molecule produces tumor [Onishi *et al.*, 2003]. Recent studies have also explored the involvement of AMFR E3 ubiquitin ligase activity in mounting innate immune responses inside the cells by polyubiquitinating and modifying functions of the stimulator of interferon genes (STING), which senses the foreign genetic materials and responds by triggering the production of interferon proteins [Wang *et al.*, 2014a]. Selective mitochondrial degradation occurs inside the cells to remove old and defective mitochondria, and AMFR regulates this process by targeting mitochondrial proteins, mitofusins, for proteasomal degradation, inducing mitochondrial fragmentation [Fu *et al.*, 2013]. Purification and microsequencing of AMF demonstrated it as neuroleukin and enzyme phosphohexose isomerase, which catalyzes glucose 6-phosphate to fructose 6-phosphate isomerization, thus playing an indispensable role in glycolysis [Watanabe *et al.*, 1996].

Apart from glycolytic pathway, AMFR is also found to be implicated in the regulation of lipid and cholesterol metabolic pathways [Song *et al.*, 2005; Timar *et al.*, 1993; Wang *et al.*, 2012]. AMFR also facilitates the ubiquitination and degradation of cytochrome P450s of the 3A subfamily4 (CYP3A4), a major enzyme involved in drug metabolism, taking place inside the liver, and also has a crucial regulatory control over metabolism of drugs [Wang *et al.*, 2012]. Gp78 as an E3 ubiquitin ligase, mainly participate in the degradation of ERAD substrates, and it is well explored, once CD3-δ was established as its putative ERAD substrate protein [Fang *et al.*, 2001]. Apart from playing these crucial roles inside the cells (as shown in Figure 2.1), AMFR has also been reported for a plethora of roles and responsibilities in various pathways and disease. Cells regulate the level and functions of AMFR very precisely by multiple mechanisms so that a static level of AMFR could be maintained. The most important mechanism used for this is targeting of AMFR by other ER-resident E3 ubiquitin ligase Hrd1 or synoviolin, which ubiquitinates and degrades it in a proteasome-dependent manner [Shmueli *et al.*, 2009]. Similarly, investigation of other E3 ubiquitin ligases involved in ERAD, suggests that tripartite motif-containing protein 25 (TRIM25), which assists Gp78 in polyubiquitination of AMF, also participates in maintaining a steady-state level of Gp78 by its ubiquitination and degradation [Wang *et al.*, 2014b].

Recently, Gp78 was also identified as a substrate of mahogunin RING Finger 1 E3 ubiquitin ligase. Under normal cellular conditions MGRN1 ubiquitinates Gp78 at the K11 position and degrades it to regulate mitophagy; however, in mitochondrial stress condition, the cytosolic level of calcium increases, which interferes with the interaction of these two ligases [Mukherjee and Chakrabarti, 2016]. Self-ubiquitination is another interesting way of regulating cellular levels of protein, which is an interesting feature found in many RING finger-containing E3 ubiquitin ligases [Metzger *et al.*, 2014].

Gp78 has also shown similar RING-dependent self-ubiquitination, through binding with E2 enzymes Ube2G2 and Ubc7, via its G2BR domain, and transfering ubiquitin molecules with the assistance of CUE domain [Chen *et al.*, 2006; Fang *et al.*, 2001]. Another way to regulate the functionalities of this ER-resident E3 ubiquitin ligase is its Derlin1-mediated functional inhibition by uncoupling of p97/VCP and Gp78 [Ballar *et al.*, 2007]. A schematic overview of all these mechanisms, controlling the functions and turnover of AMFR has been represented in Figure 2.1.

2.3 ENDOPLASMIC RETICULUM ASSOCIATED DEGRADATION (ERAD) LINKED E3 UBIQUITIN LIGASE Gp78: A STRONG EARLY DEFENDER AGAINST ABERRANT PROTEINS ACCUMULATION

Cellular proteins reside in various compartments of the cells, at different stages of their lifespan. Several nascent polypeptides are subjected to the endoplasmic reticulum (ER) for their maturation and post-translational modifications [Braakman and Bulleid, 2011]. In doing so, they always remain prone to misfolding events [Hebert and Molinari, 2007]. Therefore, to avoid accumulation of such obnoxious non-functional proteins inside ER; membrane of ER is equipped with a complex of proteins, which is capable of translocating these aberrant proteins from ER lumen to the cytoplasm [Tsai *et al.*, 2002]. These excluded toxic elements are later degraded by cytoplasmic QC components, like 26S proteasome [Kopito, 1997]. A brief overview of ERAD components and the associated mechanism has been presented in Figure 2.2, to provide a better understanding of this pathway. Gp78, RING finger domain containing protein is located on ER membrane and has shown E3 ubiquitin ligase-like activity. Gp78 along with MmUBC7, an E2 conjugating enzyme, degrades CD3-δ specifically from the CD3 complex, the T-cell antigen receptor [Fang *et al.*, 2001]. As stated earlier, the process of ERAD involves retro-translocation of misfolded proteins from ER to the cytosol. To carry out this process, AMFR needs to associate with AAA ATPase p97/VCP by a unique VIM; however, siRNA-based knockdown studies suggested that Gp78 may also degrade its ERAD substrates by an Ufd1-independent pathway [Ballar *et al.*, 2006]. The presence of Gp78 at ER membrane allows it to work in complex with other components of ERAD pathway to identify and exclude misfolded proteins inside the ER lumen [Zhang *et al.*, 2015b]. Transfection studies on HepG2 cell and cell-free system confirm that the components of low-density lipoproteins (LDL) and very low-density lipoproteins (VLDL), apolipoprotein B, is ubiquitinated in a VCP-dependent manner by the ER-resident E3 ubiquitin ligase Gp78 and is degraded through proteasome [Fisher *et al.*, 2008; Liang *et al.*, 2003]. Gp78, apart from being an E3 ubiquitin ligase, may also exhibit E4 like activity, as has been found in ERAD of a mutant form of cystic fibrosis transmembrane conductance regulator (CFTRΔF508), where it recognizes already conjugated ubiquitin molecules to substrate protein mediated by another upstream E3 ubiquitin ligase Ram 1 homolog (RMA1) [Morito *et al.*, 2008; Vij *et al.*, 2006]. Hrd1 mediated ubiquitination of Gp78 causes inhibition of CFTRΔF508 degradation; similarly, gene silencing of Gp78 by RNAi or its inhibition by small p97/VCP interacting protein (SVIP) also results in accumulation of this mutant protein [Ballar *et al.*, 2010].

The α-1-antitrypsin, serine proteinase inhibitor that protects tissues from an attack of neutrophil elastase, generally have a Z mutation (Glu 342 Lys); and this mutant form is ubiquitinated by Gp78 in conjugation with a mammalian Ubc7 E2 enzyme, and translocated to the cytoplasm and degraded by the proteasome [Shen *et al.*, 2006]. Diacylglycerol acyltransferase isoform 2 (DGAT2), an enzyme involved in the synthesis of triacylglycerol, interacts directly with Gp78 for polyubiquitination and proteasomal degradation [Choi *et al.*, 2014]. The process of ERAD and stability of E3 ubiquitin ligases involved in this process is differentially regulated by the ER stress, as it increases the stability of Gp78, with no significant effect on the level or stability of Hrd1 [Shen *et al.*, 2007]. The interaction of AMFR and its ligand AMF also provides protection in ER stress condition, by regulating ER calcium release in the cytosol [Fu *et al.*, 2011]. ER stress-induced homocysteine-induced ER protein (HERP) is recently identified as proteasomal degradation substrate of Ube2g2–gp78-complex [Yan *et al.*, 2014]. The in vivo study, carried out in zebrafish, for Gp78 expression levels during ER stress, indicates its protective functions against ER stress in liver [Chen *et al.*, 2014]. Descriptive schematic of the roles of Gp78 in ERAD degradation pathways of various substrates is drawn in Figure 2.2.

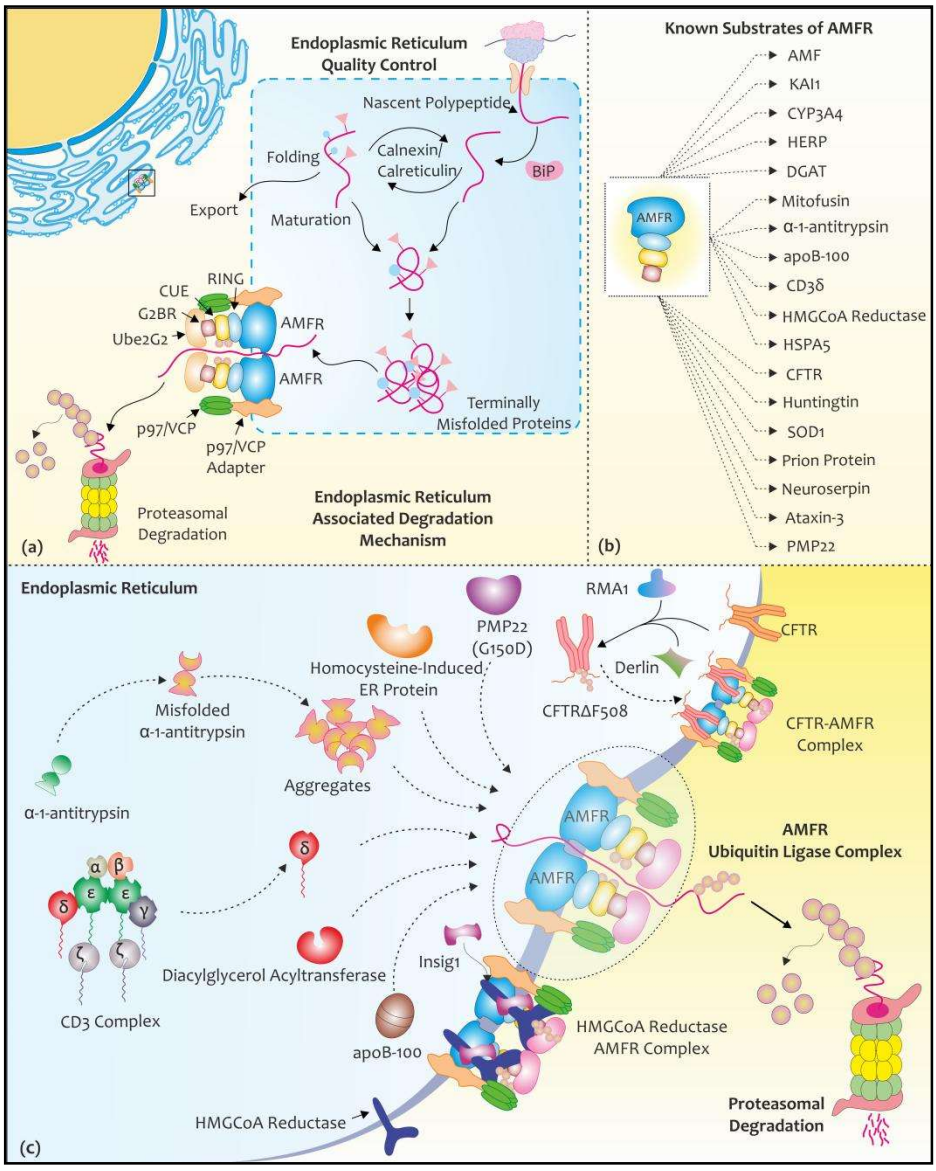

Figure 2.2 : *Gp78, as a vital E3 ubiquitin ligase of endoplasmic reticulum-associated degradation:* AMFR/Gp78 is a crucial component of ERAD, the special protein quality control (PQC) system of the endoplasmic reticulum. (a) The figure represents a basic mechanism of protein folding, misfolding and targeting of these misfolded proteins for proteasomal degradation from the lumen of endoplasmic reticulum (ER), with the aid of specific ER chaperones and E3 ubiquitin ligases like Gp78. (b) Summary of substrates regulated by Gp78 E3 ubiquitin ligase function. (c) A mechanistic overview of how Gp78 mediates retro-translocation of various ER proteins, like mutated CFTR, CD3-δ, etc., for their proteasomal degradation, to maintain the ER proteostasis and reduce the state of ER stress. Published as Joshi and Mishra et al., 2017.

2.4 NEUROBIOLOGICAL FUNCTIONS OF Gp78: IMPAIRMENT MAY LEAD TO PROTEIN AGGREGATION AND NEURODEGENERATION

E3 ubiquitin ligases play an important role in overall development and maintenance of a healthy set of neurons throughout life, since early neonatal periods and up to the late onset neurodegenerative changes taking place in our brain [Upadhyay *et al.*, 2017]. In recent years, few studies have been done to investigate the roles of Gp78 in the development of the brain. The study performed on rat cerebellum spotted higher expression level of AMFR at postnatal state in comparison with an adult, showing the probable role of AMFR in granule cells migration; however, localization study showed AMFR expression in neurites, cell body and growth cones of neurons to regulate neuroleukin activities [Leclerc *et al.*, 2000]. A novel role of AMFR has also been postulated in strengthening the learning and establishing memory, as

hypothesized by increased expression of the AMFR in hippocampus region that is also affirmed by conducting several tests on rats and mice [Luo *et al.*, 2002; Yang *et al.*, 2012].

There are very limited number of studies, which have been done to establish a direct link between Gp78 and neurodevelopment. Still, many groups have shown its involvement in neuroprotection against various stresses generated by inclusions formed of several disease-associated proteins. The E3 ubiquitin ligase activity of AMFR has given it a considerable importance in recent past for its implication in a number of neurodegenerative diseases. The involvement of Gp78 in neuroprotection came into existence with studies based on disease-associated aggregatory proteins superoxide dismutase-1 (SOD1) and ataxin-3, which are targeted by Gp78 for ubiquitination and proteasomal degradation [Ying *et al.*, 2009]. Another study shows that expanded polyglutamine containing huntingtin protein interacts with CUE domain of Gp78 and this interaction interferes with the interaction of Gp78 and ER chaperones, causing increased ER stress. However, Gp78 ameliorates such obnoxious condition by ubiquitinating and degrading the mutant huntingtin through ERAD [Yang *et al.*, 2010]. The neurodegenerative disorder familial encephalopathy occurs due to inclusion body formation by secretary glycoprotein neuroserpin, mainly in ER of neurons [Miranda *et al.*, 2004]. Hrd1 and Gp78 were identified as ERAD E3 ubiquitin ligases, which polyubiquitinate mutated forms of neuroserpin and target them for degradation in the association of VCP to abrogate toxicity, generated by their aggregates inside cortical and sub-cortical neuronal population [Ying *et al.*, 2011].

Bovine spongiform encephalopathy and Creutzfeltd-Jacob diseases are other forms of neurodegenerative diseases, which are caused by a special class of proteins, called prions (PrP) [Prusiner, 1991]. Human prion protein could be present in multiple forms inside the cells, and unglycosylated forms of PrP has a critical association with PrP aggregates [Taraboulos *et al.*, 1990]. ER-resident Gp78 specifically interacts with C-terminal region of unglycosylated prion proteins, and ubiquitinate them for their degradation in proteasome-dependent manner [Shao *et al.*, 2014]. Mutation in the peripheral myelin protein 22 (PMP22) and its accumulation in endoplasmic reticulum gives rise to Charcot-Marie-Tooth (CMT) disease, a common peripheral nervous system disorder [Roa *et al.*, 1993]. Among different mutant forms of this protein, Gp78 degrades disease-causing mutated form PMP22 (G150D) via proteasomal pathway [Hara *et al.*, 2014]. A recent study reported that Cyclin-dependent kinase 5 mediated phosphorylation of Gp78 causes its ubiquitination and degradation, which results in increased rate of neuronal death in animal models of Parkinson's disease [Wang *et al.*, 2017b].

Another novel example of Gp78 involvement in neuroprotection is its association with cholesterol homeostasis, which suggests the probable role of Gp78 in slowing down neurodegeneration by maintaining cholesterol metabolism via its well-known ERAD substrate HMG-CoA reductase [Anchisi *et al.*, 2013; Cao *et al.*, 2007; Zhang *et al.*, 2015b]. All these functions of Gp78 in degradation of misfolded forms of different disease-associated proteins and clearance of their inclusion bodies designate this glycoprotein molecule as a QC E3 ubiquitin ligase. Since the identification of RING domain in Gp78 protein and its implication in clearance of multiple target proteins, which crucially regulate several important cellular pathways, interest has generated in finding out other QC roles of Gp78 in amelioration of toxicities generated by aggregation and formation of inclusion bodies by various proteins, associated with diseases. Further work is needed to explore more about its capability to degrade other such proteins.

2.5 THE MULTIPLE ROLES OF Gp78 IN CELLULAR PROLIFERATION: NOVEL INSIGHTS INTO COMPLEX DISEASES

Discovery, purification, characterization, functional aspect and pathological mechanisms of Gp78 are linked with various types of cancers [Chiu *et al.*, 2008]. As it has been mentioned above, the protein Gp78 was first identified as surface receptor influencing the metastatic ability of B16-F1 melanoma cells [Nabi and Raz, 1987]; still, detailed characterization of its implications in affected pathways and mechanisms in melanoma, as well as different other cancer types, is yet to be accomplished. Purification of acidic and basic AMF from murine protein-free fibrosarcoma reported that metastatic properties of these cells could be affected via AMF-Gp78 signaling [Watanabe *et al*., 1994]. Expression of Gp78 is highly upregulated in bladder carcinoma tissues [Silletti *et al*., 1993]; whereas, in patients with colorectal cancer, immunohistochemical analysis proposed that patients with higher expression of Gp78 have less survival and high risk of cancer recurrence [Nakamori *et al.*, 1994]. In prostate cancer cells derived from non-metastatic nude mice (PC-3) and its metastatic variant (PC-3M) mice, expression of Gp78 is differentially upregulated in metastatic conditions [Silletti *et al.*, 1995]. The results were again confirmed in a different study based on patients of prostate cancer [Shang and Zhu, 2013].

Esophageal squamous cell carcinoma patients were examined for Gp78 expression and its association with different tumor characteristics; such as, size, growth, invasion and metastasis and again it was found that patients with higher expression of Gp78 have increased risk of cancer with lower survival rate [Maruyama *et al.*, 1995]. In patients with transitional cell carcinoma of the bladder, urine samples were tested for AMFR, and 80% of samples were found positive [Korman *et al.*, 1996]. Similarly, an upregulated expression of AMFR was reported in cutaneous malignant melanoma [Nagai *et al.*, 1996]. All these studies, done by several groups in different cancer types over the years, confirm the elevated expression of AMFR and point towards its possible association with motility and metastasis of cancer cells [Silletti and Raz, 1996]. Choriocarcinoma, a cancer of developmental tissues and tissues from oral cell carcinoma further confirmed the association of AMFR with invasiveness and metastasis potential [Niinaka *et al*., 1996; Yelian *et al*., 1996]. In gastric cancer patients, expression of AMFR reflected poor prognosis and showed a direct correlation with histopathological grades of the tumor [Hirono *et al*., 1996; Taniguchi *et al*., 1998]. Expression of Gp78 is also regulated by cell-cell contact under normal conditions, and loss of such a relation is observed during tumor progression [Silletti *et al*., 1995].

A reciprocal relationship between expressions of E-cadherin, a cell adhesion protein and Gp78 has been observed in tissues from bladder carcinomas [Otto *et al*., 1994], which was later confirmed in another study, where MSV transformed MDCK cell population has been found with lowered E-cadherin and upregulated Gp78 protein expression levels [Simard and Nabi, 1996]. This altered E-cadherin/Gp78 ratio could have lethal consequences, as has been reported in patients with bladder carcinomas and gastric cancers, in different studies [Kawanishi *et al.*, 2000; Otto *et al.*, 1997]. In lung cancer and thymoma tissues, higher expression of AMFR elevates the risk of tumor progression [Ohta *et al*., 2000]. Higher expression of vascular endothelial growth factor (VEGF) and increased AMFR worsen the disease conditions in patients with non-small cell lung cancer [Kara *et al*., 2001; Takanami *et al*., 2001]. Similar results were seen in other studies also, where high expression of AMFR was found to be implicated in mediating invasion of lung and oral squamous cell carcinomas and promoting their metastatic capabilities [Niinaka *et al*., 2002; Takanami and Takeuchi, 2003; Takanami *et al*., 2002].

A positive association between AMFR and metastasis was observed in melanoma cells also [Timar *et al*., 2002]; whereas, in pulmonary adenocarcinoma patients, AMFR positive subjects have shown a lower post-surgery survival rate, as compared to those having no

significant AMFR expression [Kaynak *et al.*, 2005]. Prognostic role of AMF-AMFR complex expression was also identified in human breast cancer by comparative study of breast cancer and non-neoplastic tissues [Jiang *et al.*, 2006]. In tongue squamous cell carcinoma and hepatocellular carcinoma (HCC) patients, higher expressions of AMFR, along with Ras homolog family member C (RhoC) and c-met coincides with increased risk of invasion and disease recurrence with low survival [Endo *et al.*, 2006; Wang *et al.*, 2007].

Small interfering RNA (siRNA) mediated knockdown and truncated AMFR expression resulted in a decrease in levels of rho-associated coiled-coil containing Protein kinase 2 (ROCK2), Cyclin D1 and B-cell lymphoma 2 (Bcl-2), suggesting a possible mechanism, by which AMFR regulates cell cycle and apoptotic pathways [Wang *et al.*, 2015]. Despite several studies indicating the correlation between Gp78 with metastasis in various cancers, the mechanism of how AMFR aids in metastasis was revealed later with identification of Gp78 mediated degradation of metastasis suppressor protein Kangai1 (KAI1), which results in induction of metastasis potential of different cancer cell lines, as well as Gp78-overexpressing transgenic mice [Joshi *et al.*, 2010; Tsai *et al.*, 2007]. Identification of another mechanism shed more light on the involvement of Gp78 in metastasis and cell proliferation, where it activates ROCK-2, an important metastasis-associated protein [Wang *et al.*, 2010]. Figure 2.3 summarizes these mechanistic findings, explaining the possible molecules and pathways affected by Gp78, postulating its involvement in transformation, development and progression of tumors.

Contrary to all the above findings, few studies have postulated an inverse correlation between AMFR/Gp78 and tumor progression; for example, microarray expression analysis of bone tumors showed AMFR among downregulated genes in giant cell tumor [Guenther *et al.*, 2005]. A recent study on Gp78 null mice showed age-related nonalcoholic steatohepatitis (NASH) and development of HCC proposing the roles of Gp78 in the maintenance of liver homeostasis [Zhang *et al.*, 2015a]. Deacetylation of heat-shock protein 5 (HSPA5) by histone deacetylase-6 (HDAC-6) is followed by Gp78-mediated ubiquitination of HSPA5, leading to suppression of invasion and migration in breast cancer cells [Chang *et al.*, 2015c]. The roles of Gp78 in metastasis and tumor-progression is yet to be fully understood; therefore further research is needed to make a detailed understanding of the molecule so that in future it could be used as a prognostic biomarker of different cancer types and might be exploited for therapeutic purposes.

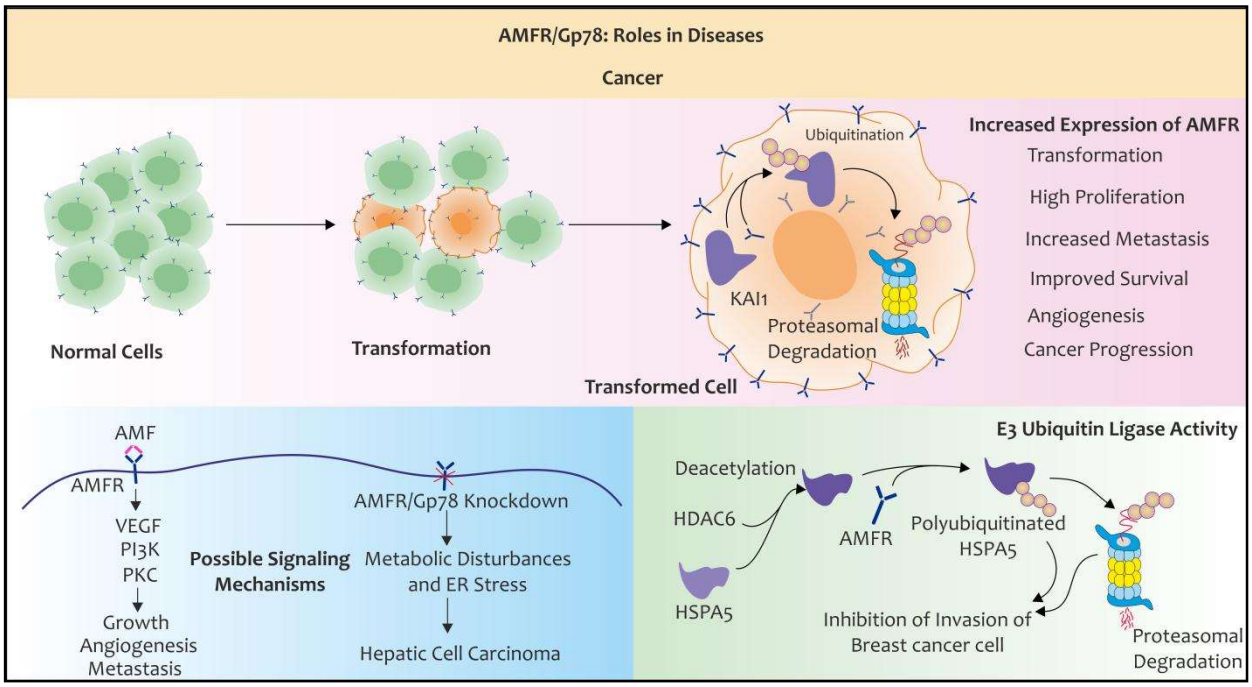

Figure 2.3 : *Functional implications of Gp78 in cancer: Miscellaneous association of Gp78 was observed in cancer:* Gp78 regulates transformation, invasion, and metastasis of tumor cells. Contrary to this, it suppresses invasion of breast cancer cells by degrading heat-shock protein 5 (HSPA5), which is deacetylated by histone deacetylase-6 (HDAC6). The Gp78 knockdown mice developed hepatic cell carcinoma because of disturbed metabolism, which also proposes tumor suppressor role of Gp78. Published as Joshi and Mishra et al., 2017.

2.6 HIDDEN PROMISING THERAPEUTIC INTERVENTIONS OF Gp78 IN PROTEIN CONFORMATIONAL DISORDERS

Overexpression of AMFR has shown important roles in overall cancer progression, which has been supported by several experimental methods and studies. Based on the reliabilities of these studies, scientists in recent past started proposing AMFR as a prognostic biomarker of tumor development and advanced stages of disease progression, as shown in Figure 2.4. Although enough data have accumulated, still acquiring complete knowledge about understanding the overall functional aspects of this protein is underway. Several attempts have also started to modulate the expression or function of AMFR inside the cells to exploit its therapeutic potential in various diseases. Natural compounds, like beta-all-trans-retinoic acid (RA), have been studied to decrease the levels of Gp78 in murine and human melanoma cell lines and to suppress the cell motility [Hendrix *et al.*, 1990; Lotan *et al.*, 1992].

Post-transcriptional suppression strategies, e.g., using micro RNAs (miRNAs), were also developed against AMFR to suppress invasion and metastasis. miR-139-5p in colorectal cancer cells and edited miR-376a* in glioblastoma target AMFR showing different effects; while the edited form of miR-376a* leads to increase in AMFR expression, miR-139-5p suppresses metastasis by downregulating AMFR [Choudhury *et al.*, 2012; Song *et al.*, 2014]. The tumor cell-specific drug delivery system was developed by conjugation of AMF/PGI paclitaxel, which get internalized by the raft-dependent endocytic pathway and hence it could be developed as a new therapeutic approach, where AMF acts as a carrier for chemotherapeutic drugs in tumor cells, expressing AMFR under in vitro and in vivo conditions [Kojic *et al.*, 2008].

Blocking the AMF/AMFR signaling pathway could be of significant therapeutic importance, as this may result in downregulation of metastatic abilities of cancer cells [Iizumi

et al., 2008]. Using the similar approach, siRNA-mediated downregulation of AMF leads to decreased ability to form tumor mass in human lung fibrosarcoma cells, which might be due to the overall suppression of AMF-mediated signaling [Funasaka *et al.*, 2007]. Based on PCR array analysis, another study on Varicella zoster virus (VCZ) infected HeLa cells reported increased mRNA levels of AMFR, Insig and BiP, along with upregulated autophagy, whereas several ERAD-associated components were significantly downregulated causing ER stress and unfolded protein response (UPR) inside the cells. Despite the increase in expressions of AMFR and BiP, upregulated UPR and autophagy, and an increase in ER size draw an elusive line between all these components and pathways, which further need to be explored [Carpenter and Grose, 2014].

These are the various available therapeutic approaches, which have been tested over the years in the context of medicinal properties of the gene AMFR and its product Gp78 which are chiefly targeted in therapeutics of various cancers. These findings have been represented and their outcomes in Figure 2.4 for a better understanding. Like molecular chaperones, QC E3 ubiquitin ligases also have the roles of surveilling the misfolded or accumulated forms of proteins and degrade them through UPS or autophagy [Chhangani *et al.*, 2012]. AMFR is a promising receptor molecule with its QC E3 ubiquitin ligase like abilities to sense cellular stresses and mediate appropriate cellular responses to counter the obnoxious changes in cellular proteins [Fang *et al.*, 2001; Shen *et al.*, 2006]. As many of Gp78 substrates are components of proteinaceous aggregates, involved in neurodegenerative diseases; it could, therefore, be targeted for therapeutic applications in these diseases [Yang *et al.*, 2010; Ying *et al.*, 2009]. Other than its association with protein misfolding-related diseases, it is clinically important for metabolic disorders, as it also maintains homeostatic conditions inside the cell [Zhang *et al.*, 2015b].

Despite so many studies on the association of Gp78 with cancer, neurodegeneration and metabolic disorders, the major challenge for researchers and clinicians remains the formulation of a successful therapeutic strategy targeting this gene. Hence, there arises a need to explore Gp78 for its pharmacological significance and drug development in future. A delicate balance of these components of PQC machinery is required for a cell to maintain a homeostatic condition. While a slight imbalance may result in several kinds of obnoxious intracellular changes, leading to unwanted disease conditions. Further exploration of functional aspects of QC E3 ubiquitin ligase Gp78 will benefit us in future therapeutic applications of this molecule, which is an indispensable part of multiple cellular pathways.

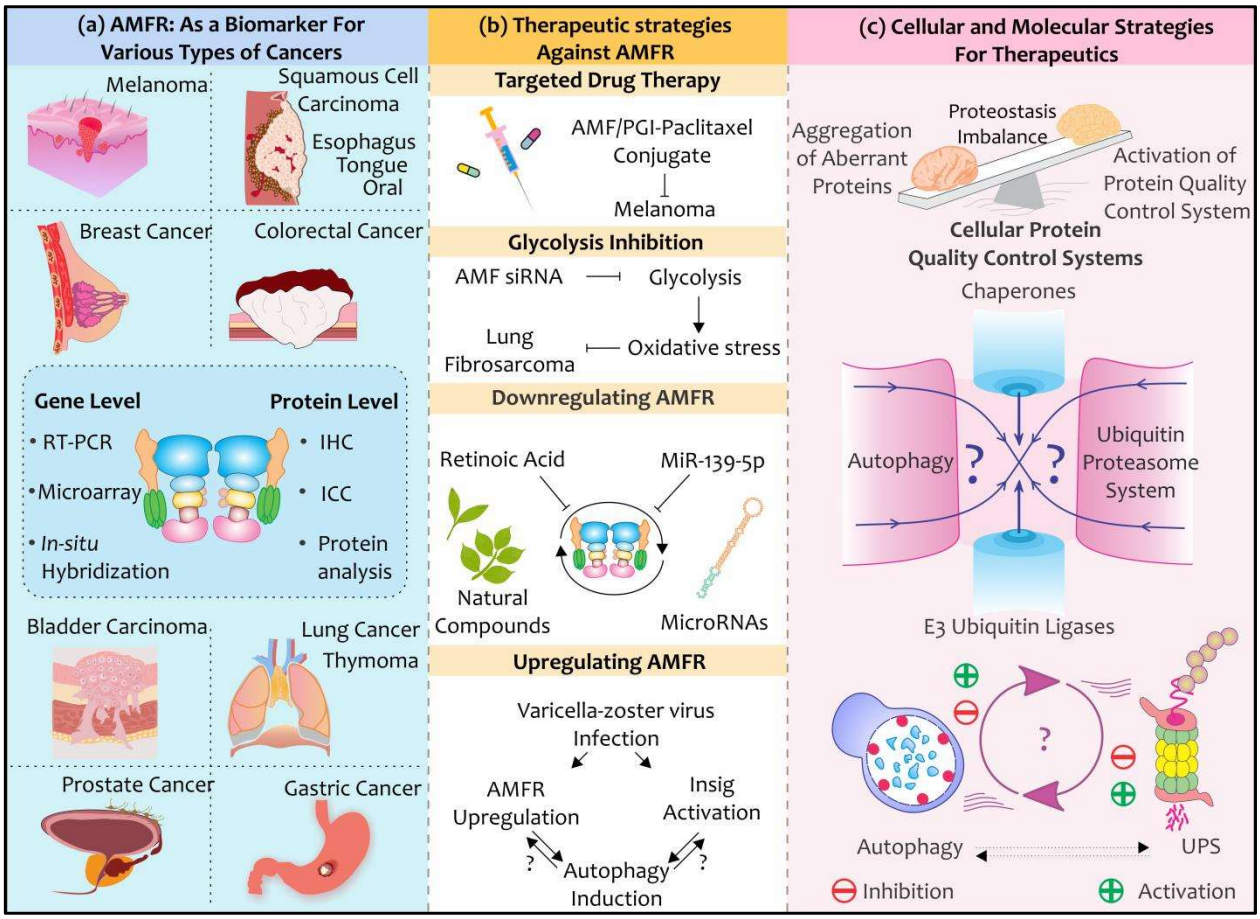

Figure 2.4 : *Targeting AMFR/Gp78 for remedial exploration of various maladies:* Schematic representation of various approaches developed to utilize multifaceted molecule Gp78 for therapeutic purposes. (a) Multiple studies on several cancer types have shown an upregulated expression level of Gp78 proposing it as a crucial molecule to be targeted for further research. (b) Few microRNAs (miRNAs) and natural compounds have been used to downregulate Gp78, having a notable delay in metastasis. Targeted therapy and upregulation strategies were also proposed for Gp78 and its ligand autocrine motility factor (AMF). (c) Still, targeting a molecule like Gp78, which is known to be involved in multiple pathways, to maintain proteostasis is a delicate function to be accomplished and it further, requires a detailed knowledge of its functional aspects. Published as Joshi and Mishra et al., 2017.

2.7 FLAVONOIDS, NATURAL PLANT PRODUCTS WITH MULTIPLE BENEFITS

Flavonoids are the products of plant secondary metabolism, mainly involved in the pigmentation of flowers, fruits, and seed [Bohm, 1998]. In the year 1664, Robert Boyle found the plant pigment components flavonoids [Haslam, 1975]. The presence of flavonoid in paprika and citrus peel was reported by Dr. Albert Szent-Gyorgyi and co-workers, which gives a new direction to research on bioflavonoids [Szent-Gyorgyi, 1955]. Flavonoids have C_6-C_3-C_6 carbon framework or phenylbenzopyran structure. On the basis of linkage between C ring attached with B ring, the level of unsaturation and C ring oxidation, flavonoids are divided into six subclasses [Grotewold, 2006]. The subclasses of flavonoids are flavones, flavonols, flavanones, flavanonols, anthocyanidines, isoflavones [Takano-Ishikawa *et al.*, 2006].

These polyphenolic compounds with benzo-γ-pyrone structure are synthesized via phenyl propanoid pathway [Wagner and Farkas, 1975; Winkel-Shirley, 2001]. Enzymes control the whole process of flavonoid biosynthesis [Hahlbrock and Grisebach, 1979]. Dietary sources of flavonoids are different types of fruits, vegetables, spices, nuts, and seeds; these flavonoids function as a modulator of enzymatic pathways and antioxidant [Yao *et al.*, 2004]. However, the amount and subclass of flavonoid vary in different natural dietary resources but the health

benefit remains the same as it prevents occurrence of inflammation, heart diseases and cancer [Kozlowska and Szostak-Wegierek, 2014; Middleton *et al.*, 2000].

2.8 MYRICETIN: STRUCTURE, FUNCTIONS AND FUTURE THERAPEUTIC PROSPECTS

Myricetin is a natural molecule, derived from plants and has a pharmaceutical importance as it is known to provide protection against various neurodegenerative disorders, cancer, and diabetes [Semwal *et al.*, 2016]. Structural analysis of Myricetin reported it as 3,5,7,3′,4′,5′-hexahydroxyflavone cannabiscetin, a natural flavonol obtained from edible fruits, vegetables, berries and red wine [Li and Ding, 2012]. Myricetin term was used for yellow crystals obtained from the bark of *Myrica nagi* that belongs to plant family Myricaceae and was proposed as yellow dye stuff [Perkin and Hummel, 1896]. It was synthesized in 1925 from ω-methoxyphloroacetophenone by Kalff and Robinson [Kalff and Robinson, 1925]. A good concentration of Myricetin is obtained from edible berries, such as cranberry, crowberry, etc. and edible tropical plants, like garlic, black tea, broccoli and few more [Miean and Mohamed, 2001; Winkel-Shirley, 2001].

Flavonoid like Myricetin have diverse cellular functions, among which antimicrobial activity was first identified in year 1986 [El-Gammal and Mansour, 1986]. It is also explored as antioxidant, prooxidant, anticarcinogen, mutagen, antiviral, antidiabetic and inducer of DNA degradation [Ong and Khoo, 1997]. The anitoxidant and prooxidant activity of Myricetin was observed in presence and absence of ascorbic acid; iron chelation and reactive oxygen scavenging properties make it as antioxidant [Chobot and Hadacek, 2011]. Due to involvement in multiple pathways, Myricetin is discovered as therapeutic target for prevalent diseases like diabetes mellitus [Li and Ding, 2012]. It interacts with multiple oncoproteins, such as protein kinase B, Janus kinase–signal transducer and activator of transcription 3 (JAK1-STAT3) and prevents transformation of cancer cells; it also shows antimitotic effect and targets mitochondria for cell death, hence considered as an anticancer molecule [Devi *et al.*, 2015].

Oxidative stress is the main cause for many cerebrovascular and neurodegenerative disorders. Natural antioxidants like flavonoids (quercetin, Myricetin, etc.) provides neuroprotection from number of brain diseases [Dajas *et al.*, 2003]. The roles of Myricetin in rotenone induced neurotoxicity as well as in Parkinson's associated apoptotic processes were explored and found to have wide variations [Molina-Jimenez *et al.*, 2004; Molina-Jimenez *et al.*, 2003]. The oxidative stress generated by free radicals give rise to pro-oxidants, which helps in neurodegenerative diseases progression; whereas dietary antioxidants, like Myricetin reduce this oxidative stress and hence are proposed for therapeutics of neurodegenerative disease [Singh *et al.*, 2004]. The common neuropathologies, like Parkinson's and Alzheimer's disease are associated with mutated tau protein, which forms aggregates on hyperphosphorylation and generate disease condition [Selkoe, 2004].

The major hallmark of Alzheimer's disease is deposition of amyloid β-peptide (Ab) in cerebral region, hence, destabilizing ability and anti-amyloidogenic property of polyphenols including Myricetin has been used for therapeutic implications in Alzheimer's disease [Ono *et al.*, 2003]. An inhibitory effect of Myricetin was also observed on tau filaments assembly by binding with filamentous, soluble and aged tau, in comparison with monomeric units [Taniguchi *et al.*, 2005]. Myricetin have crucial neuroprotective role in Alzheimer's diseases and ischemia as it reduces neuronal cell death by affecting three pathways, (i) phosphorylation of N-methyl-D-aspartate (NMDA) receptor (NMDAR) hence reduced Ca^{2+} overload, (ii) inhibition of reactive oxygen species production by glutamate, and (iii) interaction of Myricetin with caspase-3 thereby inhibiting its activity [Shimmyo *et al.*, 2008]. The *Rosa damascene* obtained Myricetin provide neuroprotection by two pathways, one is activation of α-secretase and inhibition of β-secretase to prevent its binding with amyloid precursor protein which reduce amyloid-β formation and other by direct binding with amyloid-β to alleviate cytotoxicity

associated with Alzheimer's disease [Esfandiary *et al.*, 2015]. Myricetin was also reported to perform dual functions in human neuroblastoma cells, first metal chelation and second interaction with amyloid-β, which reduces neurotoxicity generated by aggregation of metal-induced amyloid-β [DeToma *et al.*, 2011].

Different brain areas of Alzheimer's disease were investigated for effects of Myricetin and it was observed that number of hippocampal Cornu Ammonis 3 pyramidal neurons increases after Myricetin treatment to provide improvement in memory and learning processes [Ramezani *et al.*, 2016]. Similar effects were also observed in mice deficient in learning and memory due to stress and it was found that it also decreases adenocorticotrophic hormone and improves memory functions in these mice [Wang *et al.*, 2016]. Further, neuroprotective mechanism of Myricetin was investigated and it was shown that in cerebral ischemia rat models as it activates NEF-2 related factor (Nrf2) to provide protection from brain injuries and multiple kinds of cerebral deficiencies to improve neurological functions [Wu *et al.*, 2016]. Investigation of links between Myricetin and AD reported inhibition of acetylcholinestrase, which down regulate level of brain iron to provide neuroprotection in AD [Wang *et al.*, 2017a].

The rat models of Parkinson's disease having degeneration in dopaminergic neurons by exposure of neurotoxin 6-hydroxydopamine (6-OHDA) were also examined with Myricetin and reported to reduce cell death, as Myricetin restored dopamine content in striatum [Ma *et al.*, 2007]. Molecular dynamic studies reported binding of Myricetin with tau oligomers that shows its putative therapeutic application in Parkinson's disease [Berhanu and Masunov, 2015]. Further, various studies explored other Myricetin associated pathways, such as Zang et. al. observed 1-methyl-4-phenylpyridinium (MPP+)-treated mouse-rat hybrid cells develops Parkinson's disease. This effect of MPP+ attenuated by the treatment of antioxidant Myricetin, and also causes decreased phosphorylation of mitogen-activated protein kinase (MAPK) kinase 4 (MKK4) and c-Jun N-terminal kinase (JNK) [Zhang *et al.*, 2011]. Another study reported neuroprotective role of Myricetin against hydroxyl radicals and DNA damage caused by peroxynitrite in various neurodegenerative disorders [Chen *et al.*, 2011]. Hydroxyl groups in the B-ring of Myricetin plays a crucial role in neuroprotection by reducing oxidative stress and also increase Na^+K^+-ATPase, which provides protection from cognitive impairment caused by D-galactose [Lei *et al.*, 2012]. Potential clinical application was proposed for *Ampelopsis grossedentata* derived Myricetin to treat dementia in an effective manner [He *et al.*, 2014]. As amyloid formation is common hallmark of many neurodegenerative diseases, Myricetin was studied to prevent amyloid fibril formation in hen egg white lysozyme by binding β-domain, the aggregation prone region, hence suggested as neuroprotective flavonol [He *et al.*, 2014].

The study of Myricetin administration on mice undergoing repeated restraint stress showed that it helps in restoring normal level of brain derived neurotrophic factor, which represents positive effects to overcome depressant like behaviour [Berhanu and Masunov, 2015]. Release of neurotransmitter glutamate regulates multiple functions like synaptic plasticity, memory and learning. However, its excess release at cerebrocortical nerve terminals increases the Ca^{2+}, and gives rise to critical neuropathological conditions. Flavonoid Myricetin blocks the calcium channels and thus inhibits the excess release of glutamate to provide neuroprotection [Chang *et al.*, 2015a]. All these associations of Myricetin with various proteins signifies its in multiple biological activities, like neuroprotection, anti-inflammatory, anti-oxidative and anti infectious activity [Park *et al.*, 2016]. Despite so many known neuroprotective effects of Myricetin, underlying mechanisms behind their action are not explained completely, yet in comprehensive manner; therefore further studies are needed so that a detailed characterization of the compound can be done.