EUROPEAN JOURNAL OF BIOMEDICAL AND PHARMACEUTICAL SCIENCES
http://www.ejbps.com

ISSN 2349-8870
Volume: 4
Issue: 4
206-215
Year: 2017

THE ROLE OF ENDOPLASMIC RETICULUM STRESS IN TRIPLE NEGATIVE BREAST CANCER

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Article Received on 23/01/2017 **Article Revised on 13/02/2017** **Article Accepted on 05/03/2017**

ABSTRACT
Triple Negative Breast cancer (TNBC) is a clinically aggressive subgroup of breast cancers characterized by lack of Estrogen receptors (ER), Progesterone receptors (PR) and Human epidermal growth factor receptor2 (HER2) receptors. TNBC is associated with poor prognosis owing to lack of defined molecular targets and resistance to conventional drug therapies. Recently, Endoplasmic Reticulum (ER) stress leading to Unfolded Protein Response (UPR) is regarded as an upcoming strategy against solid malignancies like TNBC for seeking defined molecular targets. The role of ER stress has been fairly established in TNBC malignancy in the past few years through cross-talks by ER stress elements for promoting tumor incidence and neo-angiogenesis. A major complexity still deals with the tumor recurrence and drug resistance of TNBC which may be related with tumor dormancy and Epithelial to mesenchymal transitions (EMT) leading to stem cell-like properties owing to ER stress elements. This review therefore aims to bring forth the role of ER stress in understanding future research prospects and therapeutic approaches against TNBC.

KEYWORDS: Breast Cancer, TNBC, ER Stress.

UNFOLDED PROTEIN RESPONSE
Accumulation of unfolded or misfolded proteins inside the Endoplasmic Reticulum(ER) lumen leads to stress conditions which can be extrinsic owing to hypoxic and Nutritional stress or intrinsic due to oncogenic response activation and chromosomal alterations.[1] The concept of ER stress to ensure proper protein folding and maintain an unique environment to establish a balance of ER protein load capacity initiates a series of adaptive mechanisms collectively termed as Unfolded Protein Response (UPR).[2] Activation of the UPR affects the secretory pathway responses leading to cell survival whereas UPR signaling can also result in cell death by apoptosis if the stress response exceeds a threshold.[3]

There are three classes of mammalian ER stress receptors: inositol-requiring enzyme 1α (IRE1α and IRE1β), protein kinase RNA-like ER kinase (PERK) and activating transcription factor 6 (ATF6α and ATF6β).[2] These transmembrane receptors are activated upon dissociation of molecular chaperones Binding immunoglobulin protein (Bip) from the N-terminal of these receptors, upon sensing unfolded or misfolded proteins.[4] (**Fig.1**).

Activation of IRE1α involves its dimerization and *trans*-autophosphorylation, which leads to splicing of X-box binding protein (XBP1) mRNA leading to the expression of a more active form known as XBP1s (spliced).[5] XBP1s translationally activates a subset of target genes that are involved in ER-associated protein degradation (ERAD) and protein secretion.[6] High levels of chronic ER stress can lead to the recruitment of TNF-receptor-associated factor 2 (TRAF2) by IRE1 and the activation of apoptosis-signaling-kinase 1 (ASK1).[7,8] Activated ASK1 activates c-Jun N-terminal protein kinase (JNK), which in turn plays a role in apoptosis by regulating the BCL2 family of proteins.[9]

PERK, upon activation, phosphorylates ser51 of eukaryotic translation initiator factor 2α (eIF2α)[10], which prevents formation of ribosomal initiation complexes leading to inhibition of protein synthesis. This mechanism reduces the number of proteins entering ER, and thus imparts an important pro-survival effect on the cell. Phosphorylated eIF2α also allows the translation of Activating Transcription Factor4 (ATF4) mRNAs with short open reading frames in 5′untranslated regions. ATF4 is a b-ZIP transcription factor that regulates several UPR target genes including those involved in ER stress mediated apoptosis such as C/EBP homologous protein (CHOP) and Growth arrest and DNA damage-inducible 34 (GADD34). GADD34 uses a feedback loop to de-phosphorylate eIF2α and restore translational machinery by interacting with protein phosphatase 1C (PP1C).[11]

Unlike activation of IRE1α and PERK receptors, ATF6α translocates to the Golgi complex where it is cleaved by S1P and S2P proteases[12] and releases a cytosolic fragment of ATF6[13] which acts as a transcription factor for regulating the gene expression of the ERAD pathway[14,15] and initiating the induction of XBP1 mRNA.[16] The combined or individual action of transcriptionally active ATF6 fragment and XBP1s may also affect gene expression.[17]

ROLE OF UPR IN SOLID MALIGNANCY

The control of protein homeostasis is one of the emerging hallmarks of cancer progression.[18] High proliferation rate during tumorigenesis requires enhanced ER machinery activity for protein folding and assembly which induce physiological cytoprotective ER stress. The adaptation of cancer cells to environmental stress conditions mainly relies on bypassing the ER stress-induced apoptosis to enhance the ER stress-associated signaling.[19] High Bip expression level is a chemoresistance factor against doxorubicin in Breast cancer chemotherapy[20] and inhibition of Mitogen-activated Protein Kinase (MAPK) pathway regulating Bip expression have also shown an increase in caspase 4 levels in melanomas.[21] The phosphorylation of elf2α by PERK is necessary for adaptation of solid malignancies.[22] Phosphorylated elf2 α activates ATF4 and subsequently CHOP is expressed. CHOP enhances levels of ER oxidase 1 α (ERO1α) which promotes oxidative protein folding and can lead to increased ROS levels thus worsening stress. ERO1α is an important prognostic factor for breast cancers.[23] PERK/eIF2 pathway largely contributes to the cancer progression and metastasis under hypoxic stress, being responsible for angiogenic gene activation.[24] PERK silencing decreases tumor vascularity and reduces tumor xenografts in mice.[25] High expression of spliced XBP1 correlates with poor prognosis in cases of triple negative breast cancer[26] and glioblastoma.[27] IRE1α driven splicing of XBP1 leads to increased hypoxic tolerance and contributes to the adaptive response and survival through positive regulation of BiP.[28] Furthermore XBP1s induces proliferation through increased cyclin A1 expression.[29] ATF6 is overexpressed in many human solid tumors and contributes to cancer formation by negatively regulating genes involved in senescence.[30] ATF6 is also shown to induce unspliced XBP1 expression[16] and the activity of both ATF6 and XBP1 increases BiP expression.[31] It is thus evident how the ER sensor elements contribute to development and progression of solid malignancies by affecting angiogenesis, oxidative stress, cell proliferation and adverse microenvironment.

TRIPLE NEGATIVE BREAST CANCER

Breast cancer, constituting a widely diagnosed cancer under solid malignancy, is considerably regarded as a heterogeneous disease based on molecular subtypes, clinical behavior, sub-clonal genetics and treatment responses.[32] For convenience of research purpose and treatment therapies, these molecular subtypes have been distinguished according to presence or absence of different molecular markers like estrogen receptor (ER), Progesterone receptor(PR), Human epidermal growth factor receptor 2(HER2).[33] (**Table 1**). The classification of molecular subtypes can be done as: Luminal A, Luminal B, Triple Negative Breast Cancer (TNBC) and HER2 overexpression.[34,35] While the risks and therapies of the ER+, PR+ or HER2+ subtypes of breast cancer are well defined from the point of view of an oncologist, TNBC still continues to be a thoroughly researched area of interest owing to a) lack of targeted therapeutics for treatment due to absence of hormonal receptors and b) overlapping characteristics of Basal-like cancer gene expression profile which is defined by over expression of high molecular weight basal cytokeratin 5/6.[36,37] Triple Negative breast cancer accounts for about 10-20% of all breast carcinomas based on expression profiling studies.[38.39] at the transcriptomic level demonstrating breast cancer class heterogeneity.[40] Literature reports on the geographical and age related precedence of triple negative breast cancers show that women of African-American ethnicity and younger (<50 years) patients of the population are more frequently affected. Moreover among the other subtypes, TNBC is more significantly aggressive and has a greater invasive rate with shorter survival chances following the first metastatic event.[41] As TNBC lacks hormonal receptor targets, endocrine therapies or HER2 therapies do not benefit patient. Conventionally, pre-detected mastectomy and chemotherapy are the only effective treatment approaches.[37] Recent advancements in the field of TNBC gene expression profiles have put forward Basal-like (BL1 and BL2), Mesenchymal (M), Mesenchymal stem-like (MS), immunomodulatory (IL), and a luminal androgen-receptor (LAR) subtype with preferable molecular targets to come up with various therapeutic approaches against TNBC.[42] These new discoveries have opened up a plethora of research areas pertaining to molecular targets and therapies against Triple Negative Breast Cancers.

ROLE OF ER STRESS IN TNBC MALIGNANCY

Endoplasmic Reticulum stress and Hypoxia may be the long searched cancer hallmarks for Triple Negative Breast cancer[43] with reports of spliced XBP1 co-transcriptionally activating (Hypoxia Inducible Factor 1α) HIF1α and thus promoting neo-angiogenesis and cancer stem cell regulation.[26] The role of Hypoxia has been quite prominent in cases of Epithelial to mesenchymal Transition (EMT)[44,45,46] and Metastasis.[47,48] The member e41 of basic helix loop helix family, SHARP1, has also been reported to regulate HIFs in an oxygen independent manner in TNBC owing to its more invasive and metastatic nature.[49] SHARP1 regulates hypoxia inducible factors by serving them to proteasomal degradation and may also inhibit the complex formation of HIF1α and HIF1β. Reports of Genome wide analysis in TNBC cell lines show a low SHARP1 level to correlate with a higher metastatic

advantage whereas depletion of HIF's along with SHARP1 overexpression prevents metastasis and colonization of the cancer cells.[50] Comparisons of SHARP1 and XBP1s have shown their partially distinct roles in metastasis and angiogenesis.[26] Future research can establish probable interactions between XBP1s and SHARP1 in regulating HIFs for Triple negative breast cancers for better understanding the role of ER stress elements in HIF regulation.

Besides Hypoxia, Reactive Oxygen Species (ROS) stands as an important target for cancer therapies. In ER stress conditions, oxidative stress forces calcium out of ER and is taken up by mitochondria. Thereby, calcium concentration increases metabolic activities and ROS generation in mitochondria.[51] Reports of increased Warburg effect in TNBC cell lines exhibited lower mitochondrial respiration and a higher ROS production with an increase in electron leakage due to a dysregulated electron transport chain.[52] As a feedback, these oxidative stress conditions pose a potent threat of sensitizing Ca+2 channels in Endoplasmic reticulum where protein folding takes place. Peroxiredoxins (PRDXs I-VI), a class of antioxidant enzymes, is strongly induced for ROS regulation in cases of oxidative stress in cytoplasm and nucleus. Reports show that PRDX IV is shown to overexpress in cases of TNBC along with keap1 of Nrf2 pathway.[53] PRDXIV being mainly present in endoplasmic reticulum, it shows the importance of ER stress in regulating the prognosis of TNBC by regulating ROS levels to enhance proliferation and survival of the cancer cells [Fig.2]. Furthermore, it has been reported by Hashimoto et.al, 2014[54] that activated pAKT or pERK is a good prognostic marker in node-positive TNBC due to their upregulated levels and S. Ghosh et.al, 2014[55] showed potential anti-cancer treatment against TNBC with pERK/ MAPK downregulation by nifetepimine. Downregulated pERK levels lead to decrease in translated grp78 levels, which in turn enhance ER stress in TNBC cells and activate caspase dependent apoptotic pathways with higher ROS levels.[56] On the other hand, reports of NOS inhibition in TNBC showed decrease in hypoxia inducible factor HIF1a and ER stress receptors like PERK and ATF4/6.[57] Irrespective of the heterogeneity in TNBC cohorts, various signaling pathway denominators like ROS and NOS have inherent cross-talk with ER stress elements, responsible for proliferation and metastasis of tumor cells.

ROLE OF ER STRESS IN TNBC CHEMORESISTANCE

Although in TNBC, Standard chemotherapy remains the backbone of systemic treatment, the cure for cancer continues to escape oncologists largely due to chemoresistance, accounting for 90% of drug failures.[58] Tumor cells evade apoptotic stress by undergoing alternative fates of cellular senescence or autophagy to maintain cell viability following chemotherapeutic exposure. High levels of expression of autophagy-related microtubule-associated proteins like beclin-1, light chain (LC) 3A and 3B in TNBC cells compared to the other breast cancer subtypes[59], indicate that TNBC is more prone to chemoresistance than other cancer subtypes. In TNBC and other solid malignancies, Tumour microenvironment and cellular conditions like ER stress play a significant role in chemoresistance by autophagy. Bcl2 in mitochondria and endoplasmic reticulum can regulate autophagy induction by directly binding to Beclin1 or by IP3 regulating calcium levels.[60] Reports show that ER stress enhancing agents simultaneously added with autophagy inhibitors like choloroquine have proved to be an effective anti-cancer therapy for TNBC and basal like breast cancers.[61] The Bcl2 binding to Beclin 1 is regulated by JNK (c-Jun N-terminal kinase) downstream of PERK, an ER stress receptor marker. In cell signaling cascades related to JNK and p38, JNK kinases have their activators at the MAPK level[62], which is upregulated in TNBC breast cancer subtypes. This also shows how ER and MAPK cross-talks affect resistance of triple negative breast cancers to prevalent chemotherapy practices. Induced MAPK phosphatase-1 (MPK1) level has also been reported to be a contributing factor for resistance to chemotherapy in TNBC against drugs like paclitaxel and cisplatin, although the underlying mechanisms need better research approach.[63]

TNBC chemoresistance can be also related to tumor dormancy although the mechanistic pathways which lead to survival during prolonged dormancy are yet to be sought. Denis M. Schewe and Julio A. Aguirre-Ghiso showed the role of ATF6α, an ER stress marker, to be crucial for cellular adaptation to stress and chemotherapy drugs in squamous epithelial cells with MKK6 and p38 as contributing factors for its translational activation.[64] As TNBC heterogeneity may be attributed to its origin from mammary epithelial luminal progenitor cells, such reports of tumor dormancy by ATF6α in squamous epithelial cells can be supposedly considered for research on specific TNBC cohorts.

ROLE OF ER STRESS ON TNBC RELAPSE

When compared to other breast cancer subtypes, Triple-negative breast cancers have been shown to have higher chemoresistance and are more likely to relapse.[65] The major cause of tumor relapse till date remains metastasis and stem-like characteristics, which opens up a wide area of controversial research under cancer stem cells[66]. Epithelial to Mesenchymal Transition (EMT) helps in up-regulation of specific mesenchymal markers to bring about adhesion junction diassembly and is considered as an alternative by cells to evade apoptotic stress.[67] The EMT phentotype is usually associated with stem-like cell phenotype[68] and drug resistance has also been increasingly reported for the same.[69] Reports of EMT induction in immortalized mammary epithelial cells (HMLEs) have shown downregulation of epithelial markers like E-cadherin and upregulation of mesenchymal markers like N-cadherin, vimentin and fibronectin along with CD44high/CD24low expression

supporting evidence of mammary stem-cell like characteristics.[68] As TNBC heterogeneity may be attributed to its origin from mammary epithelial luminal progenitor cells[70], EMT is considered an essential factor for TNBC metastasis and tumour relapse. TGFβ promotes EMT upon regulation of Ras pathway by inducing Snail, Slug, ZEB1and 2 and Forkhead factors resulting in E-cadherin suppression.[71,72,73] In TNBC, Functional protein-protein-protein interaction analyses showed an interaction between ATF4/ATF3, downstream of ER stress receptors, with TGFβ[74]. This confirms the crosstalk between ER stress and TGFβ through ATF4/ATF3 and indirectly shows ER stress to be a factor for EMT, although the underlying mechanisms are yet to be researched upon.

Evidence of Epithelial to Mesenchymal Transition upon ER stress induction has been shown in alveolar epithelial cells.[75] Although the pathways leading to EMT are not clearly defined, upregulation of MAPK and βcatenin upon ER stress induction may be contributing factors.[76] Similar research on mammary epithelial cells may reproduce the idea of ER stress playing a major role in TNBC tumor relapse, owing to its heterogeneity as discussed earlier.

UPR TARGETS FOR THERAPEUTIC RESEARCH

Besides the majorly investigated role of mitochondrial pathway, the role ER stress in TNBC and other solid malignancies prove to be an area of thorough research. The discrepancy between tumor cells having the 'stress' conditions more prominent than the quiescent UPR pathway in normal cells, offer an advantage for the UPR targeting agents in cancer therapy. Therapeutic targeting of UPR components in cancer can be approached as: (i) Accumulation of misfolded proteins to overload the UPR and (ii) Inhibition of UPR adaptive pathways leading to cell death.

Proteosomal inhibitor drugs like Bortezomib.[77,78] increases ER load and regulates PERK, ATF4 other proapoptotic target.[79] Combination chemotherapy practices with Bortezomib have been shown high remission rates in the frontline cancer treatment and relapse scenario.[80] Other ERAD pathway inhibitors like Eeyarestatin I inhibits retrotranslocation of polyubiquinated proteins from ER to the cytosol for degradation.[81] Under stress conditions in solid malignancies, cells upregulate HSP90/ Grp78 chaperones which stabilizes ER stress markers like PERK and IRE1α, to prevent protein misfolding and ensure cell survival.[82] Cancer development-associated proteins such as Akt, Bcr-Abl, and CDKs (cyclin-dependent kinase) are regulated by the HSP90 inhibitors[83] like PU-H71[84] and Tanespimycin.[85] Furthermore, treatment with Grp78 inhibitors like epigallocatechin gallate (EGCG) target the ATP binding domain of Grp78 and sensitizes cancer cells to chemotherapy.[86] Other potent ER stress inhibitors like 4pba, which is also used as a deacetylase inhibitor in cancer treatments[87], reduces ER load of misfolded proteins by acting as a molecular chaperone in endoplasmic reticulum.[88] Potent analogues of 4-pba have shown progressive results in inhibiting IRE1α and ATF6 activation.[89] Besides therapies to regulate misfolded protein load by inhibiting chaperones in ER, specific ER stress receptors are also a considerable therapeutic target. Irestatin and Salicylaldehydes (3methoxy-6bromosalicylaldehyde) bind to IRE1α and inhibit XBP1 splicing and RIDD activity.[90,91] On the other hand, PABA/NO enhances UPR by activation of PERK, eIF2α, XBP1 splicing and BiP in ovarian cancer cells[92] and to support similar research on TNBC, recent studies have also shown 'triple negative' phenotype in ovarian and endometrial cancer.[93] Other than chemical drug therapies, natural compounds like Baicalein and Celastrol can also be used for cancer treatments owing to their effects on regulating ER stress.[94,95]

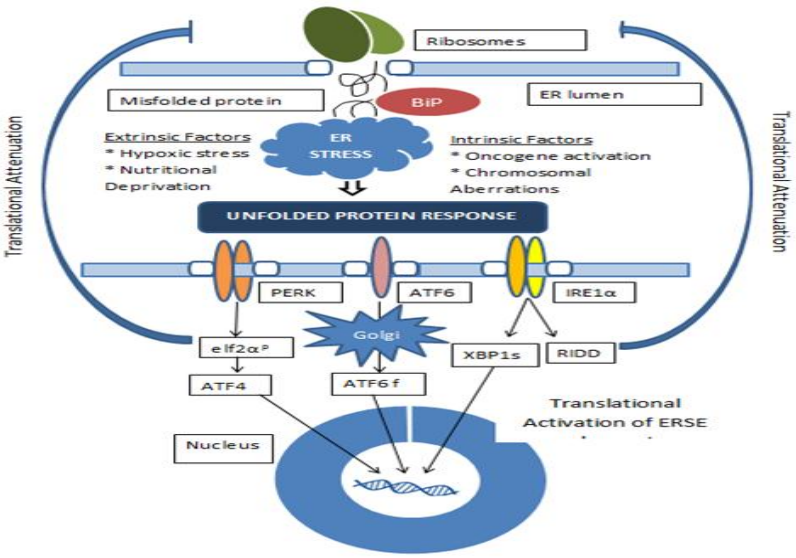

Fig1:- Endoplasmic reticulum stress machinery: Under stress conditions due to genetic or hypoxic factors, the translated misfolded proteins enter the ER lumen and the molecular chaperones bind to them and in turn,

activates the ER receptors PERK, IRE1α and ATF6. Downstream ER stress markers like ATF4 or spliced XBP1activates the Endoplasmic reticulum stress response elements (ERSE) whereas elf2α and RIDD helps in the ERAD pathway and translational attenuation.

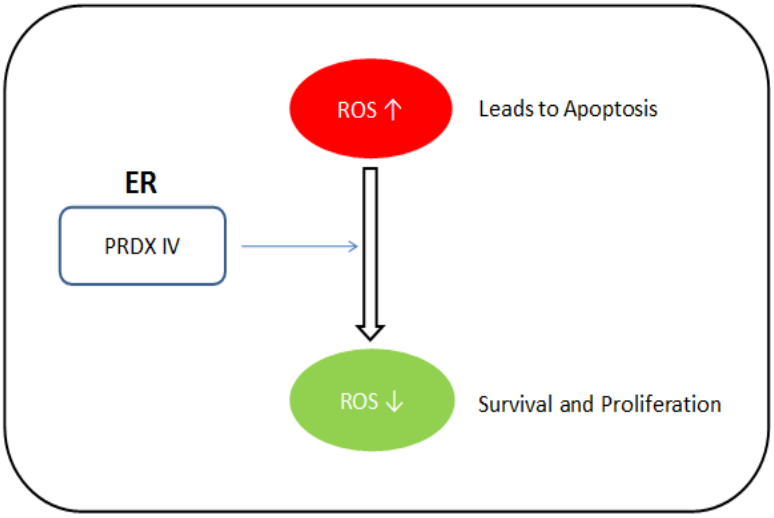

Fig2:- Role of Peroxiredoxins in ROS regulation: A higher amount of ROS level can lead to apoptotic death of tumor cells. A class of antioxidant enzymes named peroxiredoxins (PRDX I- VI) help in tumor survival by reducing the ROS levels. Peroxiredoxin IV (PRDX IV), generally found in Endoplasmic reticulum, is found to be upregulated in TNBC and shows the prominent role of ER stress in cancer regulation.

Table 1:- Breast Cancer subtypes along with their %incidence and specific endocrine receptor markers.

Molecular Subtype	% of incidence	Molecular markers
1. Luminal A	35% - 40%	$ER^+/PR^+/HER2^-$ $ER^-/PR^+/HER2^-$ $ER^+/PR^-/HER2^-$
2. Luminal B	10% - 20%	$ER^+/PR^+/HER2^{+/-}$ $ER^+/PR^-/HER2^{+/-}$ $ER^+/PR^-/HER2^{+/-}$
3. TNBC	10% - 20%	$ER^-/PR^-/HER2^-$
4. HER2 enriched	10%	$ER^-/PR^-/HER2^+$

CONCLUSION

Endoplasmic Reticulum stress response allows a balance between cell survival and cell death. In context of anti-cancer therapies, a lot of work has been done to investigate approaches of enhancing ER stress to switch the cell towards cell death conditions. Although Chemotherapy has been more recommended in TNBC and high risk luminal tumors, besides strategies dealing with cytotoxic Platinum agents or PARP inhibitors[96, 97] as therapeutics, ER stress has been lately regarded as a potential target for efficient cancer against Triple Negative Breast cancer owing to the failure of endocrine therapies. Targeting UPR pathway in TNBC and other solid malignancies represent a novel targeted anti-cancer approach with initial successes in research but further understanding of the pathways regarding ER stress involved in metastasis and Cancer stem cell development should provide additional therapeutic opportunities in future.

COMPETING INTERESTS

The authors declare they have no competing interests.

ACKNOWLEDGEMENT

The authors thank all those researchers whose works have inspired the review and apologize to all those investigators whose works were not cited due to space constraint or oversight.

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