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Original Article

The clinical relevance of unfolded protein response signaling in breast cancer

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Abstract: Protein homeostasis regulated by the Endoplasmic Reticulum (ER) is a recognized process involved in cancer progression. ER stress activates the Unfolded Protein Response (UPR) and has been implicated in a variety of cancers. Given the role of the UPR activation in carcinogenesis, we hypothesized that UPR activation could be associated with pathological progression, higher clinical stage, and worse survival in breast cancer. A total of 4,416 breast cancer patients from multiple independent cohorts were analyzed. We defined the UPR pathway score by the degree of enrichment by Gene Set Variant Analysis and median was used to divide high vs. low score groups in each cohort. High UPR breast cancer significantly enriched not only cell proliferation-related but also other pro-cancerous gene sets consistently in both METABIC and GSE96058 cohort. Majority of UPR pathway score high cells in the bulk tumor were tumor cells compared to other cells, including stromal, T-, B-, and myeloid-cells (P<0.001). UPR score was significantly associated with advanced stage, high grade, and triple negative breast cancer (TNBC) (all P<0.001). High UPR breast cancer was associated with worse patient survival in both cohorts (all P<0.001). Among breast cancer subtype, ER-positive/HER2-negative breast cancer with high UPR was significantly associated with worse survival, but neither HER-positive nor TNBC. High UPR ER-positive/HER2-negative breast cancer was infiltrated with high level of Th1 and Th2 cells, M1 macrophage, and plasma cells. On the other hand, they were significantly infiltrated with high level of several types of stromal cells in tumor microenvironment (all P<0.001). Finally, high UPR metastatic breast cancer was also associated with worse patient survival (P=0.041). UPR signaling is associated with cancer aggressiveness, and worse survival, especially ER-positive/HER2-negative breast cancer subtype.

Keywords: Biomarker, breast cancer, gene expression, hormonal, unfolded protein response

Introduction

Breast cancer (BC) is the most commonly diagnosed cancer globally with an incidence of 2.3 million new cases in a year and it is still the primary cause for cancer mortality among women [1]. With early detection and advancements in treatment the 5-year relative survival rates in localized, regional BC have improved to 99% and 85.8%, respectively; however, it is only 29% in metastatic BC according to recent data from the SEER database [2]. Detailed understanding of the tumor microenvironment, immune responses, and signaling pathways involved in the cancer cell proliferation would help with arresting or delaying cancer progression which would eventually improve survival.

Cell homeostasis is maintained by proper folding of the proteins and the misfolded proteins undergo proteolysis by the ubiquitin-dependent proteolytic degradation process of the Endoplasmic Reticulum (ER) [3-5]. Physiological processes such as aging and other factors like

hypoxia, viral infections, glucose deprivation, toxins, acidosis, irradiation, poor vascularity disrupt the ER homeostasis and lead to unfolding or misfolding of proteins. If the degradation of the unfolded proteins is not enough, this leads to accumulation of the unfolded proteins in the ER which is called ER stress and leads to up-regulation of a signaling pathway called as ER stress response or the unfolded protein response (UPR) [4, 6, 7]. The signal-transduction cascade, UPR has been designed primarily to protect the ER by counteracting the damage from the accumulation of unfolded or misfolded proteins and to limit the damage to other cellular structures by eliminating the cells experiencing prolonged stress [8]. Along with the cytoprotective role, the UPR pathway can lead to apoptosis and proliferation of tumor cells in the presence of prolonged ER stress. Cancer cells adapt to the stressful tumor microenvironment by multiple mechanisms. Tumor cells are subjected to various stressors such as hypoxia, inadequate angiogenesis, decreased glucose and amino acid supply, oxidative stress, lactic acidosis as the metabolic requirements are rapidly increasing [3]. This leads to prolonged and severe ER stress and activates the protein kinase RNA-like endoplasmic reticulum kinase (PERK) -dependent UPR downstream signaling pathway which promotes tumor cell proliferation by limiting oxidative DNA damage [4, 10-13]. The aerobic glycolysis due to low glucose levels and the subsequent lactic acidosis leads to downregulation the pro-apoptotic transcription factors and helps the tumors cells to escape apoptosis [14, 15].

The role of UPR in cancer proliferation has been studied in multiple types of cancer. We previously reported that elevated UPR measured by our newly developed transcriptomic based score using gene set variation analysis (GSVA), was significantly associated with cell proliferation and worse survival in hepatocellular carcinoma (HCC) [9]. As the prevalence of drug resistant tumor clones in breast cancer is increasing, there is an emergent need for identifying and targeting drug resistant mechanisms [10]. As the UPR signaling pathway mediates cell proliferation, UPR can be a potential target for treatment.

In this study, we investigate the clinical relevance of UPR in breast cancer. Given the carcinogenetic role of UPR, we hypothesize that UPR is associated with cell proliferation in breast cancer, aggressive breast cancer types, and poor survival. To investigate the study, we used the UPR score and total 5,176 samples from two large sample cohorts METABRIC (n=1,903), and the GSE96058 (n=3,273) cohorts to study the function and clinical implications of UPR in breast cancer [11, 12].

Materials and methods

Cohorts used for analyses

For the main analysis, we used The METABRIC (n=1,903), and the GSE96058 (n=3,273) cohorts, which have a large number of BC samples with transcriptome and clinicopathological data [13, 14]. The cBio Cancer Genomic portal and Gene Expression Omnibus (GEO) repository were used to obtained these data in the METABRIC [15] and GSE96058 cohort [16]. GEO repository was also used to obtained transcriptomic and clinical data of GSE75688 [17] andGSE124647 [18] cohorts.

Infiltration fraction of stromal and immune cells in tumor microenvironment of BC

The infiltration fraction of stromal and immune cells in each sample was predicted by the xCell score, an algorithm with mRNA gene expression data in bulk tumor [19].

Gene set expression analyses

Gene Set Enrichment Analyses (GSEA) (Java version 4.0) [20] with MSigDB Hallmark gene sets collection [21] was used to investigate biological function of breast cancer, as we previously reported [22-31]. Statistical significance was used a false discovery rate (FDR) of 0.25 following the recommendation of the GSEA software.

Other

All analyses were performed using the R software (version 4.1.0). Fisher's exact, Kruskal-Wallis, and Mann-Whitney U test were used to group comparison. The Kaplan-Meier method with log-rank test was used to survival analyses.

Results

High unfolded protein response (UPR) breast cancer (BC) enriched cell proliferation-related signaling as well as other precancerous signaling gene sets

Since we previously showed that high UPR HCC was significantly associated with proliferation-related gene sets, we were interested to study the association between UPR and other cancer-related signaling in breast cancer [9]. To investigate the association, we performed gene set enrichment analysis (GSEA) with the molecular Signatures Database (MSiDB) hallmark gene sets [20, 21]. The MSiDB Hallmark defines six gene sets as cell proliferation related in GSEA. As we expected, high UPR BC significantly enriched cell proliferation-related gene sets, including MYC target v1 and v2, E2F targets, G2M, checkpoint and Mitotic spindle, as well as DNA repair gene sets, oxidative phosphorylation, and glycolysis gene sets, in the METABRIC cohort (**Figure 1A** and **1B**). These results were validated by GSE96058 cohort. These results suggest that high UPR was significantly associated with several pro-cancer-related gene sets, including cell proliferation-related gene sets, in not only HCC but also in BC.

UPR was significantly correlated with clinical aggressiveness in BC

Given the findings that UPR was associated with cell proliferation related gene sets, we next checked the association between the UPR level and clinical factors, including the American Joint Committee on Cancer (AJCC) stage, Nottingham histological grade (grade 1, 2, and 3), and BC subtypes (estrogen receptor (ER)-positive/human epidermal growth factor receptor 2 (HER2)-negative, HER2-positive, and triple negative breast cancer (TNBC)). We found that UPR level was significantly correlated with AJCC stage, and Nottingham grade, and was significantly low in ER-positive/HER2-negative subtype in the METABRIC cohort (**Figure 2**; all P<0.001). In AJCC classification, UPR level was also significantly associated with T-category (T1/2 vs. T3/4), and N-category (N-negative vs. N-positive) in both cohorts (Figure S1). The results of the analysis of grade and subtype with UPR were validated by GSE96058 cohort (**Figure 2**; all P<0.001). These results suggest that high UPR was significantly associated with advanced and aggressive BC.

High UPR was significantly associated with worse survival in BC, especially ER-positive/HER2-negative BC

We have previously published that cell proliferation related scores such as MYC, G2M checkpoint and E2F target scores are associated with clinical aggressiveness and worse survival of patients with multiple cancers including breast and pancreatic cancer [26, 28, 32]. Here, we have found that high UPR BC are enriched in cell proliferation related genes and associated with higher grade and stage. Therefore, we next focused on the association of UPR with patient survival in BC. We found that high UPR was significantly associated with worse prognosis, including disease free survival (DFS), disease specific survival (DSS) and overall survival (OS), in breast cancer in the METABRIC cohort (**Figure 3**; all P<0.001). The result of OS analysis was validated in GSE96-058 (P<0.001). Furthermore, since the UPR level differs depending on the subtype, we also investigated the role of low and high UPR levels in each subtype. Although survival analysis in HER2-positive and TNBC did not show a significant difference between low and high UPR groups, high UPR in ER-positive/HER2-negative BC was significantly associated with worse survival in both cohorts (**Figure 3**; all P<0.001 in METABRC, OS; P=0.017 in GSE96058). These results suggest that high UPR was significantly associated with worse survival in BC, especially ER-positive/HER2-negative BC.

Low UPR was significantly associated high level of enrichment of several gene sets, including apical junction, coagulation, KRAS signaling up, in ER-positive/HER2-negative BC

Given the results that UPR in ER-positive/HER2-negative BC was significantly associated with worse patient outcome, it is of interest to study the biology of UPR in the subtypes. To investigate this question, we performed GSEA again for ER-positive/HER2-negative BC. We found that high UPR significantly enriched for cell proliferation-related gene sets, same as whole cohort (Figure S1), on the other hand, interestingly low UPR ER-positive/HER2-negative BC was enriched in several gene sets, including apical junction, coagulation, KRAS signaling up, hedgehog signaling, and epithelial mesenchymal transition (EMT), in the METABRIC cohort (**Figure 4**). Furthermore, these results were validated by GSE96058 cohort (**Figure 4**). These

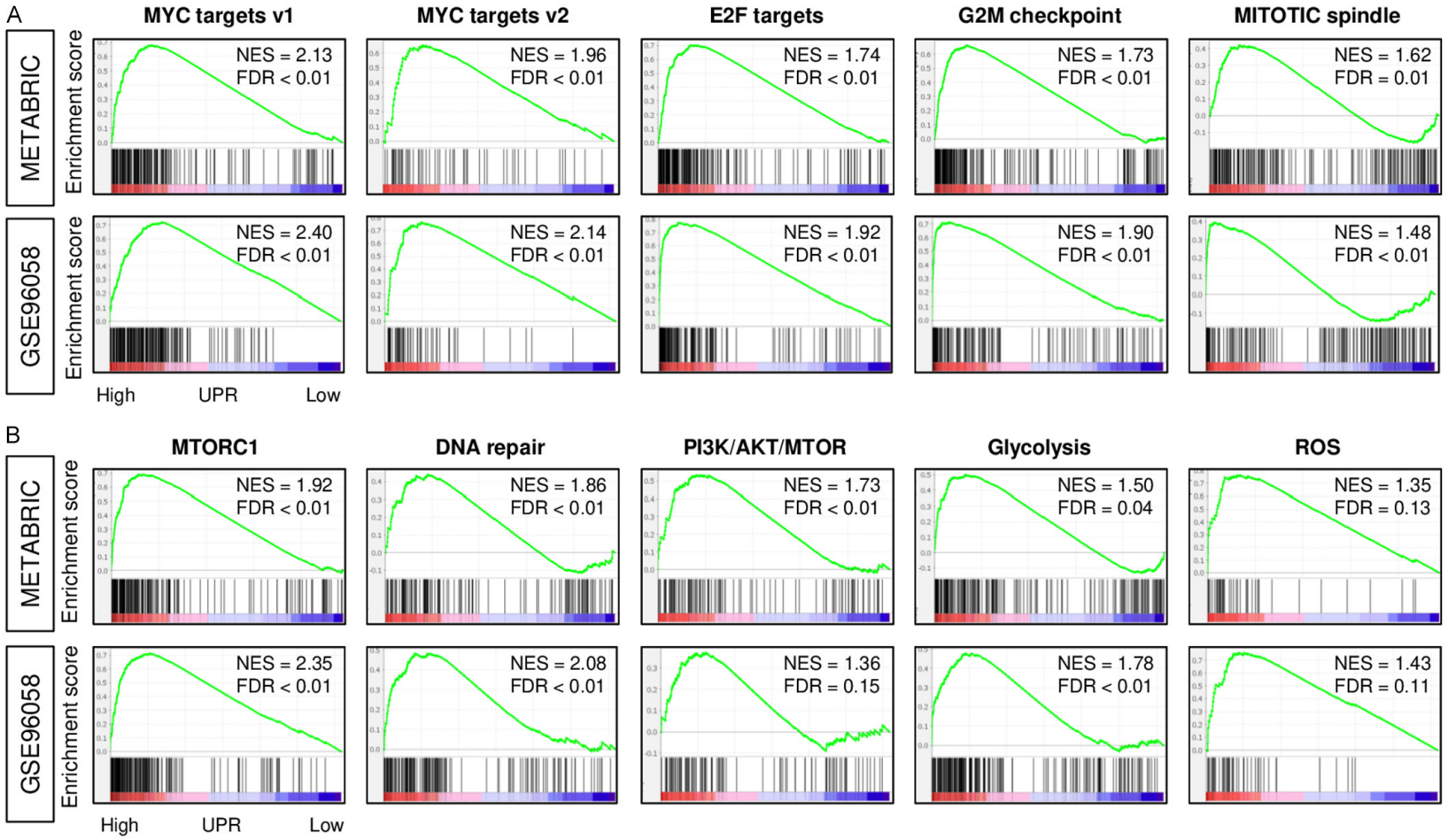

Figure 1. Biological function of high and low unfolded protein response (UPR) breast cancer (BC) by Gene set enrichment analysis (GSEA). Gene set enrichment plots with enrichment gene sets in high UPR BC in both METABRC and GSE96058 cohorts. A. Cell proliferation-related gene sets; MYC targets v1 and v2, E2F targets, G2M checkpoint, and MITOTIC spindle. B. Other gene sets; MTORC1, DNA repair, PI3K/AKT/MTOR, Glycolysis, and reactive oxygen species (ROS). Median cut-off was used to perform the analysis. As recommended by the GSEA software, FDR <0.25 defined statistical significance. FDR, False discovery rate; NES, normalized enrichment score.

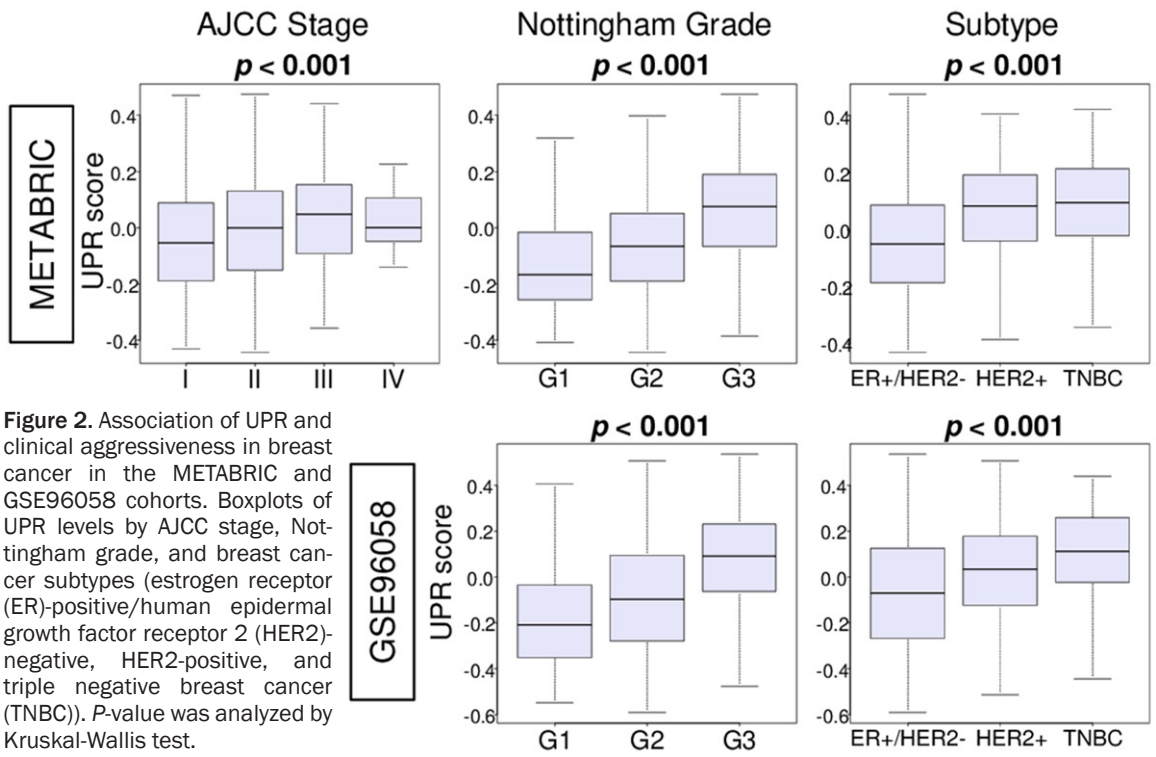

Figure 2. Association of UPR and clinical aggressiveness in breast cancer in the METABRIC and GSE96058 cohorts. Boxplots of UPR levels by AJCC stage, Nottingham grade, and breast cancer subtypes (estrogen receptor (ER)-positive/human epidermal growth factor receptor 2 (HER2)-negative, HER2-positive, and triple negative breast cancer (TNBC)). *P*-value was analyzed by Kruskal-Wallis test.

results suggest that the counterbalance of various cancer-related signaling pathways leads to a significant association between UPR level and patient outcomes in ER-positive/HER2-negative BC.

High UPR ER-positive/HER2-negative breast cancer enriched with anti-cancerous immune cells

The tumor immune microenvironment (TME) plays an important role in tumor progression, therapeutic response, drug resistance, and prognosis in multiple cancers, including breast cancer [33, 34]. Tumor infiltrating lymphocytes (TIL) have been identified as a biomarker for anti-tumor response in BC. $CD4^+$, $CD8^+$ T lymphocytes which are part of the TIL play a major role in recognizing tumor antigens and destroying tumor cells. T helper type I cells (Th1 cells) secrete interferon gamma (IFN-γ), tumor necrosis factor alpha, and interleukin -12 which exhibits anticancer activity. Th2 cells secrete interleukin 4 which inhibits the secretion of IFN-γ, thereby promoting cancer progression [35, 36]. The tumor associated macrophages: M1 distinguishes tumor cells from normal cells and kills them through its cytotoxic mechanisms whereas M2 macrophages promote tumor cell proliferation and invasion [37]. The T cell lineage γδT cells also has established anti-cancer effects with interferon-γ production [38]. As we found that the high UPR ER-positive/HER2 negative BC is associated with worse survival, we investigated the TME in this group. Interestingly, we found that high UPR ER-positive/HER2-negative breast cancer was significantly associated with high proportion of Th1 cells, M1 macrophages, and γδT cells, the anti-cancerous immune cells, as well as Th2 cells, pro-cancerous immune cells, consistently in both cohorts (**Figure 5**). Although high UPR ER-positive/HER2-negative breast cancer was significantly associated with fewer $CD8^+$ T cells, dendritic cells (DC), Tregs in the METABRIC cohort, these results were not validated by GSE96058 cohort (**Figure 5**). These findings suggest that high UPR was significantly associated with high infiltration of anti-cancerous immune cells in ER-positive/HER2-negative breast cancer.

High UPR was significantly associated with low fraction of stromal cells in BC

Next, we investigated the relationship between UPR and infiltrating fraction of stromal cells in TME of ER-positive/HER2-negative because

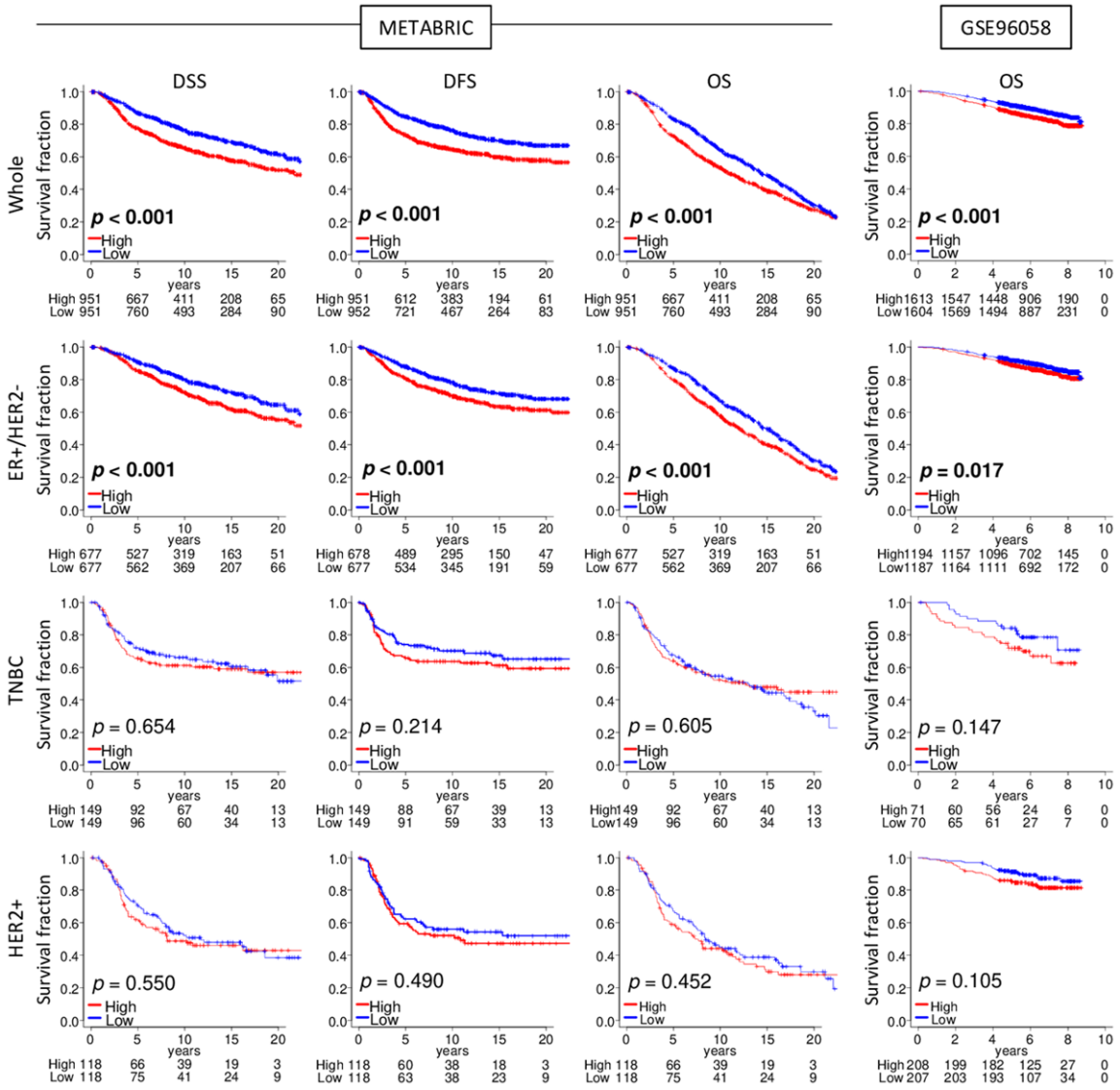

Figure 3. Clinical relevance of UPR signaling in BC in two large cohorts. Kaplan-Meier curve showing the association of high vs. low UPR on DFS, DSS, and OS in the METABRIC cohort, and OS in the GSE96058 cohort. Median cut-off was used to perform the analysis. DFS, disease-free survival; DSS, disease-specific survival; OS, overall survival.

they are also involved in patient outcomes [39, 40]. We found that high UPR ER-positive/HER2-negative BC was significantly associated with lower infiltration of several stromal-related cells, including fibroblasts, adipocytes, endothelial cells, and pericytes, consistently in both cohorts (**Figure 6A**; all P<0.001). With these results showing the association of UPR with several cells in TME, it was of interest to examine the relationship between the level of UPR score within each cell in TME. To investigate this association, we used single cell sequence data of the GSE75688 cohort. We found that UPR score in cancer cells was highest compared to other cell types, including stromal, T cell, B cell, and myeloid cells (**Figure 6B**; P< 0.001). Other cells were also involved in the expression of the UPR although at lower levels.

High UPR metastatic breast cancer was associated with worse clinical outcomes

Finally, we investigated the association of UPR with clinical outcome of patients with metastases using GSE124647 cohort, which has gene expression data of metastatic tumors. UPR can promote metastasis in several ways: by increasing angiogenesis through the expres-

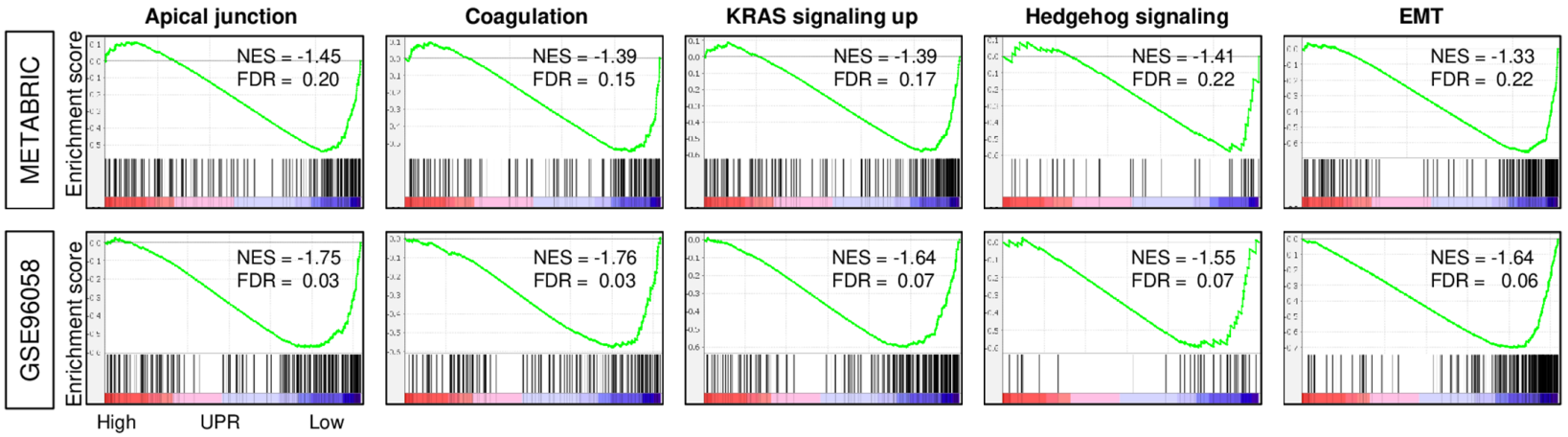

Figure 4. Biological function of high and low UPR ER-positive/HER2-negative BC by GSEA. Gene set enrichment plots with enrichment gene sets in low UPR ER-positive/HER2-negative BC in both METABRC and GSE96058 cohorts. Median cut-off was used to perform the analysis. As recommended by the GSEA software, FDR <0.25 defined statistical significance. FDR, False discovery rate; NES, normalized enrichment score.

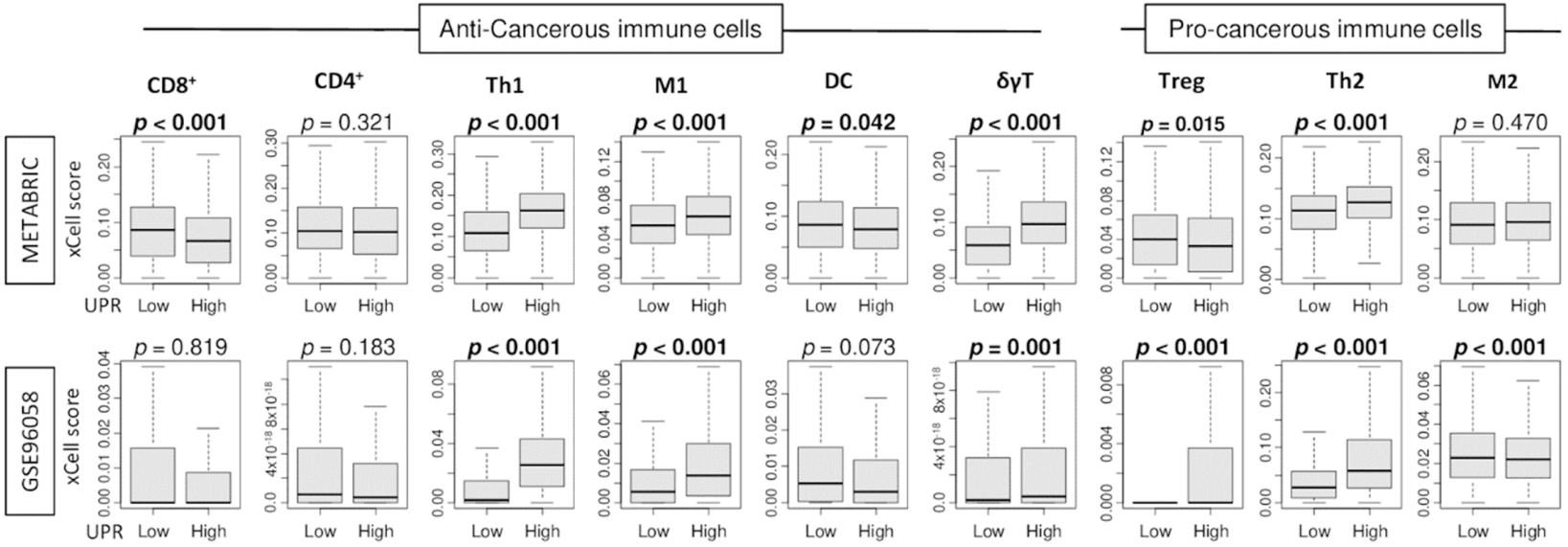

Figure 5. Association of tumor immune environment with UPR in ER-positive/HER2-negative breast cancer. Boxplots of anti-cancerous immune cells (CD8+ T cells, CD4+ T cells, T helper type1 (Th1) cells, M1 macrophages, dendritic cells (DC), and γδT cells, as well as pro-cancerous immune cells (Tregs, Th2 cells, and M2

macrophages) by low and high UPR in ER-positive/HER2-negative breast cancer in both METABRIC and GSE96058 cohorts. Median cut-off was used to perform the analysis. *P*-value was analyzed by Mann-Whitney U test.

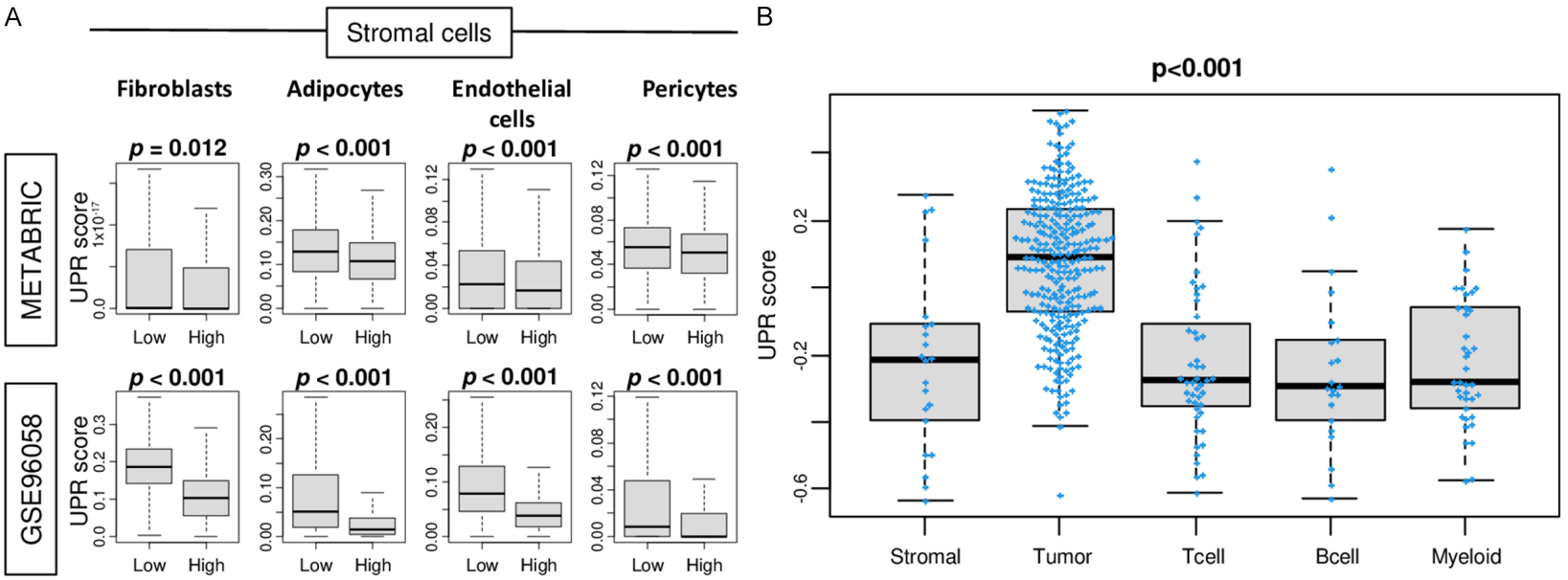

Figure 6. Association of UPR with stromal cells in tumor microenvironment (TME). A. Boxplots of stromal cells (Fibroblasts, Adipocytes, Endothelial cells, and Pericytes) by low and high UPR in ER-positive/HER2-negative breast cancer in both METABRIC and GSE96058 cohorts. Median cut-off was used to perform the analysis. *P*-value was analyzed by Mann-Whitney U test. B. Boxplots of UPR score by cell types (stromal cells, tumor cells, T cells, B cells, and Myeloid cells) in GSE75688 cohort. *P*-value was analyzed by Kruskal-Wallis test.

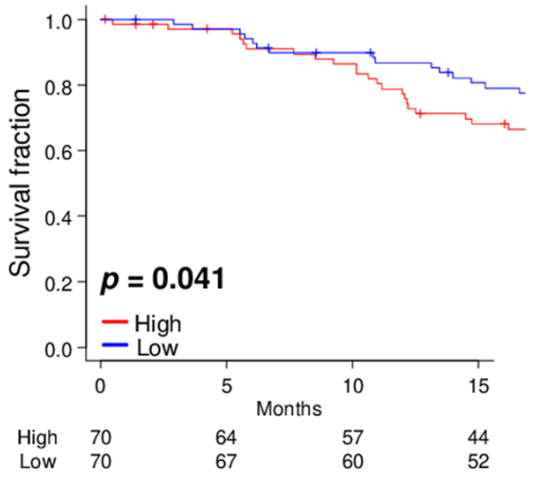

Figure 7. Clinical relevance of UPR signaling in metastatic BC. Kaplan-Meier curve showing the association of high vs. low UPR with progression-free survival in the GSE124647 cohort. Median cut-off was used to perform the analysis.

sion of vascular endothelial growth factor (VEGF), by sustaining tumor cell proliferation, and through resistance to apoptosis [41, 42]. We found that high UPR was significantly associated with worse survival (**Figure 7**; P=0.041). Furthermore, high UPR metastatic tumor was significantly associated with high infiltration of Th1 cells, Th2 cells, and γδT cells, and low infiltration of $CD8^+$ T cells and Tregs, same as primary breast cancer (Figure S2A). On the other hand, there were no association between UPR score and infiltration of several stromal cells; fibroblasts, adipocytes, endothelial cells, and pericytes, in metastatic tumor (Figure S2B). The result suggests that high UPR in metastatic tumors is associated with worse clinical outcome.

Discussion

In this study, we analyzed the association of unfolded protein response (UPR) with the cell proliferation rate, signaling pathways, clinical aggressiveness, tumor immune microenvironment (TME), and prognosis of breast cancer. We previously reported that high UPR HCC enriched cell proliferation-related gene sets and other pro-cancer signaling, including TNFα signaling via NFkB, IL6/JAK/STAT5, complement, and angiogenesis [9]. Interestingly, we found that high UPR BC was significantly enriched for not only cell proliferation-related gene sets, but also DNA repair, glycolysis and reactive oxygen species. ER-positive/HER2-negative, the least aggressive subtype, demonstrated the lowest UPR among breast cancer subtypes. In addition, high UPR was associated with higher stage and pathological grade. We have previously shown that activation of cell proliferation-related signaling was significantly associated with a poor prognosis in several cancers [23, 26, 28, 32, 43]. Our study shows consistent finding as high UPR was significantly associated with worse clinical outcomes although it was associated with high infiltrations of immune cells, particularly in ER-positive/HER2-negative subtype. Low UPR was also associated with high fraction of stromal cells including fibroblasts, adipocytes, endothelial cells and pericytes, whereas it enriched apical junction, coagulation, KRAS signaling UP, hedgehog signaling and EMT pathways. We also found that low UPR was associated with better survival in metastatic BC as well.

UPR acts as a double-edged sword by helping in maintaining cellular homeostasis but causes carcinogenesis in the presence of severe ER stress. Cancer cells are constantly subjected to a variety of intrinsic stressors such as high secretory demand despite the low availability of nutrients, hypoxia and low pH [8]. This leads to the activation of the UPR signaling pathway. Extrinsic stressors such as irradiation and pharmacological agents also subject the tumor cells to greater stress which activates the UPR signaling cascade [4, 7]. When the downstream signaling proteins of the UPR pathway, namely, PERK, inositol-requiring enzyme-1 (IRE1), and activating transcription factor-6 (ATF6) are activated, it leads to abnormal cell proliferation, oncogenesis, angiogenesis, metabolic reorganization in tumor cells, immortal replication, invasion and metastasis which are the "hallmarks of cancer" [44]. UPR is also reported to be associated with resistance to multiple chemotherapy regimens in breast cancer including paclitaxel, vinca alkaloids, cisplatin, doxorubicin, radiation therapy and endocrine therapy including tamoxifen through its downstream signaling pathways [45-49]. PERK has been reported to promote resistance to paclitaxel, doxorubicin, and radiation in breast cancer [50, 51]. IRE1 has been shown to be associated with resistance to paclitaxel and doxorubicin in TNBC and to tamoxifen in ER positive breast cancers [45, 46].

The role of UPR-related protein in cancer progression was shown in a preclinical study by Jamora et al. The authors observed that when the UPR-activated stress protein: glucose-regulated protein 78 (GRP78) was suppressed, cancer failed to progress [52]. Also, a prior study showed that downstream signal transducers in the UPR pathway were overexpressed frequently in high grade breast cancer when compared to low grade [53]. Thus, we hypothesized that high UPR is associated with clinically aggressive breast cancer. Our study indeed showed that UPR level significantly associated with advanced stage and higher Nottingham histological grade. In addition, we observed that high UPR breast cancer significantly enriched all 5-hallmark cell proliferation-related gene sets (E2F targets, G2M checkpoint, MYC target v1 and v2, and MITOTIC spindle), as well as MTORC1 signaling, DNA repair, PI3K/AKT/MTOR signaling, and reactive oxygen species pathway. The association of some of these pathways with clinical outcomes of cancers has been reported by us previously [23, 28, 43]. We have shown that that MYC targets v1 and v2 scores are associated with aggressive tumor and poor prognosis in ER-positive primary and metastatic breast cancer [32]. We have also reported that breast cancer with high activity of the G2M pathway is aggressive and likely to metastasize. It was also noticed that metastatic tumors with high activity of the G2M pathway were associated with significantly worse survival [23]. In our study in hepatocellular carcinoma (HCC), we have found that DNA repair high HCC was associated with worse survival [54]. These results highlight the prognostic value of UPR and its potential role as a therapeutic agent.

We previously reported that high KRAS signaling was significantly associated with better patient survival in breast cancer [55]. The hedgehog signaling pathway plays an important role in tissue homeostasis, embryonic development and normal stem cell differentiation and processing [56]. The acquisition of mesenchymal features from epithelial cells, which is called as the epithelial-mesenchymal transition (EMT) occurs during the tumor progression [57]. In our study, we have identified that low UPR ER-positive/HER2-negative BC was enriched in several gene sets, including apical junction, coagulation, KRAS signaling up, hedgehog signaling, and epithelial mesenchymal transition (EMT).

There is evidence to show that the tumor microenvironment (TME) plays an important role in mediating treatment response, resistance to therapeutic agents and has been evaluated as a target by multiple novel treatment options in breast cancer [33, 34]. Tumor infiltrating lymphocytes which include the $CD8^+$ T cells, $CD4^+$ helper (Th1, Th2) cells, T regulatory cells (Treg), γδT cells, macrophages (M1, M2), and mast cells play a major role in the TME. Tumor cells manipulate the TME for rapid proliferation, escaping apoptosis, invasion, extravasation, and metastasis. They secrete pro-inflammatory mediators, chemokines that attract immune cells which helps in cell proliferation, angiogenesis, tumor development, and progression [58]. The changes and adaptations in the TME are a form of ER stress which is influenced by the activity of the UPR signaling pathway sensor IRE1α [59]. The tumor progression and response are mediated by the interplay of precancerous and anti-cancerous cells. Thus, we decided to study the effect of the UPR on the TME. We found that high UPR ER-positive/HER2-negative breast cancer was significantly associated with high infiltration of Th1 cells, M1 macrophages, and γδT cells, as well as Th2 cells. Stromal cells which include vascular endothelial cells, fibroblasts, adipocytes, pericytes, and stellate cells constitute an important component of the TME [60]. Vascular endothelial cells promote angiogenesis. The cancer-associated adipocytes secrete inflammatory cytokines which contribute to pro-cancer inflammation and promote cancer progression [39]. Also, adipocytes promote angiogenesis, invasion, and metastasis by the production of leptin and interleukin-6 [61, 62]. Thus, we were interested to study the association of UPR and the stromal cells in breast cancer. Interestingly, we found that high UPR ER-positive/HER2-negative BC was significantly associated with low infiltration of several stromal-related cells.

Our study has certain limitations. As our study is a retrospective analysis of publicly available cohorts, it lacks experimental validation as we did not have access to the patient tissue samples. As the cohorts that we accessed did not contain treatment data, we assumed that all the patients received standard treatments.

Conclusions

The unfolded protein response (UPR) is associated with carcinogenesis, cell proliferation-related and pro-cancerous gene sets, clinical aggressiveness, and tumor microenvironment of breast cancer. Low UPR is associated with better survival in breast cancer, not only in primary ER-positive/HER2-negative BC, but also in metastatic BC. UPR score can be used as a biomarker to predict prognosis in breast cancer and further study is warranted to investigate its potential as a novel therapeutic target in breast cancer.

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Disclosure of conflict of interest

None.

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References

[1] Sung H, Ferlay J, Siegel RL, Laversanne M, Soerjomataram I, Jemal A and Bray F. Global cancer statistics 2020: globocan estimates of incidence and mortality worldwide for 36 cancers in 185 countries. CA Cancer J Clin 2021; 71: 209-249.

[2] https://seer.cancer.gov/statfacts/html/breast.html.

[3] Wang WA, Groenendyk J and Michalak M. Endoplasmic reticulum stress associated responses in cancer. Biochim Biophys Acta 2014; 1843: 2143-2149.

[4] Siwecka N, Rozpedek W, Pytel D, Wawrzynkiewicz A, Dziki A, Dziki L, Diehl JA and Majsterek I. Dual role of endoplasmic reticulum stress-mediated unfolded protein response signaling pathway in carcinogenesis. Int J Mol Sci 2019; 20: 4354.

[5] Stolz A and Wolf DH. Endoplasmic reticulum associated protein degradation: a chaperone assisted journey to hell. Biochim Biophys Acta 2010; 1803: 694-705.

[6] Naidoo N. ER and aging-protein folding and the ER stress response. Ageing Res Rev 2009; 8: 150-159.

[7] Chakrabarti A, Chen AW and Varner JD. A review of the mammalian unfolded protein response. Biotechnol Bioeng 2011; 108: 2777-2793.

[8] Ma YJ and Hendershot LM. The role of the unfolded protein response in tumour development: friend or foe? Nat Rev Cancer 2004; 4: 966-977.

[9] Patel A, Oshi M, Yan L, Matsuyama R, Endo I and Takabe K. The unfolded protein response is associated with cancer proliferation and worse survival in hepatocellular carcinoma. Cancers (Basel) 2021; 13: 4443.

[10] Holohan C, Van Schaeybroeck S, Longley DB and Johnston PG. Cancer drug resistance: an evolving paradigm. Nat Rev Cancer 2013; 13: 714-726.

[11] Curtis C, Shah SP, Chin SF, Turashvili G, Rueda OM, Dunning MJ, Speed D, Lynch AG, Samarajiwa S, Yuan Y, Gräf S, Ha G, Haffari G, Bashashati A, Russell R and McKinney S; METABRIC Group, Langerød A, Green A, Provenzano E, Wishart G, Pinder S, Watson P, Markowetz F, Murphy L, Ellis I, Purushotham A, Børresen-Dale AL, Brenton JD, Tavaré S, Caldas C and Aparicio S. The genomic and transcriptomic architecture of 2,000 breast tumours reveals novel subgroups. Nature 2012; 486: 346-352.

[12] Brueffer C, Vallon-Christersson J, Grabau D, Ehinger A, Häkkinen J, Hegardt C, Malina J, Chen Y, Bendahl PO, Manjer J, Malmberg M, Larsson C, Loman N, Rydén L, Borg Å and Saal LH. Clinical value of rna sequencing-based classifiers for prediction of the five conventional breast cancer biomarkers: a report from the population-based multicenter Sweden cancerome analysis network-breast initiative. JCO Precis Oncol 2018; 2: PO.17.00135.

[13] Cerami E, Gao J, Dogrusoz U, Gross BE, Sumer SO, Aksoy BA, Jacobsen A, Byrne CJ, Heuer ML, Larsson E, Antipin Y, Reva B, Goldberg AP, Sander C and Schultz N. The cBio cancer genomics portal: an open platform for exploring multidimensional cancer genomics data. Cancer Discov 2012; 2: 401-404.

[14] Curtis C, Shah SP, Chin SF, Turashvili G, Rueda OM, Dunning MJ, Speed D, Lynch AG, Samarajiwa S, Yuan Y, Graf S, Ha G, Haffari G, Bashashati A, Russell R and McKinney S; METABRIC Group, Langerod A, Green A, Provenzano E, Wishart G, Pinder S, Watson P, Markowetz F, Murphy L, Ellis I, Purushotham A, Borresen-Dale AL, Brenton JD, Tavare S, Caldas C and Aparicio S. The genomic and transcriptomic architecture of 2,000 breast tumours reveals novel subgroups. Nature 2012; 486: 346-352.

[15] Gao JJ, Aksoy BA, Dogrusoz U, Dresdner G, Gross B, Sumer SO, Sun YC, Jacobsen A, Sinha R, Larsson E, Cerami E, Sander C and Schultz N. Integrative analysis of complex cancer genomics and clinical profiles using the cBioPortal. Sci Signal 2013; 6: pl1.

[16] Edgar R, Domrachev M and Lash AE. Gene expression omnibus: NCBI gene expression and hybridization array data repository. Nucleic Acids Res 2002; 30: 207-210.

[17] Chung WS, Eum HH, Lee HO, Lee KM, Lee HB, Kim KT, Ryu HS, Kim S, Lee JE, Park YH, Kan ZY, Han W and Park WY. Single-cell RNA-seq enables comprehensive tumour and immune cell profiling in primary breast cancer. Nat Commun 2017; 8: 15081.

[18] Sinn BV, Fu C, Lau R, Litton J, Tsai TH, Murthy R, Tam A, Andreopoulou E, Gong Y, Murthy R, Gould R, Zhang Y, King TA, Viale A, Andrade V, Giri D, Salgado R, Laios I, Sotiriou C, Marginean EC, Kwiatkowski DN, Layman RM, Booser D, Hatzis C, Vicente Valero V and Fraser Symmans W. $SET_{ER/PR}$: a robust 18-gene predictor for sensitivity to endocrine therapy for metastatic breast cancer. NPJ Breast Cancer 2019; 5: 16.

[19] Aran D, Hu ZC and Butte AJ. xCell: digitally portraying the tissue cellular heterogeneity landscape. Genome Biol 2017; 18: 220.

[20] Subramanian A, Tamayo P, Mootha VK, Mukherjee S, Ebert BL, Gillette MA, Paulovich A, Pomeroy SL, Golub TR, Lander ES and Mesirov JP. Gene set enrichment analysis: a knowledge-based approach for interpreting genome-wide expression profiles. Proc Natl Acad Sci U S A 2005; 102: 15545-15550.

[21] Liberzon A, Birger C, Thorvaldsdottir H, Ghandi M, Mesirov JP and Tamayo P. The molecular signatures database (MSigDB) hallmark gene set collection. Cell Syst 2015; 1: 417-425.

[22] Oshi M, Katsuta E, Yan L, Ebos JML, Rashid OM, Matsuyama R, Endo I and Takabe K. A novel 4-gene score to predict survival, distant metastasis and response to neoadjuvant therapy in breast cancer. Cancers (Basel) 2020; 12: 1148.

[23] Oshi M, Takahashi H, Tokumaru Y, Yan L, Rashid OM, Matsuyama R, Endo I and Takabe K. G2M cell cycle pathway score as a prognostic biomarker of metastasis in estrogen receptor (er)-positive breast cancer. Int J Mol Sci 2020; 21: 2921.

[24] Okano M, Oshi M, Butash AL, Katsuta E, Tachibana K, Saito K, Okayama H, Peng X, Yan L, Kono K, Ohtake T and Takabe K. Triple-negative breast cancer with high levels of annexin A1 expression is associated with mast cell infiltration, inflammation, and angiogenesis. Int J Mol Sci 2019; 20: 4197.

[25] Oshi M, Angarita FA, Tokumaru Y, Yan L, Matsuyama R, Endo I and Takabe K. High expression of NRF2 is associated with increased tumor-infiltrating lymphocytes and cancer immunity in ER-positive/HER2-negative breast cancer. Cancers (Basel) 2020; 12: 3856.

[26] Oshi M, Newman S, Tokumaru Y, Yan L, Matsuyama R, Endo I, Katz MHG and Takabe K. High G2M pathway score pancreatic cancer is associated with worse survival, particularly after margin-positive (R1 or R2) resection. Cancers (Basel) 2020; 12: 2871.

[27] Oshi M, Newman S, Tokumaru Y, Yan L, Matsuyama R, Endo I, Nagahashi M and Takabe K. Intra-Tumoral angiogenesis is associated with inflammation, immune reaction and metastatic recurrence in breast cancer. Int J Mol Sci 2020; 21: 6708.

[28] Oshi M, Takahashi H, Tokumaru Y, Yan L, Rashid OM, Nagahashi M, Matsuyama R, Endo I and Takabe K. The E2F pathway score as a predictive biomarker of response to neoadjuvant therapy in ER+/HER2- breast cancer. Cells 2020; 9: 1643.

[29] Oshi M, Newman S, Tokumaru Y, Yan L, Matsuyama R, Endo I and Takabe K. Inflammation Is associated with worse outcome in the whole cohort but with better outcome in triple-negative subtype of breast cancer patients. J Immunol Res 2020; 2020: 5618786.

[30] Oshi M, Tokumaru Y, Angarita FA, Yan L, Matsuyama R, Endo I and Takabe K. Degree of early estrogen response predict survival after endocrine therapy in primary and metastatic ER-positive breast cancer. Cancers (Basel) 2020; 12: 3557.

[31] Gandhi S, Oshi M, Murthy V, Repasky EA and Takabe K. Enhanced thermogenesis in triple-negative breast cancer is associated with pro-tumor immune microenvironment. Cancers (Basel) 2021; 13: 2559.

[32] Schulze A, Oshi M, Endo I and Takabe K. MYC targets scores are associated with cancer aggressiveness and poor survival in ER-positive primary and metastatic breast cancer. Int J Mol Sci 2020; 21: 8127.

[33] Pages F, Galon J, Dieu-Nosjean MC, Tartour E, Sautes-Fridman C and Fridman WH. Immune

infiltration in human tumors: a prognostic factor that should not be ignored. Oncogene 2010; 29: 1093-1102.
[34] Stovgaard ES, Nielsen D, Hogdall E and Balslev E. Triple negative breast cancer - prognostic role of immune-related factors: a systematic review. Acta Oncol 2018; 57: 74-82.
[35] Zhao X, Liu JY, Ge SS, Chen C, Li S, Wu XY, Feng XY, Wang YQ and Cai DY. Saikosaponin A inhibits breast cancer by regulating Th1/Th2 balance. Front Pharmacol 2019; 10: 624.
[36] Knutson KL and Disis ML. Tumor antigen-specific T helper cells in cancer immunity and immunotherapy. Cancer Immunol Immunother 2005; 54: 721-728.
[37] Pan YY, Yu YD, Wang XJ and Zhang T. Tumor-associated macrophages in tumor immunity. Front Immunol 2020; 11: 583084.
[38] Silva-Santos B, Serre K and Norell H. γδ T cells in cancer. Nat Rev Immunol 2015; 15: 683-691.
[39] Tokumaru Y, Oshi M, Katsuta E, Yan L, Huang JL, Nagahashi M, Matsuhashi N, Futamura M, Yoshida K and Takabe K. Intratumoral adipocyte-high breast cancer enrich for metastatic and inflammation-related pathways but associated with less cancer cell proliferation. Int J Mol Sci 2020; 21: 5744.
[40] Oshi M, Huyser MR, Le L, Tokumaru Y, Yan L, Matsuyama R, Endo I and Takabe K. Abundance of microvascular endothelial cells is associated with response to chemotherapy and prognosis in colorectal cancer. Cancers (Basel) 2021; 13: 1477.
[41] Huang JJ, Pan HY, Wang JG, Wang T, Huo XY, Ma Y, Lu ZY, Sun B and Jiang HC. Unfolded protein response in colorectal cancer. Cell Biosci 2021; 11: 26.
[42] Papaioannou A and Chevet E. Driving cancer tumorigenesis and metastasis through upr signaling. Curr Top Microbiol Immunol 2018; 414: 159-192.
[43] Oshi M, Patel A, Le L, Tokumaru Y, Yan L, Matsuyama R, Endo I and Takabe K. G2M checkpoint pathway alone is associated with drug response and survival among cell proliferation-related pathways in pancreatic cancer. Am J Cancer Res 2021; 11: 3070-3084.
[44] Hanahan D and Weinberg RA. Hallmarks of cancer: the next generation. Cell 2011; 144: 646-674.
[45] Chen X, Iliopoulos D, Zhang Q, Tang QZ, Greenblatt MB, Hatziapostolou M, Lim E, Tam WL, Ni M, Chen YW, Mai JH, Shen HF, Hu DZ, Adoro S, Hu B, Song M, Tan C, Landis MD, Ferrari M, Shin SJ, Brown M, Chang JC, Liu XS and Glimcher LH. XBP1 promotes triple-negative breast cancer by controlling the HIF1alpha pathway. Nature 2014; 508: 103-107.
[46] Hu R, Warri A, Jin L, Zwart A, Riggins RB, Fang HB and Clarke R. NF-kappaB signaling is required for XBP1 (unspliced and spliced)-mediated effects on antiestrogen responsiveness and cell fate decisions in breast cancer. Mol Cell Biol 2015; 35: 379-390.
[47] Nagelkerke A, Bussink J, van der Kogel AJ, Sweep FC and Span PN. The PERK/ATF4/LAMP3-arm of the unfolded protein response affects radioresistance by interfering with the DNA damage response. Radiother Oncol 2013; 108: 415-421.
[48] Notte A, Rebucci M, Fransolet M, Roegiers E, Genin M, Tellier C, Watillon K, Fattaccioli A, Arnould T and Michiels C. Taxol-induced unfolded protein response activation in breast cancer cells exposed to hypoxia: ATF4 activation regulates autophagy and inhibits apoptosis. Int J Biochem Cell Biol 2015; 62: 1-14.
[49] Yan MM, Ni JD, Song DY, Ding ML and Huang J. Activation of unfolded protein response protects osteosarcoma cells from cisplatin-induced apoptosis through NF-kappaB pathway. Int J Clin Exp Pathol 2015; 8: 10204-10215.
[50] Del Vecchio CA, Feng Y, Sokol ES, Tillman EJ, Sanduja S, Reinhardt F and Gupta PB. De-differentiation confers multidrug resistance via noncanonical PERK-Nrf2 signaling. PLoS Biol 2014; 12: e1001945.
[51] Kim KW, Moretti L, Mitchell LR, Jung DK and Lu B. Endoplasmic reticulum stress mediates radiation-induced autophagy by perk-eIF2alpha in caspase-3/7-deficient cells. Oncogene 2010; 29: 3241-3251.
[52] Jamora C, Dennert G and Lee AS. Inhibition of tumor progression by suppression of stress protein GRP78/BiP induction in fibrosarcoma B/C10ME. Proc Natl Acad Sci U S A 1996; 93: 7690-7694.
[53] Fernandez PM, Tabbara SO, Jacobs LK, Manning FC, Tsangaris TN, Schwartz AM, Kennedy KA and Patierno SR. Overexpression of the glucose-regulated stress gene GRP78 in malignant but not benign human breast lesions. Breast Cancer Res Treat 2000; 59: 15-26.
[54] Oshi M, Kim TH, Tokumaru Y, Yan L, Matsuyama R, Endo I, Cherkassky L and Takabe K. Enhanced DNA repair pathway is associated with cell proliferation and worse survival in hepatocellular carcinoma (HCC). Cancers (Basel) 2021; 13: 323.
[55] Tokumaru Y, Oshi M, Katsuta E, Yan L, Satyananda V, Matsuhashi N, Futamura M, Akao Y, Yoshida K and Takabe K. KRAS signaling enriched triple negative breast cancer is associated with favorable tumor immune microenvironment and better survival. Am J Cancer Res 2020; 10: 897-907.

[56] Sari IN, Phi LTH, Jun N, Wijaya YT, Lee S and Kwon HY. Hedgehog signaling in cancer: a prospective therapeutic target for eradicating cancer stem cells. Cells 2018; 7: 208.
[57] Ribatti D, Tamma R and Annese T. Epithelial-mesenchymal transition in cancer: a historical overview. Transl Oncol 2020; 13: 100773.
[58] Thorsson V, Gibbs DL, Brown SD, Wolf D, Bortone DS, Ou Yang TH, Porta-Pardo E, Gao GF, Plaisier CL, Eddy JA, Ziv E, Culhane AC, Paull EO, Sivakumar IKA, Gentles AJ, Malhotra R, Farshidfar F, Colaprico A, Parker JS, Mose LE, Vo NS, Liu JF, Liu YX, Rader J, Dhankani V, Reynolds SM, Bowlby R, Califano A, Cherniack AD, Anastassiou D, Bedognetti D, Mokrab Y, Newman AM, Rao A, Chen K, Krasnitz A, Hu H, Malta TM, Noushmehr H, Pedamallu CS, Bullman S, Ojesina AI, Lamb A, Zhou WD, Shen H, Choueiri TK, Weinstein JN, Guinney J, Saltz J, Holt RA, Rabkin CS, Cancer Genome Atlas Research N, Lazar AJ, Serody JS, Demicco EG, Disis ML, Vincent BG and Shmulevich I. The Immune landscape of cancer. Immunity 2018; 48: 812-830, e14.
[59] Ramirez MU, Hernandez SR, Soto-Pantoja DR and Cook KL. Endoplasmic reticulum stress pathway, the unfolded protein response, modulates immune function in the tumor microenvironment to impact tumor progression and therapeutic response. Int J Mol Sci 2019; 21: 169.
[60] Denton AE, Roberts EW and Fearon DT. Stromal cells in the tumor microenvironment. Adv Exp Med Biol 2018; 1060: 99-114.
[61] Gonzalez-Perez RR, Lanier V and Newman G. Leptin's pro-angiogenic signature in breast cancer. Cancers (Basel) 2013; 5: 1140-1162.
[62] Gyamfi J, Eom M, Koo JS and Choi J. Multifaceted roles of interleukin-6 in adipocyte-breast cancer cell interaction. Transl Oncol 2018; 11: 275-285.

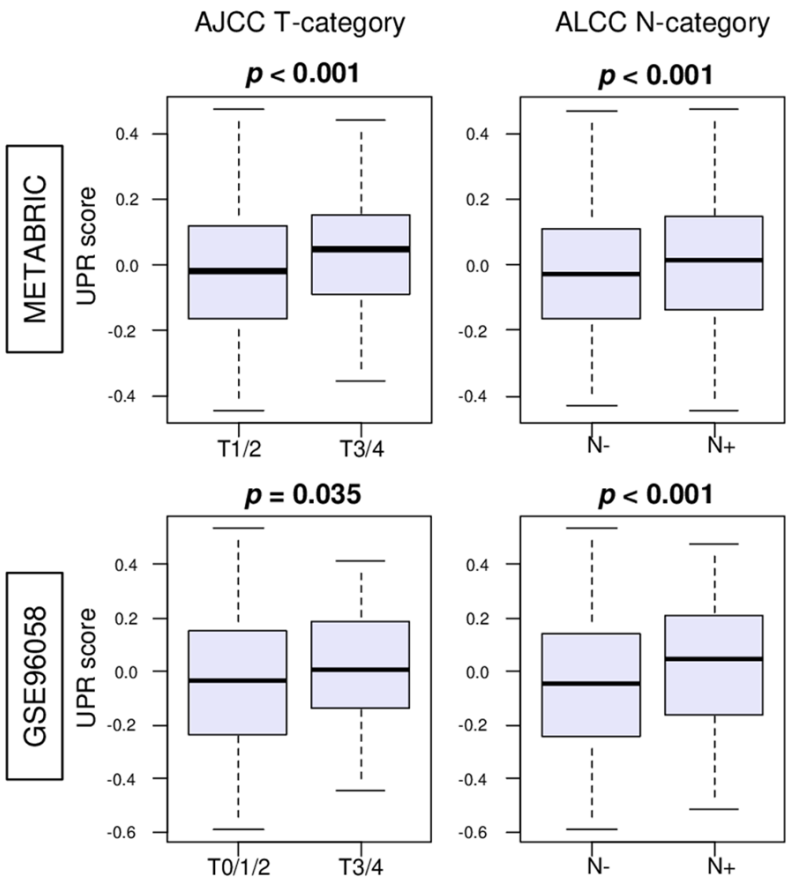

Figure S1. Association of UPR with AJCC T and N categories of breast cancer in the METABRIC and GSE96058 cohorts. Boxplots of UPR levels by AJCC T-category (T1/2 and T3/4 groups) and N-category (N-negative and N-positive). *P*-value was analyzed by Kruskal-Wallis test.

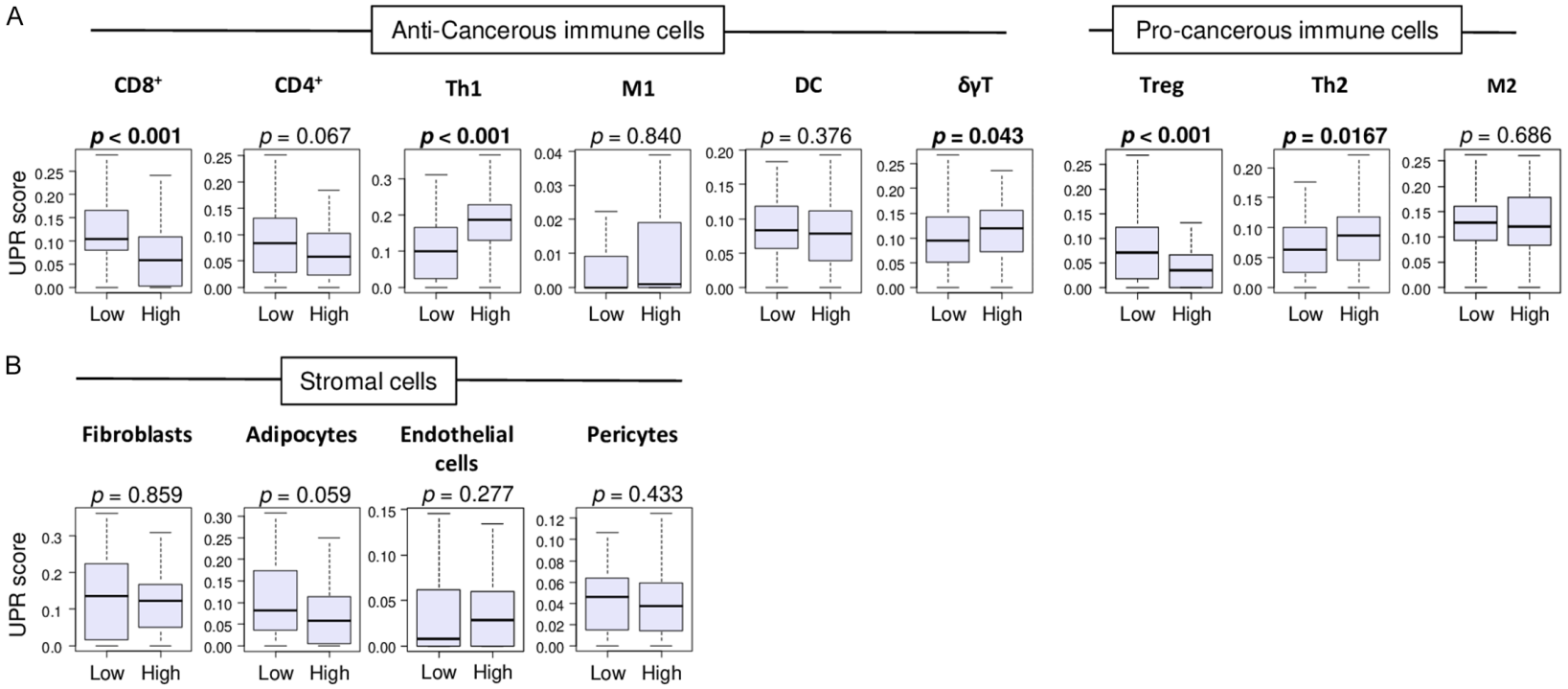

Figure S2. Association of UPR with tumor environment (TME) in metastatic breast cancer. Boxplots of immune cells; anti-cancerous immune cells (CD8+ T cells, CD4+ T cells, T helper type1 (Th1) cells, M1 macrophages, dendritic cells (DC), and γδT cells, as well as pro-cancerous immune cells (Tregs, Th2 cells, and M2 macrophages), and stromal cells (Fibroblasts, Adipocytes, Endothelial cells, and Pericytes), by low and high UPR in metastatic breast cancer in GSE124647 cohort. Median cut-off was used to perform the analysis. *P*-value was analyzed by Mann-Whitney U test.