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Original Article

The stabilization of PD-L1 by the endoplasmic reticulum stress protein GRP78 in triple-negative breast cancer

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Abstract: The immune checkpoint blockade therapy has emerged as encouraging treatment strategies in various cancer types. Anti-PD-L1 (programmed death-ligand 1) antibodies have been approved for triple-negative breast cancer, however the response rate yet to be optimized. It would be imperative to further understand and investigate the molecular mechanisms of PD-L1 regulation. Here, we identified glucose regulatory protein 78 (GRP78), a major endoplasmic reticulum (ER) stress responding protein, as a novel binding partner of PD-L1. GRP78 interacts with PD-L1 at the ER region and increases PD-L1 levels via regulating its stability. ER stress, triggered by different stimuli such as conventional chemotherapy, leads to the induction of PD-L1 in a GRP78-dependent manner. We showed that GRP78 modulates the response to chemotherapy, and dual-high levels of GRP78 and PD-L1 correlates with poor relapse-free survival in triple-negative breast cancer. Altogether, our study provides novel molecular insights into the regulatory mechanism of PD-L1 by revealing its interaction with GRP78, and offers a rationale to target GRP78 as a potential therapeutic strategy to enhance anti-tumor immunity.

Keywords: Triple-negative breast cancer, ER stress, GRP78, PD-L1

Introduction

Immune response and homeostasis within the human body help to defense pathogens, eliminate abnormal cells, and maintain self-tolerance with immunological memory [1]. To avoid hyperactive uncontrolled immune response, co-inhibitory immune proteins modulate and stabilize the T cell functions [2-4]. Among these co-inhibitory pathways, the PD-1/PD-L1 axis suppresses T cell-mediated immune responses, including cytolytic activities of CD8 T cells [5, 6]. This mechanism facilitates tumor growth by escaping host immune surveillance [7]. Tumors normally express a high protein level of PD-L1, which correlate with poor prognosis in many cancers, including melanoma, breast cancer, lung cancer, colorectal cancer, and renal cell carcinoma [8-12]. And in lung cancer, it was further shown to lead to low tumor-infiltrating T cells [9]. Additionally, PD-L1 is expressed on macrophages, tumor environmental dendritic cells, tumor-draining lymph node dendritic cells, fibroblasts, and T cells, further reducing the anti-tumor immunity [13]. Moreover, exosomal PD-L1 suppresses the T cell activity and promotes tumor growth in breast cancer [14]. Therefore, cancer immunotherapy, by exploiting the immune system to detect and eliminate tumor cells, becomes an important therapeutic approach for cancer treatment. Accordingly, these preclinical studies lead to the development of antibodies against PD-1 and PD-L1, which have 10-55% durable response rates clinically.

Genomic aberrations, transcriptional regulation of inflammatory and oncogenic signaling pathways, post-transcriptional regulation, microRNA-based control, and post-translational modifications are known regulatory mechanisms of PD-L1 [15-19]. It has been reported that the response rates of anti-PD-L1 antibodies positively correlated with the membrane PD-L1 level. Meanwhile, glycosylation is a significant post-translational modification to stabilize the PD-L1 by facilitating the folding, intracellular protein transport, and immunogenic function [20, 21]. PD-L1 is heavily N-linked glycosylated by the initial step of the insertion of PD-L1 into the ER, which is crucial for its immunosuppressive function [18]. The terminal residues of N-glycans are required for the quality control of protein folding within the ER region. This process would determine the protein turnover [22]. The regulation of PD-L1 protein to enhance its immunosuppressive function has been extensively studied, but it is not comprehensively defined. Immunohistochemical (IHC) staining of PD-L1 could not precisely predict the response rate of anti-PD-L1 therapy due to different staining methods [23, 24]. Therefore, further understanding of the post-translation modifications of PD-L1 is necessary to determine the surface expression of PD-L1 and maximize the clinical benefits of immunotherapy.

Triple-negative breast cancer has defined as lack of estrogen receptor, progesterone receptor and amplification of the human epidermal growth factor-2 gene. Among all breast cancer subtypes, triple-negative breast cancer accounts for 15-20% with chemotherapy as a mainstay in front-line therapy for treating patients in advanced stages [25]. However, with its more aggressive behavior and limited treatment options, triple-negative breast cancer patients have worse outcomes compared with other subtypes. Currently, atezolizumab, an anti-PD-L1 antibody approved by the FDA for metastatic triple-negative breast cancer (Impassion 130 trial) could provide around an additional 10% response rate than the nab (Nanoparticle albumin-bound)-paclitaxel alone group, with 16% higher response rate in the PD-L1-positive subgroup [26]. By using the combination of atezolizumab with nab-paclitaxel, this strategy provides seven months longer overall survival within the PD-L1-positive metastatic triple-negative breast cancer [27]. Despite some studies with favorable objective response rates and durable response, there is significant inconsistency regarding the clinical response by PD-L1 expression level. Consequently, a better understanding of this PD-L1 regulatory network could provide more effective, feasible alternative interventions to improve therapeutic outcomes.

Glucose regulated protein 78 (GRP78), known as Heat Shock 70kDa Protein 5 (HSPA5) and immunoglobulin heavy chain binding protein (BIP), belongs to the heat shock protein 70 (HSP70) family. GRP78 serves as a central chaperone protein within the ER, facilitating adequate protein folding, and quality control. GRP78 could bind to misfolded proteins and unassembled complexes to initiate ER-associated degradation (ERAD) which is responsible for unfolded protein responses (UPR) [28]. The UPR is a cell stress response activated by ER stress, including hypoxia, glucose deprivation, and inflammation. In cancer cells, GRP78 responds to unfolded protein response and maintains cellular homeostasis, contributing to cancer survival and progression [29]. Besides, relocalization of a fraction of GRP78 from ER to cell surface while GRP78 is overexpressed, regulates signaling functions involving proliferation, apoptosis, and immunity [30]. In several cancer types, overexpression of GRP78 has been demonstrated and usually positively correlates with poor prognosis or tumor malignancy [31, 32]. But it is not yet fully understood that how GRP78 could influence the post-translational modification of PD-L1 and the prognosis in triple-negative breast cancer. Herein, we explored the role of GRP78 in regulating PD-L1 expression among triple-negative breast cancer. We demonstrated that GRP78 positively correlated with total PD-L1 protein expression and the membrane PD-L1 expression. Furthermore, GRP78 influenced the cell response to chemotherapy and might be considered as a predictive biomarker of anti-PD-L1 antibodies in triple-negative breast cancer patients.

Materials and methods

Chemicals and antibodies

Thapsigargin, MG-132, and cycloheximide were obtained from Sigma-Aldrich. HA-15, doxorubicin, and cisplatin were purchased from Selleck

Chemical (S8299), Cayman Chemical (14595-500), and ApexBio (A8321), respectively. The following antibodies were used for western blotting: GRP78 (NBP1-06274; Novus biologicals), HSP90 (13171-AP; Proteintech), PD-L1 (GTX104763; GeneTex, 13684S; Cell Signaling Technology), α-tubulin (T5168; Sigma-Aldrich), HSPA6 (13616-1-AP; Proteintech), GAPDH (10494-1-AP; Proteintech), EGFR (C74B9; Cell Signaling Technology), HSP105B (AF4029-SP; Novus biologicals), β-actin (A2228; Sigma-Aldrich), FLAG tag (14793; Cell Signaling Technology), and His-tag (66005-1-Ig; Proteintech). Other antibodies used in the study include APC-labeled PD-L1 (catalog 329707; BioLegend), Alexa Fluor 594 and 488 (A-21203 and A-21208; 1:300; ThermoFisher), PD-L1 (66-248-1-Ig; 1:100; Proteintech), and HSP90B1 (nb300-619; 1:100; Novus Biologicals).

Cell culture

The cancer cell lines, including triple-negative breast cancer (MDA-MB-231 and BT-549), pancreatic adenocarcinoma (HPAC and BxPC-3) and HEK 293T were purchased from the ATCC (American Type Culture Collection; Manassas, VA, USA). All cancer cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM)/F12, supplemented with 10% of fetal bovine serum (FBS) and 1% of the antibiotics at 37°C and 5% CO_2, and tested negative for mycoplasma contamination.

Plasmids and transfection

Synthetic small interfering RNAs (siRNAs) specific for GRP78, 5'-GGAGCGCAUUGAUACUAGA-3' (sense) and 5'-UCUAGUAUCAAUGCGCUCC-3' (antisense) were purchased from Sigma-Aldrich. The siRNA was transfected into BT-549 breast cancer cells with an electroporator (Nucleofector II, Lonza) per the manufacturer's instructions. For stable knock-down of GRP78 cells, the shRNAs (catalog TRCN0000231124, TRCN0000231123, and TRCN0000218611) were obtained from Sigma-Aldrich. The construct, pGIPZ-shPD-L1/Flag-PD-L1, for PD-L1 knock-down and re-expression of FLAG PD-L1, was made as described in the previous study [18]. In short, we replaced the GFP cDNA within a pGIPZ-sh PD-L1 construct (3'-UTR of PD-L1, TTGACTCCATCTTTCTTCA; Thermo Fisher Scientific) with the FLAG-tagged PD-L1 WT (shRNA and ORFeome Core, MD Anderson). The pCDH-EF1α-GRP78-His plasmid was constructed by subcloning pcDNA3.1(+)-GRP78/BiP (Addgene) conjugated with His-tag into the pCDH-EF1α vector. Lentivirus against GRP78 and GRP78-overexpression was packaged into HEK293T cells and used to infect BT-549 cells to produce GRP78 knock-down cells and GRP78 overexpression cells.

Western blot analysis and co-immunoprecipitation

We lysed the whole cells in the lysis buffer containing 50 mM Tris (pH 8.0), 150 mM NaCl (Sodium chloride), 1% NP-40, 0.1% SDS (sodium dodecyl sulfate), 0.5% sodium deoxycholate, and the commercial protease inhibitor cocktail (B14001, Biotool). For co-immunoprecipitation, we lysed cells in the lysis buffer. Lysates were further incubated with PD-L1 antibodies (13684; Cell Signaling Technology) at 4°C overnight and pulled down by the protein G magnetic bead (1614023; Bio-Rad) at 4°C cold room for 6 hours with an adequate amount of IgG as the negative control. We analyzed the IP precipitants by SDS-PAGE and western blot.

Flow cytometric analysis

We prepared cell suspensions in the staining buffer (554656, BD Biosciences). We then stained cancer cells with APC-labeled PD-L1 (catalog 329707; BioLegend) and GRP78 (NBP1-06274; Novus biologicals) antibodies according to standard protocols. We performed flow cytometric analysis with the BD FACSCanto II cytometer (BD Biosciences) and analyzed the data with the FlowJo software.

Immunofluorescence staining and Duo-link proximity ligation assay

We washed cells with cold PBS and fixed the cells with by 4% paraformaldehyde solution for 15 minutes at room temperature. We incubated slides in the blocking solution containing BSA for 60 minutes after washing with PBS. We then stained the slides with primary antibodies against GRP78 (NBP1-06274; 1:100; Novus biologicals) and PD-L1 (66248-1-Ig; 1:100; Proteintech) in PBS buffer at 4°C cold room overnight. To visualize the primary antibodies, we stained cells with secondary antibodies conjugated with Alexa Fluor 594 and 488 (A-21203 and A-21208; 1:300; ThermoFisher). We fur-

ther counterstained the cells with 4,6-diamidino-2-phenylindole (DAPI) before mounting slides. Images of confocal fluorescence were captured using a Zeiss LSM710 laser microscope. To demonstrate the interaction between GRP78 and PD-L1, cells were stained using Duo-link proximity ligation assay (catalog DU092101, Sigma-Aldrich) with primary antibodies to GRP-78 (NBP1-06274; 1:100; Novus biologicals) and PD-L1 (66248-1-Ig; 1:100; Proteintech) per the manufacturer's protocol. After proximity ligation assay, we stained cells with an antibody (nb300-619; 1:100; Novus Biologicals) to HSP90B1, which is an ER marker. Images were acquired using a Zeiss LSM710 laser microscope, as described above.

Cell viability assay

We measured cell viability using the MTS assay [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt] according to the instruction (Promega, USA). In short, we seeded the breast cancer cells (2×10^3 cells) in 96-well plates with 100 µl of culture medium for each well. We treated the cells with serially diluted concentrations of cytotoxic chemotherapy for 48 hours. Then, we added 20 µl of the MTS reagent to each well and incubated for 2 hours. We measured the absorbance using a microplate reader at 490 nm.

Statistical analysis

We performed statistical analyses with the GraphPad Prism software program, and the data are shown as means ± SD. We used the student's t-test to compare two groups. A comparison of the relapse-free survival curve was demonstrated as the log-rank test. *P* values < 0.05 were accepted as statistically significant.

Result

GRP78 is a novel binding partner of PD-L1

In an effort to look for novel PD-L1 interacting proteins by IP-mass spectrometric analysis, we identified several heat shock proteins (HSPA5, HSPA6, HSP105B, and HSPA1A) as the potential binding partners of PD-L1 (**Figure 1A**). Next, we validated the findings by IP-WB. As shown in **Figure 1B**, among these HSP proteins, HSPA5 (also known as GRP78), an ER stress regulator, was found to bind PD-L1 in both MDA-MB-231 and BT549 triple-negative breast cancer cells. Furthermore, this interaction was more prominent with the increase of GRP78 levels induced by thapsigargin, an ER stress inducer (**Figure 1C**, right panel).

GRP78 not only locates at the ER but also on the cell surface. Interestingly, we detected an increase in GRP78 levels on the cell surface upon thapsigargin stimulation by flow cytometric analysis (**Figure 1D**). We next determine the location of GRP78/PD-L1 interaction in the cell. The immunofluorescence (IF) result showed that PD-L1 (green) and GRP78 (red) mainly colocalized in the ER region and the signals of both proteins were enhanced after thapsigargin treatment, which were validated by proximity ligation assay (**Figure 1E** and **1F**). The colocalization of GRP78 and PD-L1 (red dots) within the ER (HSP90B1, green dots) but not on the cell surface (**Figure 1F**), and their interaction increased upon thapsigargin stimulation. Together, these results showed that GRP78 binds PD-L1 and their interaction mainly localized in the ER region.

GRP78 upregulates PD-L1 protein levels via promoting its stability

Next, we sought to decipher the functional significance of the interaction between GRP78 and PD-L1. The findings above suggest that GRP78 might help promote protein folding of PD-L1 in the ER region and regulate PD-L1 stability. To investigate whether GRP78 affects PD-L1 protein levels, we generated GRP78 stable knock-down BT-549 cells by using three different shRNAs, and found that GRP78 depletion led to a significant decrease in PD-L1 protein levels (**Figure 2A**, left panel). Conversely, GRP78 overexpression significantly increases PD-L1 levels in BT-549 cells (**Figure 2A**, right panel). To determine whether GRP78 could also have effects on the PD-L1 level on the cell membrane where it interacts with PD-1 to suppress T-cell response, we examined the membrane PD-L1 level by flow cytometric analysis in GRP78-knockdown or overexpressing cells. Consistent with the WB results, we found lower membrane PD-L1 levels in the GRP78-knockdown cells, and higher levels in the GRP78 overexpression cells (**Figure 2B**). Together, these results indicated that GRP78 positively regulated the PD-L1 protein levels.

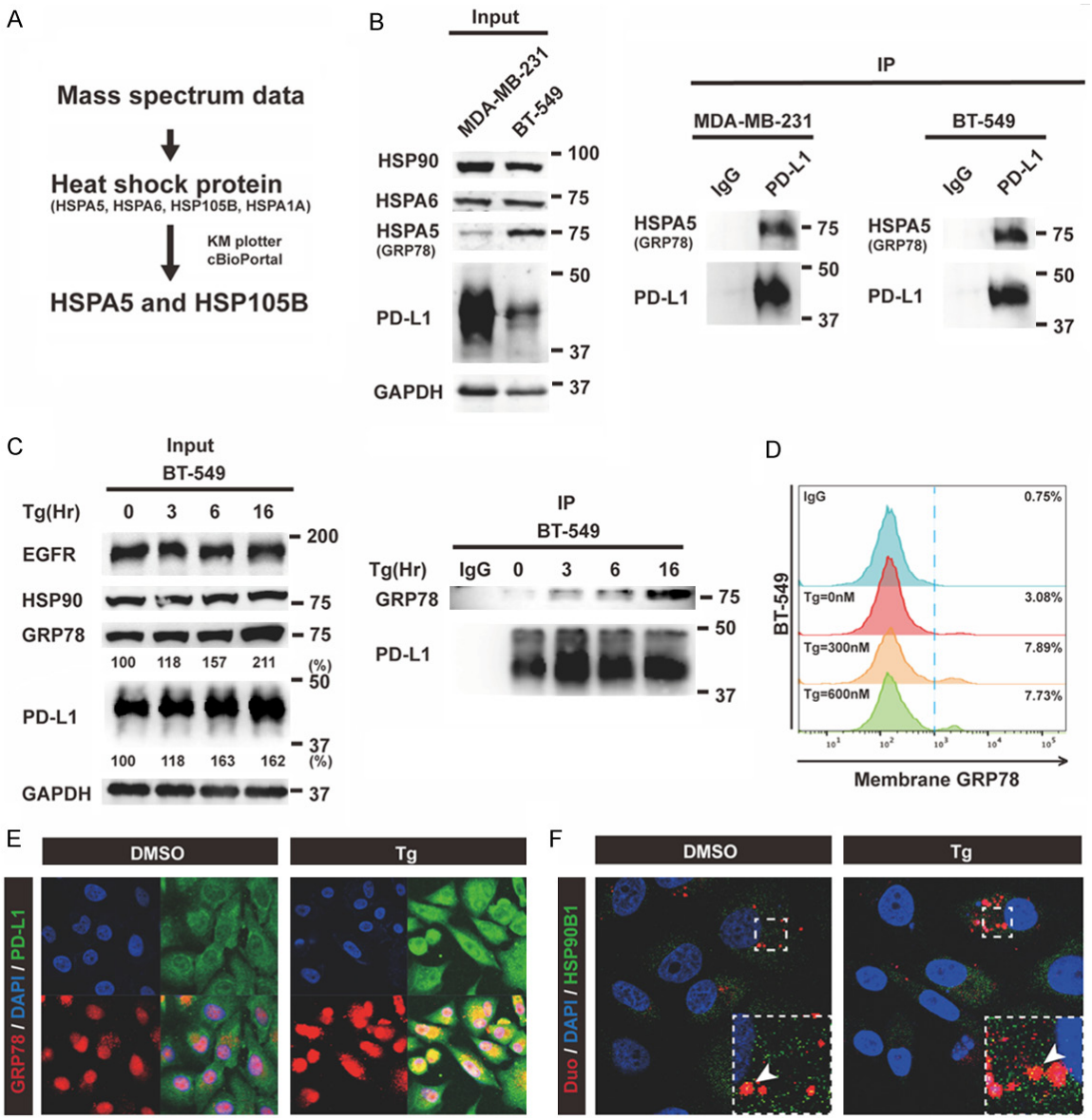

Figure 1. GRP78 is a novel binding partner of PD-L1. A. Schematic of the strategy by using these tools to identify potential heat shock proteins to affect the stability of PD-L1. B. Co-immunoprecipitation of western blot was shown to determine the interaction of GRP78 and PD-L1 in MDA-MB-231 and BT-549 cells. C. Co-immunoprecipitation of western blot was shown to determine the interaction of GRP78 and PD-L1. Quantification of indicated proteins were adjusted by GAPDH. D. Flow cytometric analysis of the effect of thapsigargin (16 hours) on surface GRP78 in BT549 cells. E. BT-549 cells treated with or without thapsigargin (16 hours) and immunostaining of BT-549 cells with antibodies against GRP78 and PD-L1. Scale bar, 100 μm. F. GRP78 interacts with PD-L1. Representative images of immunofluorescence staining of GRP78 and PD-L1 interaction in ER region in BT-549 cells treated with thapsigargin (16 hours) by Duo-link assay. The red dots (GRP78/PD-L1 interaction) indicate their interaction. Green fluorescence (HSP90B1) was used as ER marker, and DAPI as a nuclear marker.

Next, to investigate whether the PD-L1 regulation by GRP78 is mediated by post-transcriptional regulation, we treated GRP78 knock-down cells with MG132, a proteasome inhibitor. We found that MG132 could partially rescue the reduction of PD-L1 resulted from GRP78 depletion (**Figure 2C**, left panel), suggesting that GRP78 may affect the PD-L1 stability through the inhibition of the proteasome degradation process. To further validate the results, we knocked down endogenous PD-L1 in HEK 293T cells and then re-expressed exogenous

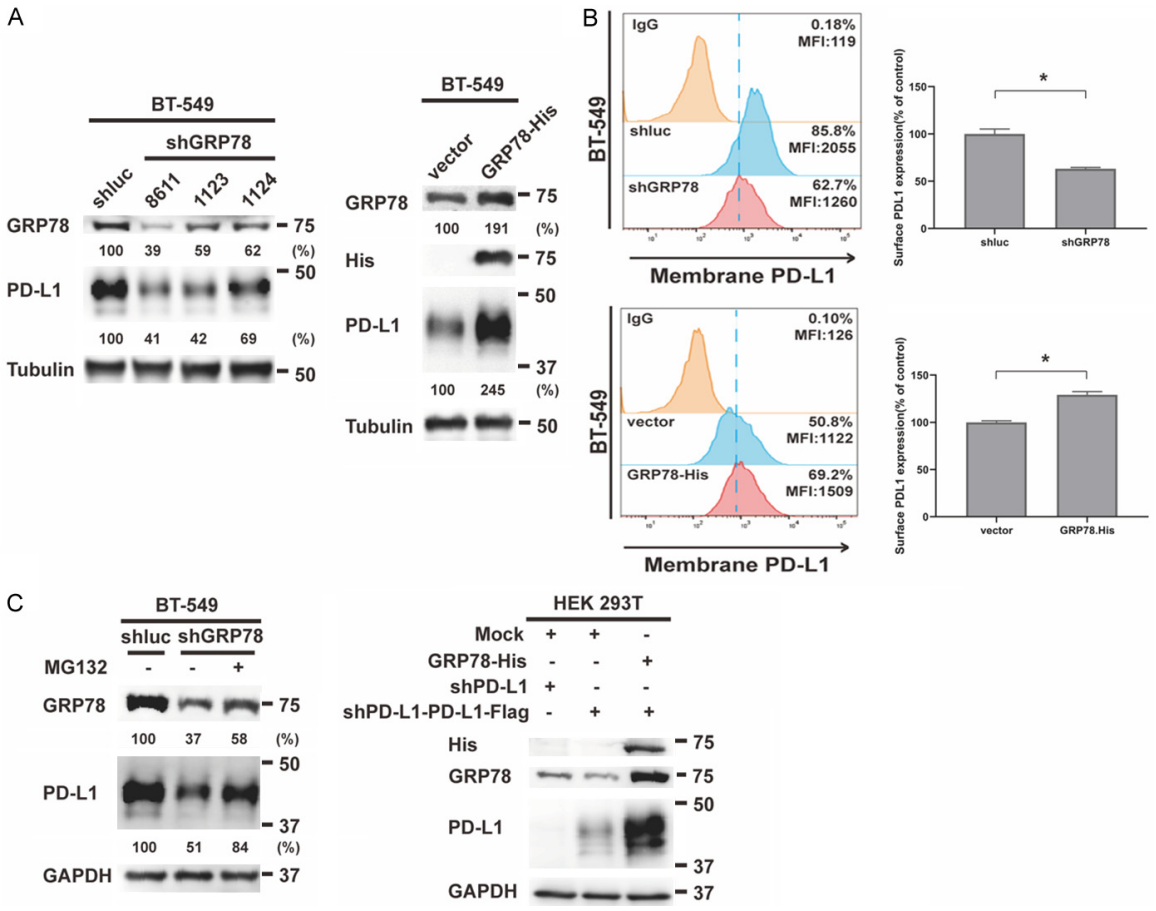

Figure 2. GRP78 upregulates PD-L1 protein levels. A. Western blot analysis of indicated protein in GRP78 knockdown and overexpression cells. B. Flow cytometric analysis of the surface PD-L1 expression in BT-549 cells. The mean fluorescence intensity (MFI) of each cell population was quantified by FlowJo and compared. Error bars represent S.D. (n = 3). C. Western blot analysis of indicated protein in BT-549 cells treated with 10 μm MG132 for 6 hours, and western blot analysis of GRP78 protein in GRP78-His and PD-L1 protein in PD-L1-Flag expressing HEK 293T cells. Quantification of indicated proteins were adjusted by tubulin or GAPDH. *P < 0.05, Student's t-test.

FLAG-tagged PD-L1, which is driven by CMV promoter. We demonstrated that overexpression of GRP78 also increased the exogenous PD-L1 protein level (**Figure 2C**, right panel). Collectively, these results showed that GRP78 could positively regulate PD-L1 protein levels via maintaining the stability of the PD-L1 protein.

ER stress induces PD-L1 levels in a GRP78-dependent manner

Since GRP78 functions as an essential master regulator for ER stress response and protein quality control [30], we asked whether ER stress could affect PD-L1 expression. To this end, we treated breast cancer cells, BT-549 and MDA-MB-231, with two ER stress inducers, thapsigargin (Tg) and HA-15. Thapsigargin causes calcium depletion within the ER, while HA-15 inhibits the ATPase activity of GRP78 to induce ER stress [33]. As expected, both drugs could induce the protein expression of GRP78. Meanwhile, we also found the upregulation of PD-L1 protein levels after treating cells with these two ER stress inducers (**Figure 3A**). Additionally, flow cytometric analysis showed that upon ER stress, membrane PD-L1 levels also increased (**Figure 3B**). To further validate these results, we treated other cancer types, including head and neck cancer (HN5 cells) and pancreatic adenocarcinoma (HPAC and BxPC-3 cells), with thapsigargin. We detected a similar up-regulation of both GRP78 and PD-L1 protein levels, whereas other heat shock proteins, including HSPA6 and HSP90, were not affect-

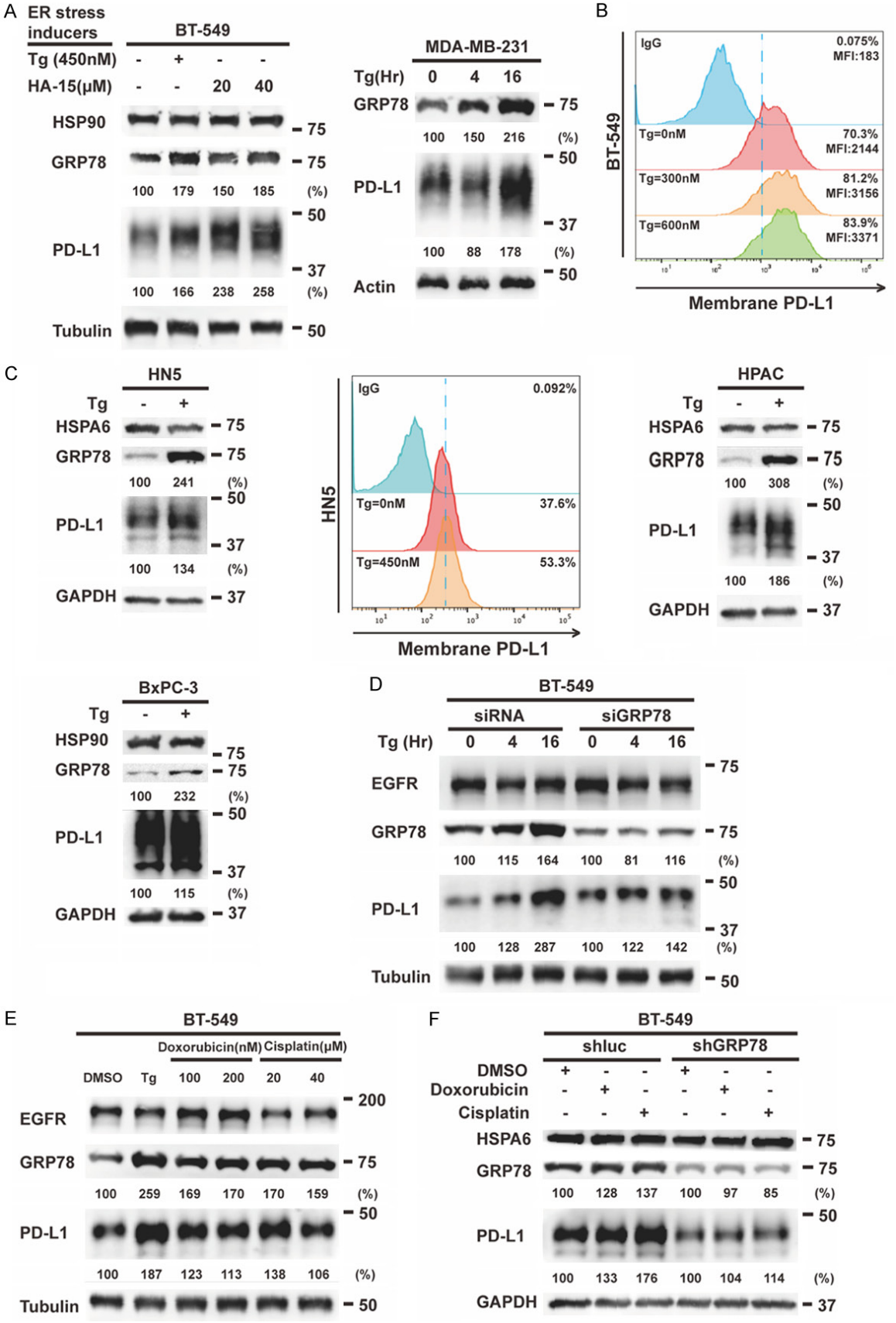
A
ER stress inducers
BT-549
Tg (450nM) - + - -
HA-15(μM) - - 20 40
HSP90
GRP78
100 179 150 185 (%)
PD-L1
100 166 238 258 (%)
Tubulin
MDA-MB-231
Tg(Hr) 0 4 16
GRP78
100 150 216 (%)
PD-L1
100 88 178 (%)
Actin
B
BT-549
IgG 0.075% MFI:183
Tg=0nM 70.3% MFI:2144
Tg=300nM 81.2% MFI:3156
Tg=600nM 83.9% MFI:3371
Membrane PD-L1
C
HN5
Tg - +
HSPA6
GRP78
100 241 (%)
PD-L1
100 134 (%)
GAPDH
HN5
IgG 0.092%
Tg=0nM 37.6%
Tg=450nM 53.3%
Membrane PD-L1
HPAC
Tg - +
HSPA6
GRP78
100 308 (%)
PD-L1
100 186 (%)
GAPDH
BxPC-3
Tg - +
HSP90
GRP78
100 232 (%)
PD-L1
100 115 (%)
GAPDH
D
BT-549
siRNA siGRP78
Tg (Hr) 0 4 16 0 4 16
EGFR
GRP78
100 115 164 100 81 116 (%)
PD-L1
100 128 287 100 122 142 (%)
Tubulin
E
BT-549
Doxorubicin(nM) Cisplatin(μM)
DMSO Tg 100 200 20 40
EGFR
GRP78
100 259 169 170 170 159 (%)
PD-L1
100 187 123 113 138 106 (%)
Tubulin
F
BT-549
shluc shGRP78
DMSO + - - + - -
Doxorubicin - + - - + -
Cisplatin - - + - - +
HSPA6
GRP78
100 128 137 100 97 85 (%)
PD-L1
100 133 176 100 104 114 (%)
GAPDH

Figure 3. ER stress induces PD-L1 levels in a GRP78-dependent manner. A. Western blots of indicated proteins in BT549 and MDA-MB-231 breast cancer cells treated with ER stress inducers (thapsigargin, Tg and HA-15) overnight (16 hours) or in time-dependent manner. B. Flow cytometric analysis of the effect of thapsigargin (16 hours) on surface PD-L1 in BT549 cells. C. Western blot analyses of indicated proteins in HN5 (head and neck cancer), HPAC, and BxPC-3 (pancreatic cancer) treated with thapsigargin overnight (16 hours). Flow cytometric analysis of the surface PD-L1 expression in HN5 cells under thapsigargin treatment. D. Western blot analysis of the effect of GRP78 using siRNA in BT-549 cells. E. BT-549 cells treated with doxorubicin and cisplatin overnight (16 hours) were analyzed by western blot analysis. F. BT-549 cells treated with doxorubicin (100 nM) and cisplatin (20 μM) overnight (16 hours) and analyzed by western blot analysis. Quantification of indicated proteins were adjusted by tubulin, actin, or GAPDH.

ed (**Figure 3C**). In addition, the cell surface PD-L1 levels were up-regulated upon thapsigargin stimulation in the head and neck cancer cell line (**Figure 3C**). To investigate whether GRP78 is essential for ER stress-mediated PD-L1 up-regulation, we transiently knocked down GRP-78 by siRNA, and showed that GRP78 protein level was decreased and ER stress-induced PD-L1 was abrogated (**Figure 3D**). Interestingly, the levels of EGFR, another cell surface membrane protein, were not affected by GRP-78 depletion, suggesting the specificity of PD-L1 regulation by GRP78. Together, these results suggested ER stress induces PD-L1 levels, and GRP78 is required for the process.

Chemotherapy drugs have been shown to induce ER stress in cancer cells. Therefore, we investigate whether cisplatin or doxorubicin, the chemotherapeutic agents commonly used in triple-negative breast cancer, could upregulate GRP78 and PD-L1 protein levels. As shown in **Figure 3E**, both chemo-drugs induced the expression of GRP78, the marker of ER stress, and this was accompanied by an increase in PD-L1 levels. To further clarify whether GRP78 is involved in chemotherapeutic agent-induced up-regulation of PD-L1 we treated GRP78 knock-down cells with cisplatin or doxorubicin, and found that the effects of PD-L1 up-regulation by chemotherapy were abrogated (**Figure 3F**). The results above indicated That GRP78 is important for chemotherapy-induced PD-L1 expression.

GRP78 modulates the response to chemotherapy in triple-negative breast cancer

Our finding that GRP78 is important for chemotherapy-induced PD-L1, which is involved in DNA damage repair [34], prompts us to investigate whether GRP78 affect the response to chemotherapy drug in triple-negative breast cancer. To test our hypothesis, we treated the cells with doxorubicin and evaluated the cell viability and the half-maximal inhibitory concentration (IC50). We found that GRP78 overexpression reduced the response to doxorubicin, with higher IC50 (IC50 = 228.4 nM) than that in control cells (IC50 = 168.9 nM) (**Figure 4A**, left panel); and GRP78 depletion increased the sensitivity of the cells to doxorubicin, with lower IC50 (IC50 = 398.8 nM, compared to IC50 = 490.1 nM in control cells) (**Figure 4A**, right panel). Similar results were obtained when these triple-negative breast cancer cells were treated with cisplatin (**Figure 4B**). Taken together, these results demonstrate that GRP78 negatively regulates the response to chemotherapy in triple-negative breast cancer.

To further investigate the clinical relevance of GRP78, we analyzed the correlation of GRP78 expression with the relapse-free survival in breast cancer patients from the Kaplan-Meier plotter (KM-Plotter) database (http://kmplot.com/analysis/) [35]. Among these heat shock proteins, we found that high HSPA5 mRNA levels were associated with poorer relapse-free survival among all breast cancer patients (n = 3951, Hazard ratio, HR = 1.35, 95% CI: 1.21-1.51, P = 1.1×10^{-7}) and triple-negative breast cancer patients (n = 255; HR = 1.57, 95% CI: 1-2.48, P = 0.05; **Figure 4C** and **4D**). Meanwhile, expression levels of HSPA6, HSP105B, and HSPA1A didn't have significant correlation in the triple-negative breast cancer. These results are consistent with previous reports that GRP78 (HSPA5) could be a marker of poor prognosis in breast cancer [36, 37]. We further analyzed the co-expression of GRP78 and PD-L1 as a prognostic marker in breast cancer. Dual-high levels of GRP78 and PD-L1 mRNA expression in the TCGA dataset predicted poor outcome in terms of relapse-free survival among all breast cancer patients (n = 1764, HR = 1.46, 95% CI: 1.23-1.73, P = 1.2×10^{-5}) and triple-negative breast cancer patients (n = 161, HR = 1.83, 95% CI: 1.03-3.24, P = 0.036; **Figure 4E**). Altogether, the

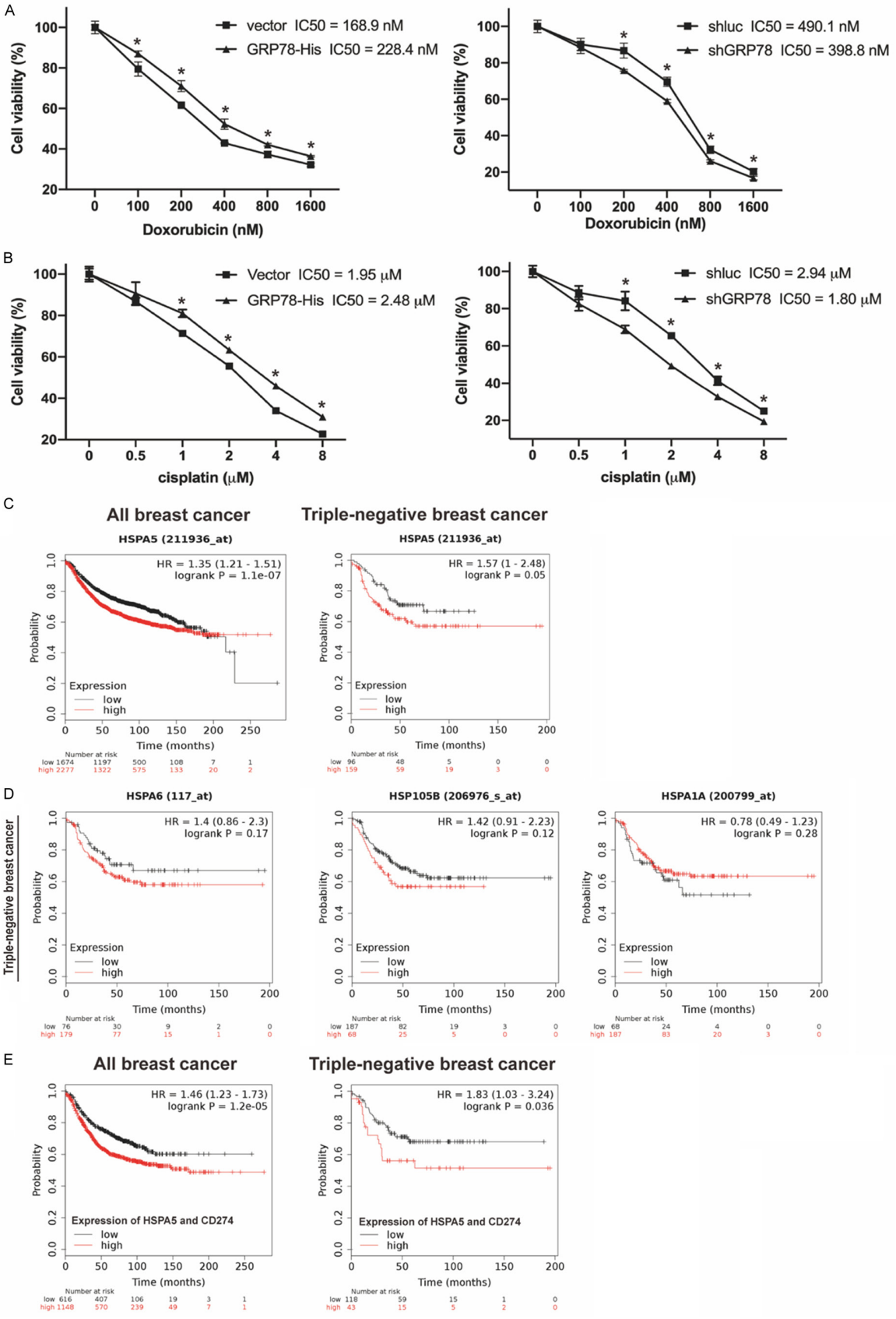
A
vector IC50 = 168.9 nM
GRP78-His IC50 = 228.4 nM
Cell viability (%)
Doxorubicin (nM)
shluc IC50 = 490.1 nM
shGRP78 IC50 = 398.8 nM
B
Vector IC50 = 1.95 μM
GRP78-His IC50 = 2.48 μM
cisplatin (μM)
shluc IC50 = 2.94 μM
shGRP78 IC50 = 1.80 μM
C
All breast cancer
Triple-negative breast cancer
HSPA5 (211936_at)
HR = 1.35 (1.21 - 1.51)
logrank P = 1.1e-07
HR = 1.57 (1 - 2.48)
logrank P = 0.05
Probability
Expression
low
high
Time (months)
Number at risk
D
Triple-negative breast cancer
HSPA6 (117_at)
HR = 1.4 (0.86 - 2.3)
logrank P = 0.17
HSP105B (206976_s_at)
HR = 1.42 (0.91 - 2.23)
logrank P = 0.12
HSPA1A (200799_at)
HR = 0.78 (0.49 - 1.23)
logrank P = 0.28
E
All breast cancer
Triple-negative breast cancer
HR = 1.46 (1.23 - 1.73)
logrank P = 1.2e-05
HR = 1.83 (1.03 - 3.24)
logrank P = 0.036
Expression of HSPA5 and CD274

Figure 4. GRP78 modulates the response to chemotherapy in triple-negative breast cancer. A. Relative survival (%) in BT-549 breast cancer cells treated with doxorubicin for 48 hours using MTS assay are shown and the half-maximal inhibitory concentration (IC50) of doxorubicin was shown. B. Relative survival (%) in BT-549 breast cancer cells treated with cisplatin for 48 hours using MTS assay are shown and the IC50 of cisplatin was shown. C. Prognostic value of relapse-free survival (RFS) by HSPA5 mRNA levels in all breast cancer (n = 3951) and triple negative breast cancer (TNBC, n = 255) patients via www.kmplot.com. D. Relapse-free survival curves of triple negative breast cancer patients (n = 255) are plotted by HSPA6, HSP105B, and HSPA1A mRNA levels. E. Relapse-free survival curves in all breast cancer (n = 1764) and TNBC (n = 161) patients by mean expressions of HSPA5 and CD274 mRNA levels. *P < 0.05, Student's t-test.

results suggest that high level of GRP78 expression correlates with the poor clinical outcome of triple-negative breast cancer.

Discussion

In this study, we identified GRP78 as a positive regulator of PD-L1 by promoting PD-L1 protein stability through physical interactions. We showed the prognostic value of GRP78 from the TCGA dataset. In addition, we demonstrated the crucial role of GRP78 in regulating PD-L1 expression and stability in triple-negative breast cancer. This effect also increased cell surface levels of PD-L1 as the main target for immunotherapy. Furthermore, our study and others confirmed that GRP78 induced chemoresistance [38], and could serve as a biomarker to predict tumor aggressiveness and PD-L1 expression.

The regulatory mechanisms of PD-L1 have been intensively studied due to its important role in immuno-oncology. Studies have shown that the expression of the PD-L1 expression is regulated by various process including gene transcription, post-transcriptional, post-translational modifications and exosomal regulation. Among these mechanisms, N-linked glycosylation begins in the ER region with subsequent glycan modifications [24]. Glycosylation is an essential post-translational modification of PD-L1 to maintain its stability and immunosuppressive function in the cancer microenvironment that is initiated in the ER region [18, 39]. However, the regulation of the initial glycosylation process is not yet clearly investigated. Sigma 1, another ER chaperone, also positively regulates PD-L1 expression levels as well as functional PD-L1 at the cell surface [40]. In a glioblastoma study, a spliced isoform of glucocorticoid receptor co-chaperone FK506-binding protein 51 (FKBP51) also regulated the glycosylation and stability of PD-L1 in the ER [41]. Therefore, ER chaperones play crucial regulatory roles in PD-L1 expression levels. In the present study, we identified the essential role of GRP78 in regulating the PD-L1 expression and maintaining its stability in triple-negative breast cancer cells.

Physiologically, GRP78 directly binds to the three ER stress sensors of the major unfolded protein response signaling pathway, including protein kinase RNA-like ER kinase (PERK), inositol-requiring kinase 1 (IRE1α), and activating transcription factor 6 (ATF6). Upon ER stress, GRP78 dissociates apart from these ER sensors and further activates the downstream signaling pathways to restore cell homeostasis. ER stress-related signaling pathways are usually up-regulated in tumor microenvironment due to the abnormal accumulation of misfolded proteins induced by gene mutations and rearrangements [42]. GRP78 has been reported to negatively modify the immune response [43]. In mouse regulatory B cells, exogenous GRP78 was reported to induce PD-L1 and Fas Ligand expression with increased IL-10 production. *In vitro*, GRP78 could down-regulate HLA-DR and CD86 expression and induce IL-10 secretion in peripheral blood monocytes [44]. In our studies, we demonstrated that GRP78 up-regulated by ER stress would further increase PD-L1 expression in the triple-negative breast cancer cells as well as other cancer types, including pancreatic adenocarcinoma and head and neck cancer. Furthermore, we detected a stronger interaction between GRP78 and PD-L1 upon ER stress stimulation. Additionally, we observed the positive regulatory effects between the endogenous GRP78 level and PD-L1 in BT-549 breast cancer cells. Furthermore, in the GRP78 knock-down system, the up-regulation of PD-L1 was attenuated despite the ER stress stimulation. These results indicated that GRP78 was required for the PD-L1 upregulation and they interacted at the ER region.

Since the membrane PD-L1 serves as the main co-inhibitory ligand of PD-1 to help cancer cells

escape from host immunosurveillance, it is obligatory to determine GRP78 regulated membrane PD-L1 change. Indeed, both ER stress and endogenous GRP78 level could regulate membrane PD-L1 expression. Moreover, we demonstrated that co-expression of exogenous GRP78 and PD-L1 would further increase PD-L1 protein, indicating the stabilization of PD-L1 by GRP78. It has been documented that the expression level of GRP78 had a positive correlation with the IL-6 expression. ER stress induced IL-6 release through the p38 MAPK/CCAAT-enhancer-binding protein homologous protein (CHOP) axis [45]. Previous study also demonstrated that IL-6 activated JAK1 and could phosphorylate PD-L1 Tyr112. This axis recruited the ER-associated N-glycosyltransferase STT3A, which further catalyzing PD-L1 glycosylation and maintaining protein stability [21, 46]. Besides, exogenous GRP78 could induce PD-L1 expression on regulatory B cells in the previous mice model [43]. These results indicated that GRP78 indeed regulated the functionally immunosuppressive PD-L1 levels.

EGFR is one of the crucial oncogenic pathways in triple-negative breast cancer. In previous studies, EGF stabilized PD-L1 through GSK3β inactivation [18]. Meanwhile, EGFR activation could up-regulate PD-L1 expression through the p-ERK1/2/p-c-Jun axis [47]. Additionally, it could be mediated through the increase of CSN6 by activation of the EGFR/ERK signaling pathway to maintain the stability of PD-L1 protein in cancer cells [48]. Meanwhile, it had been reported that EGFR expression positively correlated with PD-L1 expression [49]. Interestingly, we showed that neither ER stress nor endogenous GRP78 could affect the EGFR protein expression. Therefore, our results suggest specific regulation of PD-L1 by GRP78, and that such regulation is independent of EGFR related pathways.

GRP78 located mainly in the ER but could also exist in other cellular compartments, including nucleus, cytosol, and membrane [42]. Interestingly, the re-localization of GRP78 might have diverse functions and possess different effects on tumor growth and signaling pathways [29]. Cell membrane-bound GRP78 in breast cancer cells enhances tumorigenicity, chemoresistance, and stemness [42, 50]. Here, in our studies, we demonstrated the re-localization of GRP78 onto the cell surface upon stress. Indeed, GRP78 expression affected chemoresistance in the present study. Meanwhile, chemo-drugs induced both GRP78 and PD-L1 expression. This effect was abrogated by GRP78 knock-down, indicating that GRP78 was crucial for chemotherapy-induced PD-L1 upregulation. Therefore, our studies provide an explanation for the clinical benefit of the combination of anti-PD-L1 antibody with chemotherapy.

Altogether, we propose that GRP78 up-regulates the PD-L1 protein expression and stability within cancer cells. These results showed that high GRP78 expression would lead to immunosuppressive environments and poor clinically therapeutic response. Meanwhile, GRP78 might serve as a biomarker to select potential cancer patients for anti-PD-L1 antibodies treatment or to predict the responsiveness of systemic chemo-drugs. On the other hand, with emerging therapeutic drugs targeting GRP78, our data provide evidence that the inhibition of GRP78 or treatment with anti-PD-L1 antibody could be a promising alternative strategy to overcome chemotherapy-induced PD-L1 expression.

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Disclosure of conflict of interest

None.

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