Endoplasmic Reticulum Stress Promotes The Release of Exosomal PD-L1 From Head and Neck Cancer Cells and Facilitates M2 Macrophage Differentiation

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Abstract

Background

Endoplasmic reticulum stress (ER stress) fosters cancer cell escape from immune surveillance and upregulate PD-L1 expression, but the mechanisms remain unclear.

Methods

We analyzed protein levels by immunofluorescence and Western blotting, RNA levels by qRT-PCR. Exosomes were injected intravenously through the tail vein into 6-week-old nude mice once every other day for a total of 10 injections

Results

Expression of some ER stress markers, including GRP78 (glucose-regulated protein 78), ATF6 (activating transcription factor 6) and PERK (PKR-like endoplasmic reticulum kinase), was upregulated in OSCC tissues and correlated with poor overall survival. The level of ER stress-related proteins positively correlated with a cluster of PD-L1 expression and macrophage infiltration in OSCC tissues. PD-L1 expression in OSCC tissues was negatively correlated with cumulative survival. Incubation with Exo-ER upregulated PD-L1 levels in macrophages in vitro and vivo, and upregulation of PD-L1 promoted macrophage polarisation towards the M2 subtype.

Conclusions

ER stress induced exosome secretion by OSCC cells and PD-L1 expression in macrophages to promote M2 macrophage differentiation. A novel exosome-modulated mechanism was delineated for OSCCs-macrophage crosstalk that drove tumor growth and should be explored for its therapeutic utility.

Background

Oral squamous cell carcinoma (OSCC) is the most common cancer of the head and neck area. Despite recent advances in understanding the diagnosis, molecular biology and treatment of OSCC, the five-year survival rate has remained under 50% for the past 30 years, mainly due to metastasis or local incontrollable recurrence.[1] OSCC is mainly caused by tobacco use (chewing and smoking). Other risk factors include infections with certain types of human papillomavirus, certain workplace exposures and radiation exposure. The diagnosis is confirmed by tissue biopsy, with computed tomography and blood tests often used to check the degree of spread. Traditional treatments include surgery, chemotherapy and radiation therapy.[2, 3]

The immune system participates in anticancer activity; however, it is also involved in cancer development and progression.[4] The immunosuppressive status of OSCC has attracted increasing attention, and the effectiveness of immune checkpoint blockade and the overexpression of immune checkpoint molecules as OSCC therapy has been confirmed.[5]. Tumour cells reprogramme their surrounding cells to support cancer progression, tumorigenesis and invasion of adjacent tissues in the tumour associated environment (TME), [6] and 5–40% of the mass of solid tumours consists of macrophages.[7–9] The involvement of macrophages is now established and is becoming better understood. Macrophages serve as an interface between innate and acquired immunity, and they become polarised into M1 and M2 phenotypes depending on the expression of cytokines, receptors, and effector molecules.[10] Under physiological conditions, macrophages are normally polarised into the proinflammatory and antitumour M1 phenotype; however, tumour cells can induce macrophages to switch to the alternatively activated M2 phenotype via several pathways (CCL-2, IL-4, IL-6, IL-8, IL-10, TGF- β and PD-1/PD-L1 (Programmed cell death-ligand 1)). The M2 macrophages, in turn, secrete high levels of cytokines, chemokines, enzymes, and growth factors, such as VEGF, PDGF, TGF- β , FGF and several matrix metalloproteinases; these upregulate inflammation while also promoting immunosuppression, angiogenesis, migration, tumour progression, metastasis and treatment resistance.[11, 12]

Tumour progression also responds to endoplasmic reticulum (ER) stress, a vital cellular response that maintains cell survival by activating the unfolded protein response. ER stress acts as a point of “protein quality control” in cells and is involved in several cellular functions, including protein folding and Ca^{2+} homeostasis, by processing nascent membrane and secretory proteins in a Ca^{2+} -dependent manner.[13] ER stress is controlled by several ER stress-related proteins, including protein kinase R-like ER kinase (PERK), activating transcription factor 6 (ATF6), glucose-regulated protein 78 (GRP78) and inositol-requiring enzyme 1 α (IRE1 α). The initiation of ER stress has been reported in various tumours, and ER stress promotes further tumour progression.[14, 15] ER stress induces tumour cell escape from immunological surveillance, and activation of ER stress in immune cells affects the function of infiltrating immune cells. For example, ER stress increases a range of inflammatory factors, including interleukin (IL)-23 and IL-6, in macrophages.[16] ER-stressed tumour cells also modify immune cell functions by releasing ER stress-related molecules and subsequently promoting tumour survival, progression and metastasis.[17] However, the mechanisms by which ER-stressed tumour cells cultivate immune cells and suppress immune responses remain unclear.

One possible mechanism involves exosomes, which are 40 nm to 100 nm membrane vesicles involved in cell-to-cell communication. Exosomes are loaded with DNA, proteins, and coding and non-coding RNAs [18] and are released from living cells into the extracellular environment. Exosomes derived from cancer cells differ from the exosomes secreted by normal cells,[18] and the distinctive differences in exosome content in some cancer cells can be used as diagnostic or prognostic markers. For example, miR-21 is a well-known oncomiR that can be used as a diagnostic marker in ovarian cancer.[19] Exosomes released by cancer cells interact with myeloid-derived suppressor cells (MDSCs), tumour-associated macrophages (TAMs) or tumour-infiltrating T cells (TILs). They cause a phenotypic switch of stroma cells and tumour-infiltrating immune cells, thereby creating a tumour-permissive microenvironment. PD-L1 proteins were

recently shown to be packaged in purified exosomes, indicating that these vesicles may deliver protein information to recipient cells.[20]

These findings suggest that PD-L1 is a vital molecule involved in exosome-mediated intercellular communication. However, few studies have examined whether ER stress affects the transfer of exosomal PD-L1 and whether exosomal PD-L1 affects OSCC tumour progression. The purpose of the current study was therefore to investigate whether ER-stressed OSCC cells can transmit PD-L1-enriched exosomes to macrophages and whether these exosomal PD-L1 modulate the immunologic functions of macrophages.

Methods

Patients and samples

The present protocol conforms to the ethical guidelines of the 1975 Declaration of Helsinki and was approved by the ethics committee of the Stomatology Hospital of Nanjing Medical University (Nanjing, China). Written informed consent was obtained from all patients in accordance with the ethical principles of the Declaration of Helsinki. Primary OSCC tumour tissues were obtained from 100 OSCC patients who were hospitalised in the Stomatology Hospital of Nanjing Medical University between 2019 and 2020. Tumour samples were collected during the surgery, and non-tumour samples were cut at least 5 cm away from the tumours. Tumours were categorised based on the World Health Organization (WHO) classification and the International Union Against Cancer tumour-node-metastasis (TNM) classification system. The OSCC tumour tissues were formalin-fixed and paraffin-embedded for histopathological diagnosis and construction of paraffin-embedded tissue microarrays.

Cell culture

The HN4 human head and neck squamous cell carcinoma cell line was acquired from the Laboratory of the Stomatology Hospital of Nanjing Medical University. The cell line was authenticated using short tandem repeat-based DNA fingerprinting and multiplex polymerase chain reaction (PCR). The HN4 cells were cultured in DMEM (Gibco, USA) supplemented with 10% foetal bovine serum (Gibco, USA) at 37°C in a 5% CO₂ atmosphere. Penicillin (100 U/ml) and streptomycin (100 mg/ml) (Invitrogen, Grand Island, NY, USA) were added to the culture medium. HN4 cells were seeded at 1×10^5 cells/dish in 100 mm cell culture dishes and passaged every 6–7 days.

Small interfering RNA (siRNA) oligos

The sequence of the siRNA oligo against pd-l1 (siPD-L1; MW = 13,788.9 g/mol) was 5'-ThioMC6-D/GGUCAACGCCACAGCGAAUUU-3' (sense sequence) and 5'-PAUUCGCGUGGCGUUGACCUU-3' (anti-sense sequence). The sequence of the scrambled siRNA oligo (siSCR; MW = 13,728.8g/mol) was 5'-ThioMC6-D/UGGUUUACAUGUCGACUAAUU-3' (sense sequence) and 5'-PUUAGUCGACAUGUAAACCAUU-3' (anti-sense sequence). Both siRNAs were designed and synthesised by Dharmacon (Lafayette, CO).

Western blot analysis

HN4 cells were seeded at 1×10^5 cells/dish in 100 mm cell culture dishes. At 24 hours after seeding, the cells were washed with 10 mL PBS 3 times and then 10 mL ER-HN4/6-CM or control medium was added. After 48 hours, cells were washed with PBS and cell lysis was performed with RIPA lysis buffer (Thermo Scientific, Waltham, MA). Protein concentration was measured by Bio-Rad protein assay. Proteins were analysed by western blotting by separating by 10% SDS-PAGE, blotting onto PVDF membrane, blocking with 5% nonfat dry milk (Bio-Rad, Hercules, CA) or 5% BSA in TBS-T buffer (10 mM Tris, 150 mM NaCl and 0.2% Tween-20 [pH 8.0]). The membrane was incubated with a primary antibody overnight at 4°C and then with a secondary antibody conjugated with alkaline phosphatase (1 h at room temperature); the signal was detected by using a chemiluminescence method. The following primary antibodies were used: anti-PERK, anti-GRP 78, anti-ATF 6 and anti-GAPDH and anti-PD-L1. Immunoreactive bands were visualised using Super Signal West Dura substrate (Thermo Fisher).

Quantitative RT-PCR

Total RNA was isolated using the TRIzol reagent, and cDNA was synthesised using a Superscript VIL0 cDNA synthesis kit (cat#11754; Life Technologies, Carlsbad, CA). Quantitative real-time PCR experiments were carried out with the Step-One PCR System (Applied Biosystems, Foster City, CA). Real-time RT-PCR data analysis was conducted according to the $2^{-\Delta\Delta C_t}$ method using the threshold cycle (C_t) values for target genes and Gapdh as an endogenous control gene. All assays were performed in triplicate.

Immunohistochemistry.

An automated immunostainer (Ventana, Strasbourg, France) and standard protocols were used for immunohistochemical stainings. Briefly, 4 μ m thick sections were heated to 100°C, incubated with Inhibitor D (Ventana) and then incubated with the primary antibodies: PD-L1 (rabbit monoclonal antibody no. 13684, Cell Signaling Technology, Inc., Leiden, Netherlands); PERK (rabbit polyclonal antibody no. ab65142; Abcam Cambridge, UK); ATF6 (rabbit polyclonal antibody no. ab203119, Abcam Cambridge, UK) or GRP 78 (rabbit polyclonal antibody no. ab21685 Abcam Cambridge, UK) The sections were rinsed and then incubated with secondary antibody solution, followed by sequential incubation with Blocker D (Ventana) and SA-HRP D (Ventana). Visualisation was accomplished using DAB D (diaminobenzidine) and DAB H2O2 D (Ventana). The sections were counterstained with Hemalaun and mounted. The detailed immunohistochemical staining procedure was described previously. Staining quality and specificity were assured using established protocols and antibodies, negative controls prior to staining and on-slide positive controls for each tissue microarray slide.

Immunofluorescence

Patient tissue slides were deparaffinised and rehydrated, and the paired mixtures of lumican with CD68, CD63 or PD-L1 antibodies (5 ng/mL) were incubated overnight at 4°C. Secondary antibodies were conjugated with Alexa Fluor 488 or Alexa Fluor 594 of different species, depending on the primary antibodies (#A11008, A11012 and A11005, from Life Technologies, Grand Island, NY, USA). A 2 ng/ml concentration of these antibodies was incubated with the slides for 30 min at ambient temperature. The slides were counterstained with DAPI (#D8417, Sigma-Aldrich) for 10 min and mounted with DAKO

fluorescent mounting medium (#S3023, Dako, Burlingame, CA, USA). IgG was added to slides as a negative control. Images were captured with an Olympus FV1000 laser scanning confocal microscope and analysed using FlowView software at the Flow Cytometry and Cellular Imaging Facility of MD Anderson. All images were captured with the same exposure time for all samples.

Exosome isolation

Exosomes from 48-hour culture supernatants of HN4 tumour cells were extracted using Total Exosome Isolation Reagent (Invitrogen, USA). The supernatants were thawed in a 25°C water bath and centrifuged at 2000 × g for 30 minutes to remove cells and debris. A 100 µL volume of Total Exosome Isolation reagent was added to 500 µL supernatant, and the mixture was vortexed until homogeneous. After incubation at 4°C overnight, the sample was centrifuged at 10000 × g for 60 minutes at room temperature. The exosome pellet was dissolved in 100 µL PBS. Isolation of exosomes from the supernatants was confirmed by transmission electron microscopy (TEM), nanosizer, and zeta-potential analyses. Exosomes were fixed in 2.5% glutaraldehyde solution for at least 2 hours and then 10 µL of the diluted mixtures were transferred to a cleaned copper grid. Images were obtained by TEM (JEM-1010, Jeol, Japan) after dyeing with 2% phosphotungstic acid solution. For nanosizer analysis, isolated exosome samples were diluted 2000-fold and resuspended in PBS for size distribution analysis using a Nano series-Nano-ZS zetasizer (Malvern Instruments Ltd, UK), according to the manufacturer's instructions. The zeta-potential of the exosomes diluted in PBS (25 µg/mL) was determined using an ELSZ-DN2 zeta-potential analyser (Otsuka Electronics, Osaka, Japan), according to the manufacturer's instructions.

Animal experiments

The animal experiment strictly adhered to the principle of minimising pain, suffering, and discomfort experienced by the experimental animals. All protocols were approved by the Animal Research Committee, Graduate School of Medicine, Nanjing Medical University. Exosomes were injected intravenously through the tail vein into 6-week-old nude mice once every other day for a total of 10 injections. Then peritoneal macrophages were isolated 12 hours after the final injection. PD-L1 levels were analyzed by western blot and qPCR. Four mice were used per group in the animal experiments.

Statistical analysis.

Data are expressed as means ± SE. Statistical differences between the two groups were determined by Student's t-test. Differences between multiple groups were tested using ANOVA and checked for significance using Fisher's protected least-significant difference test. The influence of prognostic factors on tumour-related survival was assessed by Kaplan-Meier estimates, and subgroups were compared by the Breslow test for univariate analysis. The multivariate Cox proportional hazard model was applied using a stepwise forward method to detect independent predictors of survival. Two-tailed P values of 0.05 or less were considered statistically significant. The data are presented as the mean ± standard error or deviation.

Results

ER stress is upregulated and positively correlates with poor survival in OSCC patients

The function of ER stress in OSCC promotion was first evaluated by determining if ER stress is upregulated in OSCC patients by comparing the difference between the expression of ER stress-related proteins in surgically resected OSCC tissues and paracarcinoma tissues by IHC and western blotting. The ER stress-related proteins include GRP78, ATF6 and PERK.[21] The IHC results verified higher expression of ER stress-related protein expression in freshly resected OSCC tissues than in the associated paracarcinoma tissues (Fig. 1A). Western blotting also confirmed a much higher expression of these ER stress-related proteins in tumour tissues than in para-carcinoma tissues (Fig. 1B&C). The qPCR analysis also showed higher expression of GRP 78, ATF6 and PERK mRNAs in the tumour tissues (Fig. 1D). Comparison of GRP78, ATF6 and PERK expression versus overall survival of OSCC patients revealed that overall survival was shorter in patients with high expression of ER stress-related proteins than with low expression of these proteins (Fig. 2).

ER stress-related protein levels correlate with macrophage infiltration and PD-L1 expression in OSCC patients

In antitumor immune response and TME (tumor microenvironment) macrophages have been found to be instrumental in modulating immune cell acts. Therefore, in OSCC patients we carried out immunofluorescence to analyze macrophage infiltration. The immunofluorescence results shown in Fig. 3A confirm that expression of CD 68 was higher in tumour tissues than in normal tissues. Immunofluorescence double-staining revealed a frequent co-localisation of PD-L1 with CD68 + macrophages in the tumour stroma and that the expression of PD-L1 was higher than in normal tissues. The qPCR and western blot analyses showed that PD-L1 mRNA and protein were markedly upregulated in tumour tissues compared with paired paracarcinoma tissues. Together, these results suggest that ER stress-related protein levels correlate with macrophage infiltration and PD-L1 expression in OSCC patients.

Exosomes from ER-stressed OSCC cells upregulate PD-L1 expression in macrophages in vitro

The above data revealed that ER stress status is associated with upregulated PD-L1 expression in macrophages on OSCC patients, suggesting that ER-stressed OSCCs may promote PD-L1 expression in macrophages. We hypothesised that ER-stressed OSCC cells may release exosomes and subsequently upregulate PD-L1 expression on macrophages because exosomes are an important communicator between different cell types. Optimisation of the in vitro cell culture system to mimic the ER stress status by interferon- γ (IFN- γ) treatment (500U/ml for 48 hours) generated significant ER stress in the HN4 OSCC cell line. The purity of exosomes derived from IFN- γ -treated (Exo-ER) or untreated (Exo-con) OSCC cells

was confirmed by TEM, which revealed a homogeneous population of rounded membrane-bound vesicles with a diameter ranging from 30 to 200 nm. Western blot analysis showed the expression of the exosomal marker proteins CD63, and the zeta potential confirmed the stability of the exosome particles in suspension (Fig. 4A-C).

A co-culture experiment with Exo-ERs derived from HN4 cells or normal HN4 cells showed a significantly upregulated expression of GRP78, ATF6, or PERK proteins compared to co-culture with Exo-con (Fig. 4D). Co-culture of macrophages with PKH26-labeled exosomes showed that mTHP-1 cells effectively engulfed the exosomes (Fig. 4E), and western blotting showed that upregulation of PD-L1 expression in mTHP-1 cells was significantly greater following co-culture with HN4-derived Exo-ERs than with Exo-cons (Fig. 4F-G). This PD-L1 upregulation in macrophages by Exo-ERs was also confirmed by qPCR analyses (Fig. 4H). Treatment with up to 500U/mL of IFN- γ did not upregulate PD-L1 levels in macrophages (Fig. 4I-K), thereby confirming that the Exo-ER-induced ER stress in macrophages was not a consequence of IFN- γ contamination.

Collectively, these results indicated that Exo-ER-mediated upregulation of PD-L1 in macrophages was not due to IFN- γ contamination.

Exosomes from ER-stressed OSCC cells upregulate in vivo PD-L1 expression in macrophages

We examined whether Exo-ER also enhances PD-L1 expression in vivo by injecting nude mice with Exo-ER, Exo-ER-siPD-L1, Exo-Con and Exo-siNC once every 2 days for a total of 10 injections. The peritoneal macrophages were isolated 12 hours after the last injection. As illustrated in Fig. 5A-C, western blotting and qPCR analyses demonstrated that the levels of PD-L1 were significantly higher in the peritoneal macrophages isolated from Exo-ER-injected mice than from the other groups. The levels were slightly lower in the Exo-ER-siPD-L1 group and there was no significant difference between the Exo-con and Exo-siNC groups.

Exosomes from ER-stressed OSCC cells induce macrophages to polarise towards the M2 subtype and upregulate PD-L1 expression

As shown in Fig. 6A, the levels of PD-L1 were higher in Exo-ERs than in Exo-Cons. We determined whether PD-L1 would be transferred via the HN4 exosomes to macrophages, thereby accelerating macrophage polarisation to the M2 subtype, by first testing which subtype of macrophages was more prevalent in tumour tissues. The immunofluorescence double-staining results in Fig. 6B show that tumour tissues expressed higher levels of the M2 marker (CD 163) than of the M1 marker (CD 11C), indicating a greater importance of the M2 subtype in OSCC progression. The qPCR analysis showed the same results in tumour tissues (Fig. 6C).

Coculture of mThp1 cells with Exo-ER, Exo-Con, Exo-ER-siPD-L1 and Exo-siNC for 48 hours and subsequent qPCR analysis showed a significantly higher expression of the M2 macrophage marker

CD163 in the Exo-ER group than in the other groups (Fig. 6D). The peritoneal macrophages isolated from nude mice showed a similar trend (Fig. 6E), as the CD163 level was the highest in the Exo-ER group, whereas the levels in the Exo-siPD-L1 group were nearly 1/3 lower.

Collectively, these results suggested that ER stress can upregulate the expression of PD-L1 in HN4-derived exosomes and exosomes can transfer PD-L1 from ER stress cancer cells to macrophages and upregulate PD-L1 expression in the macrophages. PD-L1 could also accelerate macrophage polarisation towards the M2 subtype.

Discussion

Multiple studies have shown that ER stress can dysregulate antitumour immunity through various mechanisms, including the stimulation of protumour inflammatory factors and the induction of MDSCs. [13, 16, 22] In the current study, we identified a novel mechanism by which ER stress promotes the release of PD-L1-enriched exosomes by OSCC cells. These exosomes then upregulate PD-L1 expression by the macrophages and subsequently promote the polarisation of the macrophages towards the M2 subtype. Most of the human OSCC samples examined here showed high expression of ER stress-related proteins and genes, and their expression levels were negatively correlated with the patients' overall survival. The tumours also had greater numbers of CD163 + macrophages and PD-L1 + cells in the stroma, and the PD-L1 expression was also correlated with poor overall survival of the OSCC patients. We also found that ER-stressed OSCC cells upregulated macrophage PD-L1 expression and consequently promoted M2 macrophage differentiation through the transmission of exosomal PD-L1, resulting in elevation of PD-L1 levels. Together, our results suggest that OSCC cells can transmit ER stress signals to infiltrating macrophages through the release of PD-L1-enriched exosomes, thereby promoting tumour progression.

ER stress promotes the release of exosomes from OSCC cells and modulates PD-L1 expression in macrophages. ER stress has been reported to promote immune escape by tumour cells by constructing an immunosuppressive microenvironment, but the precise mechanism remains unclear. Exosomes have been reported to play an important role in intercellular communication by delivering their contents (such as proteins) to the "recipient" cells. [23, 24] For example, Kanemoto et al. reported that ER stress promoted exosome release in a PERK-dependent manner. [15] However, the role of exosomes released by ER-stressed OSCC cells in immune cell function and the potential immunomodulation by specific exosomal proteins are not clear.

A number of recent studies have shown that exosomal PD-L1 plays an important role in tumour progression and evasion [20, 26], and we found that exosomes from ER-stressed OSCC cells have a high level of PD-L1. Macrophages are the predominant cells in the tumour stroma, and a high macrophage density has been associated with unfavourable prognosis in many types of tumours. [22] Several studies have reported that macrophages promote tumour progression through direct communication with the tumour cells. [27] We found that OSCC tissues recruited more CD68 + macrophages into the stroma than was observed in normal tissues. These exosomes were also effectively transferred into macrophages and

transmitted ER stress signals to macrophages, suggesting that ER stress influences OSCC cell exosome release and promotes the transmission of PD-L1-enriched exosomes to macrophages residing in the tumour microenvironment.

Macrophages that express high levels of PD-L1 polarise towards the M2 subtype. PD-L1, also called B7-H1 or CD274, inhibits T-cell proliferation and promotes T-cell dysfunction by binding to programmed death 1 (PD-1).[28] PD-L1 is expressed by both hematopoietic cells and nonhematopoietic cells, and upregulation of PD-L1 has been observed in various tumours, where it contributes to immune evasion. High levels of PD-L1 have been observed in macrophages, where PD-L1 suppresses antitumour immunity.[29–31] Macrophage infiltration into the tumour microenvironment has been reported to promote tumour progression by impairing the immune responses of cytotoxic CD8 + T cells. Furthermore, blocking PD-L1 using a specific antibody improved the T-cell immune response, suggesting that increased PD-L1 expression in macrophages helps OSCC cells escape from cytotoxic T cells. PD-L1 can inhibit T-cell activation and contribute to tumour immune evasion in many cancers, like liver cancer and mammary cancer, and macrophages that infiltrate into liver cancers can express higher levels of PD-L1; therefore, we assumed that the same trend would occur in OSCC.[32–34]

In this study, we found that the macrophages that infiltrated into ER-stressed OSCC tissues expressed high levels of PD-L1 and that the PD-L1 levels were negatively correlated with overall survival. Macrophages are classified into M1 and M2 macrophages, where M1 macrophages play a protective role and M2 macrophages promote tumour growth. 23, 7 Our in vitro and in vivo studies demonstrated that Exo-ERs polarised macrophages to the M2 phenotype, suggesting that ER-stressed OSCC cells may “teach” macrophages to assume the M2 subtype, thereby facilitating disease progression. Our results also indicated that ER stress promotes OSCC cells to release PD-L1-enriched exosomes, and these exosome-treated macrophages significantly upregulate PD-L1 expression and polarise towards the M2 subtype to promote tumour growth.

Conclusion

The current study provides evidence indicating that exosomes released by ER-stressed OSCCs induce immunosuppression through inducing M2 macrophage polarization. Meanwhile, our findings provide the groundwork for ER-stressed OSCCs exosomes research aimed towards a greater understanding of their pathological importance. In addition, a novel exosome-modulated mechanism was delineated for OSCCs-macrophage crosstalk that drove tumor growth and should be explored for its therapeutic utility.

Abbreviations

OSCC: Oral squamous cell carcinoma; TME: tumour associated environment; ER: endoplasmic reticulum; PERK: protein kinase R-like ER kinase; ATF6: activating transcription factor 6; GRP78: glucose-regulated protein 78; IRE1 α : inositol-requiring enzyme 1 α ; Exo: exosomes; Exo-con: exosomes isolated from untreated OSCC cells; Exo-ER: exosomes isolated from IFN- γ -treated OSCC cells; MDSCs: myeloid-derived

suppressor cells; TAMs: tumour-associated macrophages; TILs: tumour-infiltrating T cells; WHO: World Health Organization; TNM: tumour-node-metastasis; PCR: polymerase chain reaction; TEM: transmission electron microscopy; IFN- γ : interferon- γ ; PD-L1: Programmed cell death-ligand 1.

Declarations

Acknowledgements

Not applicable.

Authors' contributions

YY, PFJ and ZYW contributed to the acquisition of data, analysis and interpretation of data. YY and YQZ wrote the manuscript. YY, MQC, HMD and HMW were involved in revising the article. LX, JYX and YJD analysed data and edited the manuscript. FGW, YQZ and HMW contributed to analyse data and revise the article critically for important intellectual content. YQZ contributed to the conception and the study design. All authors read and approved the final manuscript. YQZ and HMW are the guarantor of this work and take full responsibility for the content of the manuscript.

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Availability of supporting data

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Ethical Approval and Consent to participate

The study was approved by the the Stomatology Hospital of Nanjing Medical University and in accordance with the Declaration of Helsinki. The patients provided their written informed consent to participate in this study.

Consent for publication

All the co-authors consent to publish the work in Cell Communication & Signaling.

Competing interests

The authors declare no competing interests.

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Figures

Figure 1

ER stress is upregulated and positively correlates with poor survival in OSCC patients. (A) Representative low and high expression of ER stress markers PERK, ATF6 and GRP78, in OSCC tissue samples (scale bar = 50 μ m). (B) Representative western blot analyses of PERK, ATF6 and GRP78 protein expression in human OSCC tissues. (C) Grey-level intensity analysis of PERK, ATF6 and GRP78. (D) qPCR detected the mRNA levels of PERK, ATF6 and GRP78 in 12 cases of freshly resected human OSCC tissues. ** $P < 0.01$

Figure 2

Activation of ER stress correlates with poor survival in human HCC. Kaplan-Meier curves demonstrated significantly shorter overall survival in patients with overexpression of (A) PERK, (B) ATF6, and (C) GRP78 than with low expression levels.

Figure 3

ER stress-related protein levels correlate with macrophage infiltration and PD-L1 expression in OSCC patients. (A) The expression pattern of CD68+ cells in OSCC tissues and normal tissues measured by immunofluorescence (scale bar = 50µm). (B) The expression pattern of PD-L1 on CD68+ cells in OSCC tissues and normal tissues measured by immunofluorescence (scale bar = 50µm) (C) qPCR analyses of

PD-L1 mRNA levels in 12 cases of freshly resected OSCC tissues. (D) Western blot analysis of PD-L1 protein levels in human OSCC tissues and normal tissues. (E) Grey intensity analysis of PD-L1

Figure 4

Exosomes from ER-stressed OSCC cells upregulate in vitro PD-L1 expression in macrophages. (A) TEM image of HN4-derived exosome formation (scale=200 nm). (B) Presence of exosome marker CD63 determined by western blotting. (C) Zeta potential of HN4-derived exosomes (D) HN4 cells were incubated

with Exo-ER and Exo-Con and protein levels of PERK, ATF6 and GRP78 were determined by western blotting. (E) PKH26-labelled exosomes were co-incubated with mTHP-1 cells and examined by confocal microscopy (scale bar = 50 μ m). Representative images are shown. (F-G) Macrophages were co-incubated with HN4-derived Exo-ERs and Exo-cons, and the PD-L1 protein level was detected by western blotting. (H) The mRNA levels of PD-L1 determined by qPCR. (I) Macrophages were co-incubated with HN4-derived Exo-ERs and IFN- γ , followed by detection of the PD-L1 protein level by western blotting. (J-K) mRNA levels of PD-L1 determined by qPCR

Figure 5

Exosomes from ER-stressed OSCC cells upregulate in vivo PD-L1 expression on macrophages. Exosomes were intravenously injected into nude mice through the tail vein. Peritoneal macrophages were isolated from mice treated with Exo-ER, Exo-ER-siPD-L1, Exo-Con and Exo-siNC, and the expression of PD-L1 was evaluated by (A) qPCR analysis, (B) western blotting. (C) Grey-level intensity analysis of PD-L1.

Figure 6

Exosomes from ER-stressed OSCC cells induce macrophage polarisation towards the M2 subtype and upregulate PD-L1 expression. (A) The protein level of PD-L1 in exosomes derived from ER stressed and normal HN4 cells measured by western blotting. (B) The expression pattern of CD163 and CD11C on CD68+ cells in OSCC tissues measured by immunofluorescence. (C) The qPCR results showing the mRNA levels of CD163 and CD11C in tumour tissues. (D) Macrophages were co-incubated with HN4-derived Exo-ER, Exo-ER-siPD-L1, Exo-Con and Exo-siNC, and the CD163 mRNA levels were determined by qPCR. (E) Exosomes were injected to nude mice intravenously through the tail vein, and the peritoneal macrophages were isolated from mice treated with Exo-ER, Exo-ER-siPD-L1, Exo-Con and Exo-siNC. The expression of CD163 was evaluated by qPCR analysis.