Development of a multifunctional luciferase reporters system for assessing endoplasmic reticulum-targeting photosensitive compounds

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Abstract Photodynamic therapy (PDT) is a recently developed antitumor modality utilizing the generation of reactive oxygen species (ROS), through light irradiation of photosensitizers (PSs) localized in tumor. Interference with proper functioning of endoplasmic reticulum (ER) by ER-targeting PDT is a newly proposed strategy to achieve tumor cell death. The aim of this study is to establish a multifunctional model to screen and assess ER-targeting PSs based on luciferase reporters system. Upregulation of GRP78 is a biomarker for the onset of ER stress. CHOP is a key initiating player in ER stress-induced cell death. Here, the most sensitive fragments of *GRP78* and *CHOP* promoters responding to ER-targeting PDT were mapped and cloned into pGL3-basic vector, forming -702/GRP78-Luc and -443/CHOP-Luc construct, respectively. We demonstrated that -702/GRP78-Luc expression can be used to indicate the ER-targeting of PSs, meanwhile estimate the ROS level induced by low-dose ER-targeting PDT. Moreover, the luciferase signaling of -443/CHOP-Luc showed highly consistence with apoptosis rate caused by ER-targeting PDT, suggesting that -443/CHOP-Luc can evaluate the antitumor properties of PSs. Hypericin, Foscan® and methylene blue were applied to verify the sensitivity and reliability of our model. These results proved that GRP78-CHOP model may be suitable to screen ER-targeting

photosensitive compounds with lower cost and higher sensitivity than traditional ways.

Keywords Photodynamic therapy (PDT) · Endoplasmic reticulum targeting · *GRP78* promoter activity · *CHOP* promoter activity · Antitumor property

Introduction

Photodynamic therapy (PDT) relies on the selective retention of photosensitizers (PSs) in tumor, followed by light-dependent production of reactive oxygen species (ROS) and oxidative stress in these tissues (Robertson et al. 2009). In addition to photosensitizer concentration and light fluence, the precise subcellular localization of the photosensitizer is the most important factor in determining the effect and mechanism of PDT (Castano et al. 2004, 2005). Photosensitizer can selectively accumulate in cellular compartments, including mitochondria, endoplasmic reticulum (ER), lysosomes, and Golgi apparatus (Peng et al. 1996). Although the significant role of mitochondria in apoptosis is well verified, ER is becoming gradually recognized as another crucial cell death regulator (Boyce and Yuan 2006; Szegezdi et al. 2006). Pharmacological interference with proper functioning of ER has become a newly proposed strategy to achieve tumor cell death (Logue et al. 2013; Schönthal 2013), and the ER-targeting PDT is an attractive approach for cancer therapy.

A large number of correlative studies have established that ER-locating PDT can result in dramatic changes in ER homeostasis, and activate a cell death program (Teiten et al. 2003; Ali and Olivo 2002). However, there are few effective methods to evaluate the primary photodamage to ER induced by photodynamic compounds. Traditionally, the subcellular localization of photosensitizers is determined by co-staining with organelle-specific probes, such as Mito-Tracker, ER-

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Tracker, and Lyso-Tracker (Moserova and Kralova 2012). Nevertheless, the assumption that an ER-localizing drug could cause an ER stress, which may not always be the case, furthermore mild ER stress could not effectively trigger cell death (Boyce and Yuan 2006). With this purpose, the present study focuses on establishing a fast and sensitive drug screening model to obtain effective ER stress-inducing PSs and evaluate oxidative cytotoxicity induced by PSs.

Glucose-regulated protein GRP78/HSPA5 is an ER-resident chaperone which involves in protein folding and controlling the activation of ER stress sensors (Dudek et al. 2009). The expression of GRP78 is upregulated in ER stress, for its function is to alleviate ER stress and maintain cell survival (Lee 2005). Overexpression of GRP78 protein has always been observed following ER-targeting PDT (Teiten et al. 2003; Gomer et al. 1991). However, persistent or intense ER stress will finally cause apoptotic cell death. CHOP, also known as growth arrest- and DNA damage-inducible gene 153 (GADD153), is mostly absent, but strongly emerges responding to intense ER stress (Oyadomari and Mori 2003). The induction of CHOP triggers the critical early events leading to the initiation of ER stress-induced cell death (Zinszner et al. 1998). It has been previously reported that PDT induce CHOP expression and CHOP induction contribute to the anti-tumorigenic activity of PDT (Buytaert et al. 2007; Wong et al. 2004).

Bioassays utilizing reporter vectors regulated by promoters of cellular stress-responsive gene have been suggested as promising tools to assess photocytotoxic (Cheng et al. 2010). A recent study has shown that MCF-7 cells containing a human HSPA1A promoter-driven luciferase reporter could be used for assessing oxidative stress associated with low-dose photodynamic therapy (Zheng et al. 2013). Our research developed cancer reporter cells expressing the firefly luciferase reporter under the control of the functional human *GRP78* or *CHOP* promoter, respectively, and provided a comprehensive analysis of *GRP78* and *CHOP* promoter-driven luciferase activity regulated by ER-targeting PDT. We demonstrated that the reporters of *GRP78* gene can be used to evaluate the photoreaction on ER. While, to what extent photodamaged-ER contribute to apoptosis can be evaluated by reporters of *CHOP* gene.

It is widely reported that human breast cancer cell line MCF-7 is sensitive to ER-targeting PDT (Marchal et al. 2007; Li et al. 2010; Xue et al. 2001). So this study applied MCF-7 as model cell. Classical ER-localizing photosensitizer Hypericin (Ritz et al. 2008; Davids et al. 2008) was applied to form this reporter system. Foscan® and methylene blue, two PS exhibiting different intracellular localization properties, were used to verify the sensitivity and reliability of our model. Our GRP78-CHOP luciferase reporter system can be a complementary method to the co-staining with standard organelle probes when screen ER-targeting antitumor lead compounds with ideal photo-activity.

Materials and methods

Cell culture

MCF-7 cells purchased from the Cell Bank of Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China) were cultured in Roswell Park Memorial Institute (RPMI) 1,640 medium, supplemented with 10 % heat-inactivated bovine serum, penicillin (100 U/ml), and streptomycin (100 U/ml). Cells were incubated at 37 °C in a humidified atmosphere of 95 % air and 5 % CO₂.

PDT treatment

PDT treatment was performed as described previously (Zheng et al. 2013). Cells were co-incubated with certain concentrations of photosensitizers in the dark at 37 °C. After an indicated time of incubation, cells were illuminated with a specific light fluency (7.2 J/cm²), then harvested 4 h after illumination. Light was emitted from a 100-W quartz-halogen lamp, and the light intensity was measured by a photo radiometer (Delta Ohm, Padua, Italy).

Assessment of photocytotoxicity

MCF-7 cells (1×10^4) were seeded into a 96-well plate, treated with different concentrations of hypericin, Foscan®, or methylene blue. After 24 h incubation, cells were exposed to the PDT treatment. Cell viability was measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay 4 h after PDT treatment.

Flow cytometric analysis of apoptosis

Extent of apoptosis was measured through Annexin V-FITC Apoptosis Detection kit (Invitrogen Corporation, Carlsbad, CA, USA) as described by the manufacture's instruction. Briefly, MCF-7 cells (1×10^6 /well) were seeded in six-well plates and treated with hypericin or other photosensitizer at various concentrations. After that, cells were collected and stained with AnnexinV-FITC and then analyzed by flow cytometry using FACScan flow cytometer (Becton Dickinson, New Jersey, USA).

Real-time quantitative reverse transcription-PCR analysis

Total RNA of Hypericin-treated cells were extracted using TRIZOL™ reagent (Promega Corporation, Madison, WI, USA) according to the supplier's instruction. Reverse transcription was performed using Reverse Transcription System (TaKaRa Biotechnology Ltd., Shandong, China). Real-time polymerase chain reaction (PCR) was performed using SYBR

Green Supermix (Vazyme Biotech, Nanjing, China) with an iCycler® thermal cycler (Bio-Rad Laboratories Inc., Berkeley, CA, USA). Primers of GRP78 and CHOP were in Table 1. The data were collected and analyzed using the comparative Ct (threshold cycle) method using GAPDH as the reference gene.

Protein extraction and western blotting analysis

Proteins were obtained as Shen et al. (2013) described. Protein concentrations were determined using the BCA assay (Pierce Biotechnology Inc., Rockford, IL, USA). Equal amounts of proteins (60 µg) were prepared for western blotting assay, then resolved on 10 % SDS-PAGE and electrotransferred onto PVDF membranes (Millipore, Billerica, MA, USA). After the transfer of proteins, membranes were blocked for 1 h at room temperature, and incubated with primary antibody in dilution buffer (BSA in TBS-T) overnight at 4 °C. Membranes were then incubated with an alkaline phosphatase (AP) conjugated-secondary antibody and detected in AP buffer containing NBT/BCIP for 10 min, and then photographed. β-actin was used as loading control. The primary antibodies against GRP78 (GTX113340), CHOP (GTX112827) were all purchased from GeneTex Co. (San Antonio, TX, USA). Primary antibody against cleaved caspase-9 (SC-22182) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Construction of CHOP promoter-driven luciferase plasmid

Standard PCR was utilized to prepare the luciferase constructs used in this study. A 1,088-bp CHOP promoter construct (−1,059/+29 CHOP), corresponding to the sequence from −1,059 to +29 (relative to the transcriptional start site) of the 5'-flanking region of the human CHOP gene, was generated from human genomic DNA using F1_C (5'-GGAAGATCTTGACTACCAAGGGATTCCAG-3') and R1_C (5'-CCCAAGCTTTACGCTCAGTGCCTTAGAC-3') for forward and reverse primers, respectively. PCR product was cloned into Bgl II and HindIII sites of pGL3-basic vector. The underlined nucleotides represent the HindIII and Bgl II sites. The resulting construct was confirmed by DNA sequencing.

Table 1 Primers sequence for Q-PCR

Gene	Primer sequence	Genebank ID
GRP78	F: 5'-CTGGAAGTATTGCTGGCCTA-3'	NM_005347.4
	R: 5'-TGGTGAGAAGAGACACATCG-3'	
CHOP	F: 5'-TAAGTCTAAGGCACTGAGCG-3'	NM_001195053.1
	R: 5'-CTTTCAGGTGTGGTGATGT-3'	

The 5'-flanking deletion constructs of the CHOP promoter were similarly generated by using the −1,059/+29 CHOP construct as a template. The following oligonucleotides were used as the sense primers for construction of the following vectors: F2_C (−649/+29 CHOP-luc), 5'-GGAAGATCTCTCGAGATGTCGACAATCCC-3'; F3_C (−443/+29 CHOP-luc), 5'-GGAAGATCTAAGGCTGATAGCCGTTGG-3'; F4_C (−261/+29 CHOP-luc), 5'-GGAAGATCTCCCTCCGCGCGCGCAT-3'; F5_C (−222/+29 CHOP-luc), 5'-GGAAGATCTAAGCCTCGTGACCCAAAG-3'. The antisense primer in all cases was R1_C.

Site-directed mutagenesis of endoplasmic reticulum stress element (ERSE) sites were done by multiple rounds of PCR using (−443/+29) CHOP construct and appropriate primers with altered bases. The primers used to make ERSE mutant were pair 1, F_C-MUT (5'-aaggTGCCGGCGTGCCACTTT-3') and R1_C; pair 2, F5_C and R_C-MUT (5'-AAAGTGGCAGCCGCAcctt-3'). The first two rounds of PCR generated two fragments of DNA, with 23 bp of overlap. These two fragments were gel purified and used as templates for a third PCR with F5_C and R1_C primers. The mutated DNA was then cloned in pGL3-basic and verified by sequence analysis.

Construction of GRP78 promoter-driven luciferase plasmid

Similarly, a 981-bp GRP78 promoter construct (−933/+48 GRP78), corresponding to the sequence from −933 to +48 (relative to the transcriptional start site) of the 5'-flanking region of the human GRP78 gene, was generated from human genomic DNA using F1_G (5'-AAAGGATGGAGAAAGCCC CG-3') and R1_G (5'-CCTCTCACACTCGCGAAACA-3') for forward and reverse primers, respectively. PCR product was cloned into Bgl II and HindIII sites of pGL3-basic vector. The 5'-flanking deletion constructs of the GRP78 promoter were generated by using the −933/+48 GRP78 construct as a template. The following oligonucleotides were used as the sense primers for construction of the following vectors: F2_G (−702/+29 GRP78-luc), 5'-GGAAGATCT AAAATGGCTGGCAAGGGTG-3'; F3_G (−547/+29 GRP78-luc), 5'-GGAAGATCT ATGTGTTCTGAGGCGATCCC-3'; F4_G (−302/+29 GRP78-luc), and 5'-GGAAGATCT GGTCAG AAGTCGAGGAGA-3'. The antisense primer in all cases was R1_G.

Site-directed mutagenesis of heat shock element (HSE) sites was done by multiple rounds of PCR using −702/+29 GRP78-luc construct and appropriate primers with altered bases. The primers used to make HSE mutant were pair 1, F_G-MUT (5'-ATTCGTGGTCaggCAGAGTC-3') and R1_G; pair 2, F2_G and R_G-MUT (5'-TCTGGACTCTGcctGACCAC-3'). The first two rounds of PCR generated two fragments of DNA, with 25 bp of overlap. These two fragments were gel purified and used as templates for a third PCR with F4_G and R1_G primers. The mutated DNA was then cloned in pGL3-

Basic and verified by sequence analysis. All luciferase constructs were verified by sequencing analysis.

Transient transfection and Luciferase activity assay

Each promoter construct was transfected into subconfluent monolayer cells using lipofectamine reagent Lipofectamine^{TM}2000 (Invitrogen Corporation) according to the manufacturer's instructions. Each well was co-transfected with 100 ng promoter construct and 50 ng pRL-SV40 plasmid expressing renilla luciferase to serve as an internal control for variations in transfection efficiency. After 4 h of transfection, cells were washed by PBS and incubated with various concentrations of photosensitizer for 24 h. Luciferase activity was detected 4 h after PDT treatment using the dual luciferase reporter assay system (Promega). The relative luciferase activity was normalized with renilla luciferase activity.

Measurement of intracellular ROS level

After PDT treatment, ROS level in PDT-treated cells was assessed by DCFH-DA method. In this method, ROS can oxidize fluorogenic substrate DCFH-DA to highly fluorescent DCF, so as to monitor intracellular level of ROS. After PDT treatment, DCFH-DA was added to the media at a final concentration of 10 μ M and incubated for 30 min at 37 °C. Then, the generation of ROS was measured by fluorescence intensity using EnSpire® Multimode Plate Reader (Perkin Elmer, Boston, MA, USA) at an excitation wavelength of 485 nm and an emission wavelength of 535 nm (Zheng et al. 2013).

Biostatistical analysis

All experiments were repeated three times. The results are presented as the mean of the three independent experiments. Experimental value was expressed as means $\pm$ standard deviation (SD). Significant differences between experimental groups and control group were determined using two-tailed Student's *t* test. Statistical analysis was performed using the GraphPad prism5 software to evaluate the significance of the difference between groups, considered as *p < 0.05, **p < 0.01, and ***p < 0.001.

Result

Hypericin-mediated PDT-induced expression of GRP78 and CHOP

The photocytotoxicity of hypericin mediated-PDT (HY-PDT) in the MCF-7 cells was determined by MTT assay 4 h after the photosensitization (Fig. 1a). The cells were incubated with

various HY concentrations (0–1 μ M) for 24 h in the dark, and were irradiated with the light dose of 7.2/cm². Figure 1 showed HY resulted in a dose-dependent cell viability.

To investigate the induction of ER stress-related protein GRP78 and CHOP associated with HY-PDT-induced photocytotoxicity, we examined the dose effect of HY on the expression of GRP78 and CHOP 4 h post-PDT. As western blotting analysis show, treatment with HY-PDT induced a dose-dependent increase in protein expression of GRP78 and CHOP (Fig. 1b). Quantitative RT-PCR analysis demonstrated that, consistent with protein level, PDT upregulated GRP78 and CHOP mRNA level in a dose-dependent manner (Fig. 1c).

Localization of functional region in promoter involved in activation of GRP78 transcription by HY-PDT

Research has reported that the induction of GRP78 is regulated primarily at the transcriptional level (Li and Lee 2006). To identify the HY-PDT responsive functional region within the GRP78 promoter, series of 5'-deletion GRP78 promoter constructs were generated by PCR and fused to the coding region of the luciferase reporter pGL3-basic (Fig. 2a). These constructs were transiently transfected into MCF-7 cells, and the PDT-induced cellular stress response was determined by luciferase assay. All transfected cells were incubated with 0.25 μ M HY for 24 h in the dark, then irradiated with 7.2 J/cm² light, and re-incubated for 4 h. At this sublethal dose, the luciferase activity in treated cells could be detected without the influence of cytotoxicity.

The -702/GRP78-Luc transfected cells exposed to PDT, showed a 3.1-fold higher stress response compared with that of the -547/GRP78-Luc transfected cells (Fig. 2b, rows 1 and 2), suggesting the region between -702 and -547 contains an important *cis*-positive element involved in the transcriptional activation of GRP78 by HY-PDT. Given the relative luciferase activity generated by -302/GRP78-Luc transfected cells with or without photo-irradiation were comparable (Fig. 2b, row 4), we speculated that the ERSEs located at the nucleotide sequence of the -140 to -19 region of the GRP78 promoter does not contribute predominantly to the activation of GRP78 by HY-PDT. To identify potential transcription factor binding sites in the region between -702 and -547 of GRP78 promoter, the genomic nucleotide sequence was analyzed for known transcription factor binding consensus with TFSearch (Searching Transcription Factor Binding Sites from the Computational Biology Research Center of Japan) and TESS (Transcription Element Search System) database. Both of the database show there is a putative heat shock element (HSE) lying between -646 and -635 of human GRP78 promoter (Fig. 2a). It is well documented that induction of HSP genes is mediated by the heat shock transcription factor (HSF) binding to the HSE (Walther and Stein 2009). To

Fig. 1 Effect of HY-PDT on cell viability, mRNA and protein expression of GRP78 and CHOP gene in MCF-7 cells. Cells were incubated with various concentrations (0–1 μM) of HY for 24 h before photoirradiation at 7.2 J/cm² light dose, and re-incubated for 4 h. **a** Cell viability was assessed by the MTT assay. **b** Protein levels were detected by Western blot after PDT treatments. β-actin was used as the loading control. **c**

Quantification real-time reverse transcription-PCR was done on cDNA which was generated from total RNA by using $2^{-\Delta\Delta C_t}$ method. Data were presented as means $\pm$ SD and as representative of an average of three independent experiments per concentration. Significant differences from untreated control were indicated by * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

determine the role of the putative HSF binding site in the HY-PDT responsiveness of the GRP78 promoter, we next tested whether mutation of HSE actually affects the PDT-induced GRP78 promoter activity. Compared with wild-type construct (−702/GRP78-Luc), luciferase activity of mutation construct

(−702/+48 mut GRP78-Luc) was a 3.9-fold decrease by treatment with HY-PDT (Fig. 2b, row 5). Taken together, these results suggested the putative HSE in the distal promoter region is critical for HY-PDT mediated transactivation of GRP78 promoter.

Fig. 2 Localization of functional region in GRP78 promoter involved in activation of GRP78 transcription by HY-PDT. **a** Schematic representation of the reporter gene constructs. **b** Deletion and mutant analysis of the GRP78 promoter. MCF-7 cells were transiently transfected with progressive 5' deletion mutants and site-specific mutant of GRP78 promoter. After transfection, cells were then treated by HY-PDT with conditions

described previously. The cells were lysed and luciferase activity measured 4 h post-PDT, the bar graphs represented the relative levels of luciferase activity in each of the transfected samples. Values are shown relative to the corresponding controls without PDT treatment. Columns, duplicate determinations representative experiment of three; bars $\pm$ SD

Localization of functional region in promoter involved in activation of *CHOP* transcription by HY-PDT

The *CHOP* promoter contains several well-characterized response elements (AARE2, AARE1, and AP-1) and two ERSE in reversed orientations (Oyadomari and Mori 2003). However, the elements controlling transcription of *CHOP* gene varies depending on the kind of stress induced (Guyton et al. 1996; Woo et al. 2007; Kwok and Daskal 2008). In order to identify the functional region of *CHOP* that responds to HY-PDT, we constructed five luciferase plasmids that carry a 5'-deleted promoter of the *CHOP* gene, following the location of potential response elements. The structures of the luciferase recombinants and the PDT-induced stress response of MCF-7 cells transfected with these recombinants are shown in Fig. 3a.

According to the results of 5'-deletion analysis, it seems that the deletion of AARE2 had no negative effect on the HY-PDT-mediated activation of the *CHOP* promoter (Fig. 3b, rows 1 and 2). Deletion of AARE1 (-261/*CHOP*-Luc) partly suppressed HY-PDT-mediated transcriptional induction of *CHOP*, while further deletion of AP-1 (-222/*CHOP*-Luc) demonstrated AP-1 alone does not contribute greatly to the transcriptional induction of *CHOP* by HY-PDT (Fig. 3b). On the other hand, the -222/*CHOP*-Luc transfected cells treated with irradiation showed a 5.8-fold higher stress response compared with that of the non-PDT group (Fig. 3b, row 5). Hence, we hypothesized that ERSE played a key role in the responsiveness of the *CHOP* promoter to PDT-induced ER stress. Site-directed mutagenesis was further performed to confirm the hypothesis. As shown in Fig. 3b, row 6, HY-PDT responsive promoter activity of ERSE mutation promoter (-443/+29 mut *CHOP*-Luc) showed a nearly threefold decrease, suggesting the ERSE plays a key role in *CHOP*

promoter regulation by HY-PDT. The AARE1 or AP-1 alone is insufficient.

Correlations between *GRP78*-regulated luciferase activity and intracellular ROS level induced by HY-PDT

ER-targeting photosensitizers generate ROS which provokes photo-oxidative ER stress. The activation of *GRP78* promoter is used extensively as a biological marker for the onset of ER stress. Thus, we compared the changes in luciferase activity under the control of functional *GRP78* promoter in relation to the intracellular ROS level induced by HY-PDT. *GRP78* promoter-driven luciferase activity and intracellular ROS level were respectively examined 4 h after the transfected cells were stimulated with 7.2 J/cm² light in the presence of 0–1 μ M HY.

We observed that there was a first-increased and then-decreased tendency in *GRP78*-regulated relative luciferase activity of -702/*GRP78*-Luc transfected reporter cells with increasing HY concentration (Fig. 4a). The lowest detectable increase in relative luciferase activity occurred at 0.03125 μ M HY, with nearly three times the control level ($p < 0.05$). On the other hand, HY-PDT treatment brought about a dose-dependent increase in intracellular ROS level (Fig. 4b). The increase of ROS level in this tested concentration range are not statistically significant up to 0.125 μ M ($p < 0.05$). Maximum *GRP78*-driven luciferase activity, up to more than 10 times ($p < 0.001$) the control level, was observed at 0.5 μ M HY-PDT treatment (Fig. 4a). Under the same concentration, HY-PDT treatment has resulted in a 6.1-fold increase in ROS level (Fig. 4b). From this perspective, it suggests that the luciferase activity of *GRP78*-regulated reporter cells is more sensitive than the changes in ROS level in response to PDT mediated by low dose of ER-targeting photosensitizer.

Fig. 3 Localization of functional region in *CHOP* promoter involved in activation of *CHOP* transcription by HY-PDT. **a** Schematic representation of the reporter gene constructs. **b** Deletion and mutant analysis of the *CHOP* promoter. MCF-7 cells were transiently transfected with progressive 5' deletion mutants and site-specific mutant of *CHOP* promoter. The

cells were treated with or without HY-PDT, and lysed and luciferase activity measured 4 h post-PDT. The relative fold induction is defined as the ratio of the relative luciferase activity of PDT-treated cells to untreated cells. Data represent the mean $\pm$ SD of three independent experiments

Fig. 4 The dose-response effect of HY-PDT on *GRP78* promoter activity and intracellular ROS level in MCF-7 cells. **a** Relative luciferase activity of -702/*GRP78*-Luc transfected cells treated by PDT mediated by various concentration HY (0–1 μM) with a light dose of 7.2 J/cm² was measured

using the luciferase assay system. The untreated cells were used as control. **b** The intracellular ROS level respond to PDT are indicated as values relative to the control group. Data represent mean ± SD of three individual experiments

Correlations between *CHOP*-regulated luciferase activity and extent of photodamage induced by oxidant stress associated with HY-PDT

It has been reported that low level of ROS production has a beneficial effect to cells, which is known as hormesis, while a massive level of ROS causes oxidative damage to cells and even induce cell death (Radak et al. 2005). Therefore, we wonder to what extent the photo-oxidative stress might contribute to apoptosis. *CHOP* induction is believed to be a key element of the switch from prosurvival to prodeath signaling in ER stress. Thus, we also wonder if the luciferase activity under the control of functional *CHOP* promoter could exhibit a preference for evaluating the extent of photodamage caused by ER-targeting photosensitizers. For this purpose, dose effect of HY on *CHOP*-regulated luciferase activity and apoptosis rate were assessed 4 h post-PDT.

Increasing concentrations of HY led to a strong dose-dependent increase in relative luciferase activity linked to the functional *CHOP* promoter (Fig. 5a). Compared with protein expression (Fig. 1b), we observed that luciferase activity of -443/*CHOP*-Luc reporter cells is more sensitive than *CHOP* protein level in response to oxidative damage induced by PDT. The further study of the relationship between *CHOP*-regulated luciferase activity and photo-damage showed, the cell apoptosis rate induced by increasing dose of HY-PDT exhibited highly consistence with corresponding *CHOP*-regulated luciferase activity (Fig. 5b). Apoptotic marker cleaved caspase-9 was also examined 4 h-post irradiation (Fig. 1b). The results mentioned above indicated that the reporter gene assay using the promoter of *CHOP* gene is able to rapidly detect the photodamage induced by ER-targeting PDT in comparison to apoptosis detection and protein detection.

Validation of the luciferase reporters system to assess ER-targeting PDT

Foscan® exhibits diffuse subcellular localization involving the Golgi apparatus and ER (Teiten et al. 2003), while methylene blue (Me) localizes in the mitochondria and lysosomes (Gabrielli et al. 2004). Foscan® and Me are all well-studied photosensitizers with defined photochemical properties. For these reason, we applied them to verify the sensitivity and reliability of our luciferase reporter. Marchal et al. (2007) proved a predominant accumulation of Foscan® in the ER after 24 h incubation. The reporter cells were treated by PDT under a specific light fluency (7.2 J/cm²) with various positive compounds concentration (Me, 0–10 μM; Foscan®, 0–12 μM) after 24 h incubation.

A dose-effect relationship study showed that, consistent with the effect of HY, *GRP78* promoter-driven luciferase activity first increased and then decreased with increasing concentration of Foscan®, exhibiting a maximum at 6 μM (Fig. 6a), while Me failed to activate *GRP78* promoter (Fig. 6b). However, after photo-activation, both Foscan®- and Me-mediated PDT elicited a dose-dependent increase in intracellular ROS level (Fig. 6c, f). At the dose of 6 μM, the apoptotic population in Foscan®- and PDT-treated cell was about 55 % (Fig. 6g). This is also in agreement with the results obtained under the treatment of HY-PDT, the maximum *GRP78*-regulated luciferase activity was observed at 0.5 μM HY, at this dose the apoptotic population was about 57 % (Fig. 5b).

We further examined *CHOP* promoter induction pattern in MCF-7 cells following Foscan®- or Me-mediated PDT. As shown in Fig. 6c, induction of the *CHOP* promoter-driven luciferase activity was dramatically increase in a

Fig. 5 The dose-response effect of HY-PDT on *CHOP* promoter activity and apoptosis rate in MCF-7 cells. **a** Relative luciferase activity of -443/*CHOP*-Luc transfected cells treated by PDT mediated by various concentration HY was measured using the luciferase assay system.

Histograms show the PDT response of *CHOP* promoter activity as folds of induction. Changes in cell viability estimated by flow cytometric analysis are shown in **b**. Data represent mean $\pm$ SD of three individual experiments

concentration-dependent manner with Foscan®, while Me was ineffective to *CHOP* promoter-driven luciferase activity (Fig. 6d). And the apoptotic population with increasing concentration of Foscan® was relative to *CHOP*-regulated luciferase activity (Fig. 6g). The results shown above suggest these two ER stress-inducible gene promoter activity will not elevated following other organelle-targeting photosensitizers-mediated PDT.

Discussion

PDT efficacy relies on ROS-induced oxidative damage (Dolmans et al. 2003). However, the mechanism of cell death induced by photo-oxidative stress is tightly associated with the sites of intracellular PS accumulation (Castano et al. 2004, 2005). An important role in initiating intrinsic apoptosis following PDT treatment has been attributed to oxidative

Fig. 6 Luciferase reporters system responds to two positive photosensitive compounds. Compounds tested were Foscan® and methylene blue. The promoter activity of *GRP78* (a and b) and *CHOP* (c and d) was measured and expressed as corresponding relative luciferase activity

versus control. Oxidative stress was evaluated by ROS assay (e and f). Apoptotic population was assessed using Annexin V-FITC apoptosis detection kit (g and h). Data represent mean $\pm$ SD of three individual experiments

damage of the ER, which proceeds through disturbing ER-calcium homeostasis or promoting accumulation of misfolded proteins in ER (François et al. 2011; Grebeňová et al. 2003; Szokalska et al. 2009). Hence, it is necessary to develop a vitro model with high specificity and sensitivity for screening effective ER-stress inducing photosensitizers with good anti-tumor activity.

In ER stress response, activation of prosurvival component GRP78 and pro-apoptotic component CHOP are two key opposing representatives of the cellular struggle for survival versus cell death (Schönthal 2013). We thus developed two plasmid constructs containing ER stress-inducible transcription factor binding sequence of GRP78 and CHOP promoters, respectively, and applied GRP78-CHOP system in determining the photo-efficacy, ER stress-inducing property, as well as antitumor activity of photosensitive compounds.

At the beginning of the present study, we respectively performed 5'-deletion analysis of the *GRP78* gene and *CHOP* gene promoter to locate the functional region. It has been reported that elevated baseline levels of GRP78 are generally present in many tumor cell lines and tumor tissues, which provide a survival advantage for tumor cells to proliferate in a sub-optimal environment (Schönthal 2013). Consistent with literature reports, we observed that GRP78 protein pre-emerges in MCF-7 without the treatment of HY-PDT. Therefore in our study, when measuring the luciferase activity under the control of series of 5'-deletion GRP78 promoter constructs in PDT-treated cells, the cells transfected with the same luciferase plasmid but not treated with PDT were used as control group. In reporter cells, the endogenous GRP78 promoter drives the expression of the endogenous GRP78 gene, while the transfected plasmid containing the GRP78 promoter drives the luciferase reporter. Such that pre-existing expression of GRP78 would not affect the detection of the functional region of GRP78 promoter in response to PDT.

The results showed that the DNA sequence from -702 to +48 of *GRP78* gene and the DNA sequence from -443 to +29 of *CHOP* gene respectively contains a functional region responding to ER-targeting PDT. Furthermore, through site-directed mutagenesis, we confirmed the putative HSE within the functional region of *GRP78* promoter played a vital role in stress responsiveness to PDT. Mutation analysis also suggested that ERSE may be an important *cis*-positive element to regulate CHOP expression in response to PDT-induced ER stress. Although, it is frequently reported that the ERSEs located at the proximal region of human *GRP78* promoter with the consensus sequence of CCAAT(N₉)CCACG is found to be a *cis*-element critical for transcriptional induction of human *GRP78* (Yoshida et al. 1998), in this paper we demonstrated that ERSEs does not contribute greatly to the activation of *GRP78* by ER-targeting PDT.

PDT has a profound effect on HSPs, especially HSP70 (Gomer et al. 1996; Korbelik et al. 2005). However, the specific HSP70 upregulated after PDT varied depending on the specific subcellular target of PDT mediated by different PSs (Gomer et al. 1996). GRP78/HSPA5 is a member of HSP70 family localizing in ER (Dudek et al. 2009). The classical transcription mechanism of genes belonging to HSP family is inducing HSFs binding to specific HSEs in promoter of HSP genes (Liu et al. 1993). It is widely documented PDT-mediated oxidative stress could activate HSF binding activity almost instantaneously after PDT treatment (Gomer et al. 1996; Zheng et al. 2013; Luna et al. 2000). Furthermore, it has been reported that PDT treatment will induce thermotolerance in treated cells, and the activation of HSF binding activity after PDT is partly correlated with the induction of thermotolerance (Gomer et al. 1996; Fuchs et al. 2000). Thus, it might be the reason why the putative HSE element plays a more important role than ERSE in ER-targeting PDT-induced GRP78 expression. However, with the aggravation of oxidative damage to ER, the pro-apoptotic module of ER stress system gain dominance (Schönthal 2013), accompanied by the increasing demand of ERSE in transcriptional induction of related gene, which might explain why CHOP is controlled by ERSE.

Dose-response effect study shown that the relative luciferase activity level of *GRP78* first increased and then decreased in response to increasing level of intracellular ROS level induced by ER-targeting PDT, and the maximal luciferase activity of *GRP78* exhibited at the dose when the corresponding percentage of apoptotic cells reach the values of 50–60 %. Moreover, dose-dependent *CHOP*-regulated luciferase activity showed highly consistence with apoptosis rate induced by ER-targeting PS (Fig. 5b). We also observed the luciferase activity of -443/CHOP-Luc is more sensitive than the corresponding protein expression level in response to ER-targeting PDT (Fig. 1b). Therefore, we believe that the reporters of *GRP78* gene can be used to evaluate the photoreaction to ER in cells with low-dose PDT. And the extent, photodamaged-ER contribute to apoptosis can be evaluated by reporters of CHOP gene.

Intriguingly, the tendency of GRP78 expression and promoter activity with increasing dose of HY is not compatible in our study. The reason might be as following: the induction of *GRP78* is due to the protection mechanism of ER stress in response to ER-targeting PDT, whereas persistent ER stress ultimately cause apoptosis, and thus the activation of *GRP78* promoter no longer elevated. Although the activity of *GRP78* promoter activated by high dose of HY was no longer elevated, the slowly induction of *GRP78* is still lasting, which result in maintained increase of *GRP78* mRNA and protein expression. Moreover, the overload of HY or Foscan® or some other toxic side effects may cause the drop of the promoter activity of *GRP78*.

Collectively, we tried to associate the luciferase activity of both *GRP78* and *CHOP* promoter-driven recombinant construct with organelle targeting, intracellular ROS level, as well as lethality rate induced by ER-targeting PDT. The linkage between PDT-induced oxidative stress to ER and induction of *GRP78* and *CHOP* promoter activity revealed in this study provides the possibility that *GRP78* and *CHOP* promoter-driven luciferase activity may be suitable to screen ER stress-inducing photosensitive compounds and assess the antitumor activity of these ER-targeting photosensitizers with lower cost and higher sensitivity. Based on *GRP78*-*CHOP* system, the comprehensive photoreaction process can be simplified to a relatively mature method of dual luciferase reporter gene assay. Although, more data with other types of photodynamic compounds are necessary to confirm our findings and further validate the application of the luciferase cells in ER-targeting PS assessment.

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