Original Contribution

Overexpression of GRP94 in breast cancer cells resistant to oxidative stress promotes high levels of cancer cell proliferation and migration: Implications for tumor recurrence

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Targeting the altered redox status of cancer cells is emerging as an interesting approach to potentiate chemotherapy. However, to maximize the effectiveness of this strategy and define the correct chemotherapeutic associations, it is important to understand the biological consequences of chronically exposing cancer cells to reactive oxygen species (ROS). Using an H2O2-generating system, we selected a ROS-resistant MCF-7 breast cancer cell line, namely Resox cells. By exploring different survival pathways that are usually induced during oxidative stress, we identified a constitutive overexpression of the endoplasmic reticulum chaperone, GRP94, in these cells, whereas levels of its cytoplasmic homolog HSP90, or GRP78, were not modified. This overexpression was not mediated by constitutive unfolded protein response (UPR) activation. The increase in GRP94 is tightly linked to an increase in cell proliferation and migration capacities, as shown by GRP94-silencing experiments. Interestingly, we also observed that GRP94 silencing inhibits migration and proliferation of the highly aggressive MDA-MB-231 cells. By immunohistochemistry, we showed that GRP94 expression was higher in recurrent human breast cancers than in their paired primary neoplasias. Similar to the situation in the Resox cells, this increase was not associated with an increase in UPR activation in recurrent tumors. In conclusion, this study suggests that GRP94 overexpression may be a hallmark of aggressiveness and recurrence in breast cancers.

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Introduction

Exploiting vulnerabilities of cancer cells is a key strategy for the development of new anticancer drugs. One such vulnerability is intracellular redox alteration, consecutive to a decrease in antioxidant capacities and an increased formation of reactive oxygen species (ROS) via several mechanisms including oncogenic signaling [1–3]. Since the discovery of this susceptibility, scientists have developed strategies that specifically target cancer redox sensitivity [1,3]. Candidate drugs designed for this purpose can be separated into two main categories: the prooxidants, for example, β-lapachone [4] or the association of ascorbate/menadione (Asc/Men) [5], and the inhibitors of antioxidant capacity, for instance, phenylethyl isothiocyanate [6] or As2O3 [7]. Despite initial promise with these approaches, adaptation of cancer cells to oxidative stress may result in unwanted side effects. Indeed, oxidants can induce activation of prosurvival pathways, such as NF-E2-related factor 2 (Nrf2) [8] or the unfolded protein response (UPR) [9], which have been associated with chemoresistance [8,10]. Cancer adaptation to redox equilibrium dysregulation is also supported by the recent discovery that cancer stem cells contain a high degree of ROS defense resulting in radioresistance [11]. Consequently, there is a special need to better understand the physiological and molecular consequences of chronically exposing cancer cells to oxidative stress in order to improve anticancer oxidative strategies.

Within the tumor, malignant cells face restricted supplies of nutrients and oxygen. Therefore, to survive, they need to activate prosurvival pathways, like Nrf2 and UPR [12]. Under these stress conditions, activation of UPR leads to the upregulation of prosurvival proteins involved in angiogenesis, folding capacity, redox protection, or degradation of unfolded proteins. However, when activation of this response is prolonged, it can also result in cell death. Factors controlling the balance between proapoptotic and protective functions of the UPR are of critical

Abbreviations: ROS, reactive oxygen species; UPR, unfolded protein response; Asc/Men, ascorbate/menadione; ER, endoplasmic reticulum.

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importance for tumorigenesis [10,13]. A major mechanism of the UPR protecting arms is an increase in endoplasmic reticulum (ER) molecular chaperones and there is a large body of evidence to support a role of these chaperones in cancer features, including proliferation rate, drug resistance, and metastasis [14]. For example, GRP78 (also called BIP), one of the best-characterized ER chaperones, has been shown to be necessary for tumorigenesis of fibrosarcoma cells injected into mice [15] and to protect cancer cells from topoisomerase inhibitors [16,17], microtubule-interfering agents [18], and doxorubicin [17]. This, together with the fact that GRP78 has been found to be overexpressed in a wide range of cancers [10,14], clearly supports a role of ER chaperones in cancer biology. As for GRP78, an elevated level of GRP94 (gp96, HSP90B1) has also been reported in several cancers, including esophageal [19], lung [20], colon [21], gastric [22], and breast [23] carcinoma. Nevertheless, the biological significance of the increase in GRP94 remains unclear.

The present study was designed to identify survival pathways associated with redox protection, with a particular focus on putative changes in ER chaperones and their potential effects on cancer cell resistance, proliferation, and migration capacities.

Materials and methods

Cell lines and chemicals

The breast cancer cell line MCF-7 was purchased from ECACC (Salisbury, UK) and maintained in DMEM supplemented with 10% fetal calf serum, penicillin (10,000 U/ml) and streptomycin (10 mg/ml). The MDA-MB-231 cell line was a kind gift from Pr Akeila Bellahcene (Metastasis Research Laboratory, Giga Cancer, Liege, Belgium). These cells were maintained in DMEM supplemented with 10% fetal calf serum, penicillin (10,000 U/ml), and streptomycin (10 mg/ml) and 1% of non-essential amino acids. An MCF-7 cell line resistant to oxidative stress was established by constantly exposing cells to increasing concentrations of the pro-oxidant association of Asc/Men for 6 months, starting with 0.5 mM ascorbate/5 μM menadione to a final concentration of 1.5 mM ascorbate/15 μM menadione. Cells were first treated at 50% confluence by replacing their media with fresh media containing Asc/Men. When surviving cells reached 50% confluence, they were washed with warm PBS and treated again (every 1 to 7 days, depending on the level of resistance). To avoid the development of islets of resistance, which could arise from cooperation between cells, the cells were trypsinized approximately every 2 weeks and subcultured into new flasks. After selection, the cell line was stabilized in drug-free medium for 1 month. We will refer to these MCF7 cells resistant to oxidative stress as Resox cells. Menadione sodium bisulfite, sodium ascorbate, dimethyl sulfoxide, glucose oxidase, hydrogen peroxide, cisplatin, doxorubicin, paclitaxel, 5-fluorouracil, DCFH-DA (2,7'-dichlorofluorescein diacetate), ter-butylhydroquinone, N-acetylcysteine, and 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma (St. Louis, MO). Thapsigargin was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Small interfering RNA (siRNA) and Dharmafect 1 reagent were purchased from Dharmacon (Lafayette, CO). All other chemicals were of ACS reagent grade.

Cell culture, functional assays, and siRNA transfection

Cell viability was evaluated using a classical MTT reduction assay [24]. In all cell viability experiments, MTT assay was being completed in serum-containing media. For the cell proliferation assay, cells were seeded into 96-well plates at a density of 5000 cells per well for 24 to 96 h and the relative cell density was then measured using the MTT reduction assay. To evaluate proliferation under reducing conditions, cells were cultured in a medium containing 1 mM DTT, and media with or without DTT were changed every 24 h. For doubling time determination, 100,000 cells were seeded in 6-well plates and counted every 24 h with an automated cell counter (TC10, Bio-Rad Laboratories, Hercules, CA). Doubling time was then determined using the doubling-time.com website (Roth V. 2006; http://www.doubling-time.com/compute.php). Clonogenic assays were performed by seeding cells (500) in 6-well plates at a single-cell density and allowing them to grow for 14 days. Clonogenicity was determined by staining colonies using crystal violet. Migration was tested in wound-healing assays using Culture Inserts (Ibidi, München, DE) according to the manufacturer’s instructions. After removal of culture inserts, pictures of the remaining gaps were taken at different time periods. Dharmafect 1 was used for transfection of siRNAs into MCF-7 and Resox cells according to protocols provided by Dharmacon. Transfection was performed on cells at 1/2 confluence for 24 h, with a 0.1 μM siRNA solution. The siRNA solution was then washed with PBS and replaced with DMEM.

Immunoblotting

Immunoblotting was performed as previously described [25]. For Nrf2 immunoblotting, cytoplasmic and nuclear extracts were prepared as previously described [26]. β-Actin, HSP70 (cytoplastic control), or TBP (nuclear control) levels were used to control protein loading amounts in each sample. Rabbit antibodies to Erp72, Erp57, Erp44, phospho-eIF2α (Ser51), ERO1-Lα, PDI, and anti-GRP78 were from Cell Signaling Technology (Danvers, MA); mouse antibodies to HSP90, Nrf2, SOD, and NQO1 were from Santa Cruz Biotechnology (Santa Cruz, CA); mouse antibody to β-actin and TBP were from Abcam (Cambridge, UK); rabbit antibodies to GRP94 and catalase were from Chemicon International (Temecula, CA).

Biochemical measurements

The content of reduced glutathione was determined using the GSH-Glo glutathione assay (Promega, Madison, WI, USA) according to the procedures described by the supplier. Catalase activity was measured using the TISO4 method [27]. NQO1 (DT-diaphorase) activity was assayed spectrophotometrically by measuring cytochrome c reduction in the presence of NADH as previously described [28].

Immunohistochemistry on human breast tissues

Twenty nine formalin-fixed, paraffin-embedded breast cancer samples were retrieved from the Tumor Bank of the University of Liege (Liège, Belgium). These samples included 15 primary invasive ductal breast carcinomas and their related secondary tumors (including 5 local recurrences and 9 metastases). For the immunohistochemistry on normal human breast tissue, 10 formalin-fixed, paraffin-embedded breast healthy tissue samples originating from reduction mammoplasties were retrieved from the Tumor Bank of the University of Liege (Liège, Belgium). The protocol was approved by the Ethics Committee of the University Hospital of Liege. Clinical and pathological data were available for each patient and are summarized in supplementary Table 1. Serial sections of the breast tumor specimens were stained using antibodies directed against GRP94 (Abcam, Cambridge, UK) and P-eIF2 (Cell Signaling). Immunoperoxidase staining was performed using the LSAB-2 kit (Dako, Glostrup, Denmark) for GRP94 and CSA-II kit (Dako) for P-eIF2 according to the supplier’s recommendations. Positive cells were visualized using a 3,3’-diaminobenzidine (DAB) substrate and the sections were counterstained with hematoxylin. The immunostaining of GRP94 and P-eIF2 was evaluated on labeled tissues using a semiquantitative scoring scale based on staining. For staining intensity, score 0 represented undetectable staining in tumor cells, and scores 1+, 2+, and 3+ denoted staining of low, moderate, and strong intensity, respectively. For staining extent, score 0 represented 0% of stained tumor cells, and scores 1+, 2+, and 3+ corresponded to 6–33, 34–66, and 67–100%, respectively. Finally, the intensity score and the extent score were
multiplied, thus providing a unique global score for each immunostaining, ranging between 0 and 9.

Data analyses

All experiments (excepting immunohistochemistry) were performed at least 3 times. MTT assays, Western blot, enzymatic activities, or proliferation assays were analyzed using a paired or unpaired t test or ANOVA as appropriate, performed with GraphPad Prism software (GraphPad Software, San Diego, CA, USA). For immunohistochemistry on the breast cancer samples, the P values were obtained from a paired Wilcoxon signed rank test with continuity correction, a Welch’s t test, or a t test for unequal sample sizes with equal variance. P values less than 0.05 were considered statistically significant. All the results were computed in R language.

Results and discussion

Establishment of a MCF-7 cell line resistant to oxidative stress

To investigate the relationship between resistance to oxidative stress and biological cancer properties (chemoresistance, proliferation and migration), we established an MCF-7 cell line, namely the Resox cell line, resistant to the pro-oxidant association of ascorbate and menadione (Asc/Men). This treatment was chosen because ascorbate driven menadione-redox cycling generates mainly H₂O₂ [2] and represents a potential candidate treatment for future prooxidative-based anticancer therapies [5,29]. After 6 months of repeated exposure to Asc/Men, we obtained a quite homogeneous population (as deduced from karyotype analyses, see supplementary Figs. 1 and 2): the Resox cells. The resistance to Asc/Men was confirmed by a cell survival assay (Fig. 1A). Most MCF7 cells were not viable when they were exposed to Asc/Men (1 mM/10 μM) whereas about 60% of Resox cells were still viable. Two other pro-oxidant systems were also used to confirm tolerance of the Resox cells to oxidative stress (Fig. 1B). The first, glucose/glucose oxidase, is another H₂O₂-generating system that mimics chronic exposure to H₂O₂, and the second was acute exposure to H₂O₂ itself. With both systems, the Resox cells had increased tolerance compared to their parental cell line (Fig. 1B). Using the fluorescent probe DCFH, we also observed a lower increase of intracellular level of ROS in Resox cells following Asc/Men treatment, as compared to MCF-7 cells (supplementary Fig. 3).

Analysis of antioxidant elements in Resox cells

To characterize the Resox cell line, a first set of experiments was performed to identify survival mechanisms or constitutively activated pathways triggered by acute oxidative stress, focusing on the antioxidant capacities. When looking at the expression of antioxidant enzymes, protein abundance (Fig. 2A) and enzyme activity (Figs. 2B and C) of NAD(P)H:quinone oxidoreductase 1 (NQO1) and catalase (CAT) were enhanced in Resox cells. Finally, intracellular GSH, another critical factor associated with redox protection and chemoresistance [8], was not modified in Resox cells compared to MCF-7 cells (Fig. 2D). Similarly, no changes in the activities of glutathione peroxidase and superoxide dismutase were observed in Resox cells compared to MCF-7 cells (data not shown). These results show that some antioxidant enzymes (CAT and NQO1) were upregulated in Resox cells.

Analysis of survival pathways associated with resistance to oxidative stress

To identify other survival pathways likely activated in the resistant cells, a second set of experiments was performed. First, since ER chaperones (particularly GRP78) have been shown to be important factors for cancer resistance and recurrence, we evaluated the expression of these proteins in Resox cells. Fig. 3A shows that GRP94 was upregulated 3-fold in Resox cells, but not its cytoplasmic homologue HSP90, nor...
GRP78. A similar increase in GRP94 protein abundance was also observed in 8 Resox subclones established from the whole cell population (data not shown), thus confirming the relative homogeneity of the Resox cell population revealed by the karyotype analysis. Although GRP94 is one of the most commonly expressed ER chaperones and a member of (ER)-localized multiprotein complexes [30], its functions remain poorly characterized [31]. This is partly due to the compensatory regulation of multiple chaperones that has usually been observed when GRP94 (or another chaperone) expression is suppressed [31,32]. Indeed, Mao et al. [32] showed that only complete knockout of GRP94, but not partial depletion, was associated with ER chaperone upregulation. To study the role of GRP94 overexpression in Resox cells, we therefore reduced GRP94 protein abundance using a siRNA, to about 70% of the MCF-7 basal level in the Resox cell line and 50% in MCF-7 cells after 96 h (Fig. 3B). Silencing of GRP94 was not accompanied by changes in the expression of other chaperones, namely GRP78, HSP70, or HSP90 (Fig. 3B). Then, because GRP78 is able to sense ER protein unfolding/misfolding leading to induction of UPR, we also studied whether GRP94 overexpression was associated with a modified ability to induce this pathway. Fig. 3C shows that Resox cells did not significantly activate the UPR during oxidative stress (as indicated by the inability to phosphorylate eIF2 after Asc/Men treatment) but the UPR was activated after ER calcium depletion by thapsigargin (TG). This result shows that Resox cells still had the capacity to respond to an ER stress by activating UPR but were unable to activate this pathway when it was triggered by oxidative stress. Finally, silencing of GRP94 did not restore UPR activation after Asc/Men treatment (Fig. 3C), demonstrating that GRP94 was not associated with the inability of Resox cells to induce the UPR during oxidative stress.

Secondly, we assessed whether the activation of Nrf2, a redox-sensitive factor, was modified in the Resox cells. No significant difference in the activation of this factor was observed in these cells under basal or stress conditions (Supplementary Fig. 4). This rules out a putative acquisition of resistance by a constitutive activation of Nrf2 leading to activation of several antioxidant genes. Indeed, no differences were observed in the induction of NQO1, a well characterized Nrf2 target, after A/M treatment (data not shown).

Fig. 3. Resistance to oxidative stress is associated with Grp94 overexpression. (A) Western blot against GRP94, GRP78, HSP90, and actin in MCF-7 and MCF-7/Resox cells. Numbers 1, 2, and 3 represent 3 different independent protein extractions. Protein abundances were normalized to actin and represent the mean ± SEM of three independent experiments. (B) GRP94 silencing was not associated with HSP90, HSP70, or GRP78 protein variations. Cells were exposed to GRP94 (siGRP94) or nonrelevant (siCONT) siRNA. A classical Western blot against the indicated proteins was performed on the proteins extracted from cells exposed to siRNAs for 96 h. (C) Phosphorylation of eIF2 after Asc/Men (1 mM/10 μM, 2 h) or thapsigargin (TG, 1 μM, 6 h) treatment in cells pretreated with the indicated siRNAs for 96 h. A Western blot against the phosphorylated form of eIF2 (P-eIF2) is shown. **P < 0.01.

Fig. 4. Relationship between GRP94 upregulation and MCF-7 resistance. (A) MCF-7 and MCF-7/Resox cells treated with the indicated siRNA for 72 h were exposed to different concentrations of Asc/Men for 24 h. Then cell viability was measured by MTT reduction assay, as described under Materials and methods. (A) Cells pretreated with the indicated siRNAs for 72 h were exposed to cisplatin (Cis, 50 μM) or doxorubicin (Doxo, 1 μM), 5-fluorouracil (5-FU, 100 μM) or paclitaxel (PAC, 0.1 μM) for 24 h before cell viability measurement. Results represent means ± SEM.
Relationship among GRP94 expression, cell survival, and resistance

We investigated whether GRP94 upregulation had any effect on cell survival and Resox cell resistance to standard chemotherapy. Fig. 4A shows that there were no changes in the pattern of cellular resistance against oxidative stress in either cell line (MCF-7 and Resox) whether GRP94 was silenced or not. This indicates that GRP94 overexpression in Resox cells was not linked to the resistance to oxidative stress.

We then assessed whether GRP94 overexpression, as well as resistance against oxidative stress, could be related to a putative resistance to standard chemotherapeutic drugs. Four drugs, structurally and mechanistically unrelated, were selected: cisplatin (Cis), 5-fluorouracil (5-FU), doxorubicin (Doxo), and paclitaxel (PAC). Fig. 4B shows that Resox cells partially resisted Cis and Doxo treatments ($P_b<0.0001$ for MCF-7 versus Resox, and for both treatments) for 24 h, but GRP94 did not appear to be involved in this resistance. Indeed, the silencing of GRP94 did not result in a survival decrease after doxorubicin exposure ($P=0.62$) and appeared to induce resistance to cisplatin ($P<0.001$). This is in agreement with previous reports showing that induction of ER chaperones is associated with potentiation of cytotoxicity induced by platinating agents [33]. These results indicate that acquisition of resistance against oxidative stress may be accompanied by crossed drug resistance (i.e., cisplatin and doxorubicin), indicating the importance of selecting the right association and schedule to effectively eradicate cancer cells.

Role of GRP94 in Resox proliferation and migration capacities

Although GRP94 overexpression was not involved in the survival of Resox cells against oxidative stress or in the partial resistance to some chemotherapy agents, we decided to explore the consequences of its upregulation on two other hallmarks of cancer aggressiveness, i.e., cell proliferation and migration. Two methods were used to assess the Resox cell proliferation rate, namely the colony forming unit (CFU) and the MTT reduction assay. Fig. 5A shows that, compared to...

Fig. 5. GRP94 is important for MCF-7/Resox increased proliferation and migration capacities. (A) Clonogenic assays performed on MCF-7 and MCF-7/Resox cells. A representative image of MCF-7 and MCF-7/Resox clonogenicity is shown in the lower panel. (B) Role of GRP94 upregulation in the increase in MCF-7/Resox proliferation. Cells were incubated with the indicated siRNAs for 24 h. Cells were then trypsinized and incubated for different times under normal growth conditions. Cell proliferation was assessed as described under Materials and methods. (C) MCF-7 and MCF-7/Resox cells were incubated with the indicated siRNAs for 24 h. Cells were then trypsinized and incubated in culture insert systems. Cell migration was assessed following removal of culture inserts. Typical pictures of the remaining gaps are shown 0, 3, or 7 days after insert removal. Results represent means±SEM. * $P<0.05$, ** $P<0.01$, (ns) not significant.
their parental cell line, the Resox cells had a higher proliferation rate which was partly mediated by GRP94 overexpression, as revealed by the use of siRNA against GRP94 (Fig. 5B). Furthermore, we compared MCF-7 versus Resox migration capacity using a culture-insert system as described under Materials and methods. This experiment showed that MCF-7 cells were virtually unable to migrate 7 days after insert removal whereas the Resox cells almost completely recovered the gap during this time (Fig. 5C). This capacity to migrate is tightly associated with GRP94 overexpression since silencing GRP94 almost completely suppressed Resox migration capacity (Fig. 5C).

To confirm these results, we sought to establish the role of GRP94 in the proliferation and migration capacity of a more aggressive breast cancer cell line, the MDA-MB-231 cells. As shown in Fig. 6A, GRP94 was expressed in MDA-MB-231 to a level close to that of the Resox cells. Interestingly, Resox and MDA-MB-231 cells presented an almost similar doubling time (33 and 31 h, respectively), considerably shorter than that of MCF-7 cells (47 h, Fig. 6C). Finally, as observed for Resox cells, GPR94 silencing (Fig. 6B) completely suppressed migration (Fig. 6D), and largely reduced proliferation of MDA-MB-231 cells (Fig. 6E). Taken together, these results support the existence of a link

Fig. 6. GRP94 is important for the high proliferation and migration rates of MDA-MB-231. (A) Comparison of GRP94 protein abundance in MCF-7, Resox, and MDA-MB-231 by Western blot. Numbers 1, 2, and 3 represent three different independent protein extractions. (B) MDA-MB-231 cells were exposed to GRP94 (siGRP94) or nonrelevant (siCONT) siRNA. A standard Western blot against the indicated proteins was performed on the proteins extracted from cells exposed to siRNAs for 72 h. For (A) and (B), protein abundances normalized to actin were indicated, and represent the mean ± SD of three independent experiments. (C) The amount of 100,000 cells was incubated for different time periods and counted. The doubling time was calculated as described under Materials and methods for each cell line. (D) MDA-MB-231 cells were incubated with the indicated siRNAs for 24 h. Cells were then trypsinized and incubated in culture insert systems. Cell migration was assessed following removal of culture inserts. Typical pictures of the remaining gaps are shown 0, 6, or 24 h after insert removal. (E) Clonogenic assays performed on MDA-MB-231 cells preincubated 24 h with the indicated siRNA. Results represent means ± SEM. *P < 0.05, **P < 0.01.
between high GRP94 expression and increased aggressiveness of human breast cancers. It should be noted that GRP94 is essential for the correct processing of insulin-like growth factor [34], integrins [35], and Toll-like receptors [36]. Given the major role of these proteins in cancer cell migration, this provides new perspectives for understanding the role of GRP94 overexpression on the high migration capacity of Resox cells. Studies are now in progress to explore this particular issue.

Cell proliferation under reducing conditions

Increased ROS production has generally been associated with elevated cell growth. Therefore, it was rather unexpected that Resox cells, although having high antioxidant capacities, had a higher proliferation rate than their parental cell line. We therefore hypothesized that GRP94 is overexpressed in resistant cells to promote proliferation under an unfavorable redox context. To challenge this hypothesis, the intracellular redox homeostasis of Resox cells was modified by silencing catalase (CAT), an antioxidant enzyme which is upregulated in these cells (Fig. 2A). Fig. 7A shows that CAT silencing partially counteracted the antiproliferative effect of GRP94 silencing in resistant cells. This indicates that GRP94 overexpression is not simply the consequence of a reducing environment but that this chaperone is required by cells to proliferate under a reducing basal condition. Another element in favor of this hypothesis is the importance of GRP94 for cell proliferation under reducing conditions (1 mM DTT) in both MCF-7 and Resox cell lines (Fig. 7B). Indeed, exposing cells to DTT leads to an impairment of protein folding and to redox alterations in the ER [37]. Moreover, the identification of genetic mutations responsible for DTT hypersensitivity has been used as a method to identify genes involved in the cellular redox machinery required for disulfide-linked protein folding in the ER [38]. Given the known association of GRP94 with the protein machinery dedicated to disulfide bond formation in the ER [30], it may be suggested that GRP94 could help to maintain an optimal redox state in the ER or to transfer reducing equivalents through the ER folding machinery. In this context, since hydrogen peroxide has been proposed to directly mediate the formation of disulfide bonds in the ER [39,40], it is tempting to speculate that the ER folding machinery was increased in Resox cells in order to compensate for a high ER reducing state due to antioxidant enzyme overexpression. On this basis we decided to evaluate whether the ER chaperones involved in disulfide bond formation were also elevated in Resox cells. Western blot analyses revealed an increased abundance of the three major protein disulfide isomerases PDI, ERp72, and ERp57 in resistant cells (Fig. 7C).

GRP94 expression and breast cancer recurrence

Our previous results showed that GRP94 overexpression is involved in high cell proliferation and cell migration, suggesting that it is an important mediator of cancer cell aggressiveness. We therefore wondered whether this was also the case in human recurrent breast tumors. To this end, GRP94 expression was studied by immunohistochemistry in primary invasive ductal carcinomas and in recurrent tumors...
tumors. We first observed that GRP94 was overexpressed in tumors, as compared to normal tissues (supplementary Figs. 5A and B). Then, to avoid the effects of interindividual variability, the analysis was performed in matched primary and secondary neoplasias derived from the same patients. As exemplified in Fig. 8A and illustrated in Fig. 8C, immunostaining revealed significantly higher levels of GRP94 expression in recurrences compared to the corresponding initial breast tumors. An increase in GRP94 score was observed in the second cancer event in 87% of the patients, whereas for the remaining 13% the GRP94 scores were equivalent in the first and second events (supplementary Table 1). In tumors, GRP94 can be overexpressed as a consequence of nutrient and oxygen deprivation via UPR activation [41,42]. However, in our resistant cell model, the increase in GRP94 expression was associated with a reduced ability to phosphorylate eIF2 and, subsequently, to reduce UPR activation. We, therefore, decided to evaluate whether UPR activation was correlated to GRP94 expression in recurrent breast cancers. This experiment revealed that, in contrast to GRP94, P-eIF2 staining was not modified in secondary compared to primary tumors (Figs. 8B and C). Moreover, there was no statistical correlation between GRP94 expression and P-eIF2 staining (not shown). This suggested that, as in Resox cells, GRP94 overexpression in recurrent tumors was not due to UPR activation. These data are also supported by the bidimensional hierarchical clustering of patients and tumor features (including tumor GRP94 and P-eIF2 staining and number of chemotherapeutic classes received by the 15 patients of the study), which is presented in Fig. 8D. In this clustering, P-eIF2 staining was not grouped with GRP94 staining in the first or second event of cancer, also suggesting a lack of association between these two variables. It is noteworthy that patients are essentially grouped according to their modulation of P-eIF2 staining between the first (A) and second (B) event of tumors. Remarkably, it can be also deduced from the clustering that most of the patients that received chemotherapies presented the highest values of GRP94 levels in their corresponding recurrent tumor. This observation was confirmed statistically, as samples from patients treated with chemotherapy had higher GRP94 levels than nontreated patients ($P<0.05$). Finally, with regard to other indexes, no correlation was observed between GRP94 expression and tumor grade, KI67 staining, age, hormone-therapy, radiotherapy, HER2/neu, estrogen receptor or progesterone receptor status (supplementary Table 1 and data not shown). Taken together, these findings suggest that GRP94 expression is increased in chemoresistant tumors.

Conclusions

This study highlights a clear association between GRP94 expression and cancer aggressiveness. Indeed, GRP94 appeared to promote a high level of proliferation and migration in a cell line resistant to oxidative stress and some chemotherapy drugs. In addition, GRP94 expression was increased in recurrent breast cancers. GRP94 upregulation could, therefore, represent a central mechanism by which cancer cells acquire an aggressive phenotype after chemoresistance. A model summarizing our results and explaining the potential relationship between

Fig. 8. Overexpression of GRP94 in recurrent breast carcinomas. Representative immunostaining of GRP94 (A) and P-eIF2 (B) in the initial tumor (1st event) and the corresponding recurrence (2nd event) from a single patient. Original magnifications: ×200 and ×400 for inset. (C) Comparison of GRP94 and P-eIF2 expression score in primary and recurrent tumors. (D) Bidimensional hierarchical clustering of patients (horizontal axis) and tumor features (vertical axis). Individual data for the clinicopathological factors are shown by the color scale. Red cells indicate a score or value equal to 9 and white cells equal to 0. Chemo.: number (from 0 to 5) of chemotherapeutic classes used (taxanes, platins, anthracyclines, pyrimidines, and alkylating agents). A, 1st cancer event; B, 2nd cancer event. *** $P<0.001$, (ns) not significant.
chemoresistance and ER folding capacity in cancer cells is depicted in Fig. 9. Thus, many types of cancer cells exhibit increased intracellular levels of reactive oxygen species (ROS), which can promote a high level of cell proliferation and an increased adaptation capacity [1]. However, this can also sensitize cancer cells to prooxidative-based chemotherapy. We have shown in this study that targeting the altered redox state of cancer cells results in an increase of proteins of the ER machinery dedicated to protein folding, including GRP94 and PDIs. These proteins allowed ROS-resistant cells to maintain a high proliferation rate despite their elevated antioxidant capacity. GRP94 overexpression also leads, probably through modulation of secreted proteins, to an acquisition of a high migratory capacity. In agreement with this hypothesis, previous reports have shown that GRP94 was particularly abundant in cancer metastases [21, 22, 43, 44], disseminated tumor cells [45], and radioresistant cell lines [46]. Moreover, targeting GRP94 with a vaccination strategy suppressed cancer metastasis when combined with radiation therapy [47]. GRP94 may, therefore, constitute an important and specific potential therapeutic target to avoid cancer recurrence. Taken together, our findings provide new therapeutic perspectives in the fight against cancer recurrence.

Supplementary materials related to this article can be found online at doi:10.1016/j.freeradbiomed.2011.12.019.

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