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Phospholipid Hydroperoxide Glutathione Peroxidase (PHGPx) expression is downregulated in poorly differentiated breast invasive ductal carcinoma

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Abstract

Phospholipid Hydroperoxide Glutathione Peroxidase (PHGPx) is the only known enzyme able to reduce lipid peroxides bound to cell membranes. Moreover it has been involved in apoptosis and can influence intracellular signaling. To investigate the possible relationship between PHGPx and human cancer we have quantified PHGPx expression levels by real-time quantitative PCR and immunohistochemistry in tissue samples of human breast invasive ductal carcinoma from 34 patients compared with their own controls of benign breast tissue. PHGPx expression levels were compared with the clinical and pathological data of these patients. The results showed that PHGPx expression levels are downregulated in poorly differentiated (grade 3) breast invasive ductal carcinoma ($P = 0.0043$). PHGPx expression levels decreased gradually with tumor grade from grade 1 to grade 3.We also found a downregulation of PHGPx in cases that showed p53 accumulation compared with cases without p53 immunostaining ($P = 0.0011$). PHGPx was also downregulated in cases without progesterone receptors (PR) immunostaining compared with cases with PR immunostaining ($P = 0.0165$). Grade 3, p53 immunostaining and absence of PR immunostaining are poor prognostic factors. These results suggest that PHGPx downregulation could be related with a poorer prognosis in breast invasive ductal carcinoma.

Keywords: *PHGPx, lipid peroxides, breast cancer, p53, progesterone receptors, estrogen receptors*

Introduction

Phospholipid Hydroperoxide Glutathione Peroxidase (PHGPx) is an antioxidant enzyme that is crucial in protecting the cell against DNA-damaging induced by lipid peroxides (LPO). In fact, PHGPx is the only known enzyme able to reduce LPO bound to cell membranes [1]. Beside this role in cell membrane protection, PHGPx can also influence intracellular signaling by inhibition of lipoxygenases [2,3], by inhibition of cyclooxygenase-2 (COX-2) expression/activity [4], and by suppression of cytokine-induced nuclear factor kappa-B (NFκ-B) activation [5]. PHGPx can also inhibit apoptosis [5–7]. The different roles of PHGPx protecting DNA against LPO and influencing intracellular signaling, together with some experimental findings in cell cultures, suggest a possible influence in cancer for this enzyme. For example, a diminished level of PHGPx protein has been shown in pancreatic cancer cell lines compared

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to normal human pancreas. Moreover, an overexpression of PHGPx in these cells has growth inhibitory effects in tumors produced when these cells are injected into nude mice [8]. At the same time, a blockage of eicosanoids synthesis by PHGPx overexpression impedes tumor growth of weakly tumorigenic fibrosarcoma cells and malignant progression when highly tumorigenic melanoma cells are injected in nude mice [4]. However, there is no information about PHGPx expression levels in human cancer tissues.

To further investigate the possible relationship between PHGPx and human cancer, we have measured PHGPx mRNA by real-time quantitative PCR (QRT-PCR) and protein expression by immunohistochemistry in breast cancer samples. Breast cancer is the most common malignant neoplasia in women in the western world [9]. Many molecular events leading to the development of this neoplasia have been described in recent years (for a review see Refs [10,11]). Some studies have detected elevated levels of LPO in serum or in urine from breast cancer patients and from women at high risk for breast cancer [12–16]. We have compared PHGPx expression in breast cancer tissue samples versus benign breast tissue samples from the same patients. We have also examined the relationship between PHGPx expression levels and the clinical and pathological data of the patients, including detection of estrogen receptors (ER), progesterone receptors (PR) and p53 by immunohistochemistry. Our results give light into a possible role of PHGPx expression in breast cancer behavior and prognosis.

Material and methods

Pathological study

Breast intraoperative biopsies from 34 female patients were studied at the pathology service of our institution. Institutional approval was received to carry out the study. An experienced pathologist selected breast cancer tissue samples and benign breast tissue samples from each case. Samples were snap frozen. The remainings of each biopsy was formalin-fixed and paraffin embedded. A complete pathological study, including tumor size, tumor histopathological type, tumor grade, lymph node involvement and immunohistochemistry for ER, PR, p53 and ki-67, was performed according to standard procedures [17–19]. The 34 cases were diagnosed of breast invasive ductal carcinoma (IDC). Clinical and pathological data are summarized in Table I.

RNA isolation and QRT-PCR

Total RNA from 15 sections of 20 μm of each frozen tumor sample and its corresponding benign breast tissue sample was isolated with TrizolR reagent (Life Technologies®, NY, USA) according to manufacturer's instructions. One microgram of total RNA was reverse transcribed with Multiscribe reverse transcriptase (High Capacity cDNA Archive Kit®, Applied Biosystems®, CA, USA) and random hexamers primers in a total reaction volume of 100 μl. A relative quantization was performed in tumor samples versus its corresponding benign breast tissue samples. Commercial assay on demand gene expression products (Applied Biosystems®) was used: a specific human PHGPx assay (Hs00157812_m1) that recognizes both mitochondrial and cytoplasmic isoforms, and a human ribosomic 18S RNA assay (Hs99999901_s1) as an endogenous control to normalize cDNA levels. The reaction mix contained 2 μl of cDNA, 2 μl of 20X assay on demand gene expression product, 20 μl of 2X TaqMan Universal PCR Master Mix (Applied Biosystems®) and water to a final volume of 40 μl. The QRT-PCR equipment was ABI PRISM 7000 Sequence Detection System (Applied Biosystem®). The cycler program was: first step of 2 min at 50°C, a next step of 10 min at 95°C, 40 cycles of 15 s at 95°C, 1 min at 60°C. The relative quantification has been carried out using the Delta–delta Ct model [20] and (User Bulletin #2 ABI PRISM 7700 Sequence Detection System®, Applied Biosystems®, Foster City, CA). (User Bulletin #2 ABI PRISM 7700 Sequence Detection System, Applied Biosystems®.) Figure 1 shows the relative expression of tumor samples normalized with the ribosomic 18S RNA measurements and relative to the expression of the corresponding benign breast tissue samples that were normalized to 1. Reactions were prepared in triplicate and the complete experiment was duplicated.

Immunohistochemistry

Sections of 5 μm were cut from formalin-fixed, paraffin-embedded tissue blocks of the aforementioned patients. Blocks included IDC and adjacent benign ductal epithelium. Slides were deparaffined and endogenous peroxidase activity was blocked by incubation in 3% H_2O_2 in methanol for 10 min at room temperature (RT). Antigens were retrieved by incubation in EDTA for 45 min at 155°C. The primary antibody dilutions were: anti-PHGPx monoclonal antibody (1H11, ab16739 Abcam®, Cambridge, UK) was diluted at 1:150; anti-p53 monoclonal antibody (DO-7, NCL-p53-DO7, Novocastra Laboratories®, Newcastle, UK) was diluted at 1:100; anti-ER monoclonal antibody (NCL-L-ER-6F11, Novocastra Laboratories®) was diluted at 1:100; anti-PR monoclonal antibody (PgR 636, M3569, DakoCytomation®, Denmark) was diluted at 1:100; anti-ki-67 monoclonal antibody (MIB-1, M7240, DakoCytomation®) was diluted at 1:100. The antibodies dilution solution was 1% bovine serum albumine in TBS. Tissue slides were incubated for 2 h

Table I. Clinical and pathological data of the patients.

N	Age (Y)	Histopathological type	Histopathological grade	pT	pN	ER	PR	p53	Ki-67 (%)
1	47	IDC	G1	T2	N0	+	+	−	5
2	60	IDC	G1	T2	N0	+	+	−	3
3	36	IDC	G1	T1c	N1mi	+	+	−	5
4	43	IDC	G1	T1c	N1a	+	+	−	10
5	53	IDC	G1	T1c	N1b	+	+	−	15
6	51	IDC	G1	T2	N2a	+	+	−	5
7	33	IDC	G1	T1c	N1b	+	+	−	3
8	51	IDC	G2	T1c	N0	+	+	−	<1
9	52	IDC	G2	T1c	N0	+	+	−	5
10	41	IDC	G2	T1c	N0	+	+	−	10
11	67	IDC	G2	T2	N0	+	+	−	3
12	76	IDC	G2	T2	N0	+	+	−	3
13	73	IDC	G2	T2	N0	+	+	−	5
14	43	IDC	G2	T1c	N1m	+	+	−	10
15	66	IDC	G2	T1c	N1a	+	+	−	1
16	71	IDC	G2	T1c	N1a	+	+	−	5
17	66	IDC	G2	T1c	N1a	+	+	−	<10
18	77	IDC	G2	T1c	N2a	+	+	−	10
19	65	IDC	G2	T2	N1a	+	−	−	5
20	56	IDC	G2	T2	N1a	+	−	−	10
21	49	IDC	G2	T1b	N1a	+	−	+	10
22	59	IDC	G3	T1c	N0	+	+	+	5
23	46	IDC	G3	T1c	N0	+	+	−	5
24	48	IDC	G3	T1c	N0	+	+	+	30
25	74	IDC	G3	T1c	N0	+	+	+	30
26	75	IDC	G3	T2	N0	−	−	−	20
27	64	IDC	G3	T2	N0	+	+	−	25
28	23	IDC	G3	T1c	N0	+	−	−	70
29	74	IDC	G3	T2	N0	−	−	+	30
30	39	IDC	G3	T2	N1a	+	+	+	25
31	37	IDC	G3	T2	N1a	−	−	−	50
32	44	IDC	G3	T2	N2a	+	−	+	30
33	41	IDC	G3	T2	N3a	+	−	+	20
34	49	IDC	G3	T2	N3a	−	−	−	70

N: number of patients; Y: year; IDC, invasive ductal carcinoma; G1: well differentiated; G2: moderately differentiated; G3: poorly differentiated; pT: primary tumor in TNM staging system (International Union Against Cancer); pN: regional lymph node involvement in TNM staging system (International Union Against Cancer); ER: estrogen receptor; PR: progesterone receptor.

at room temperature. Slides were then rinsed in TBS and incubated with the peroxidase based EnVision™ kit (DakoCytomation®) according to manufacturer's instructions. Specimens were then incubated with diaminobenzidine chromogenic substrate (DakoCytomation®) for 5 min at room temperature. Sections were counterstained in hematoxylin, stepwise dehydrated through graded alcohols and cleared in xylene.

Statistical analysis

The Kruskal–Wallis test was used to assess the statistical significance of the PHGPx expression levels obtained by QRT-PCR compared with histopathological grade. Mann–Whitney test was used to assess the statistical significance of PHGPx expression levels versus PR, ER, and p53 immunostaining. A possible

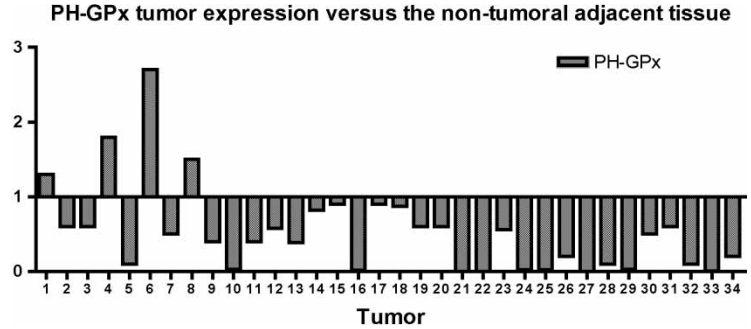

Figure 1. PHGPx mRNA expression levels in 34 cases of invasive ductal carcinoma. The results of each case are relative to the expression of their benign breast tissue samples that are normalized to 1. All the cases with a value lower than 1 correspond to invasive ductal carcinoma showing a repressed expression of PHGPx mRNA compared with their controls.

significant correlation between the expression of PHGPx and the other clinical and pathological data, summarized in Table I, was also studied using both tests.

Results

PHGPx expression levels were determined by QRT-PCR analysis in 34 cases of breast IDC compared to their own controls of benign breast tissue from the same patients. PHGPx transcripts were downregulated in 30 of 34 cases (88%) (Figure 1).

A significant association between PHGPx expression levels obtained by QRT-PCR and tumor grade was found. PHGPx was downregulated in poorly differentiated grade 3 (G3) tumors compared with well-differentiated grade 1 (G1) tumors ($P = 0.0043$). Moreover, PHGPx expression decreased gradually from G1 to G3 tumors (Figure 2A). PHGPx expression was further investigated by immunohistochemistry, revealing similar intensity of immunostaining between G1 tumor cells and benign ductal epithelium (Figure 3A). In contrast, PHGPx immunostaining was lower in G3 tumor cells compared with benign ductal epithelium (Figure 3B).

We found another significant association between PHGPx expression levels and p53 immunohistochemistry. Cases that showed p53 immunostaining showed lower PHGPx expression levels compared with cases without p53 immunostaining ($P = 0.0011$) (Figure 2B).

A third significant association was found between PHGPx expression levels and hormonal status. Interestingly, cases that showed immunostaining for PR showed increased levels of PHGPx compared with cases without PR immunostaining ($P = 0.0165$) (Figure 2C). A tendency of higher PHGPx expression levels in cases showing immnostaining for ER was also observed (Figure 2D). IDC cells showed a stronger intensity of PHGPx immunostaining compared with benign ductal epithelium in cases showing ER and PR immunostaining (Figure 3C). Lower immunostaining was found in tumor cells compared to benign ductal epithelium in cases without ER and PR immunostaining (Figure 3D).

No significant association was found between PHGPx expression levels and patient's age, tumor size, lymph node involvement and the mitotic index estimated by ki-67 immunohistochemistry.

Immunostaining with the anti-PHGPx antibody was detected in epithelial and myoepithelial cells (Figure 3E). The staining was in all cases cytoplasmic and occasionally grainy or granular in appearance. Noteworthy, a strong immunostaining was found in the epithelial cells showing apocrine metaplasia; these cells have a cytoplasm enriched with mitochondrias (Figure 3F).

Discussion

The present study shows a downregulation of PHGPx expression levels measured by QRT-PCR in human

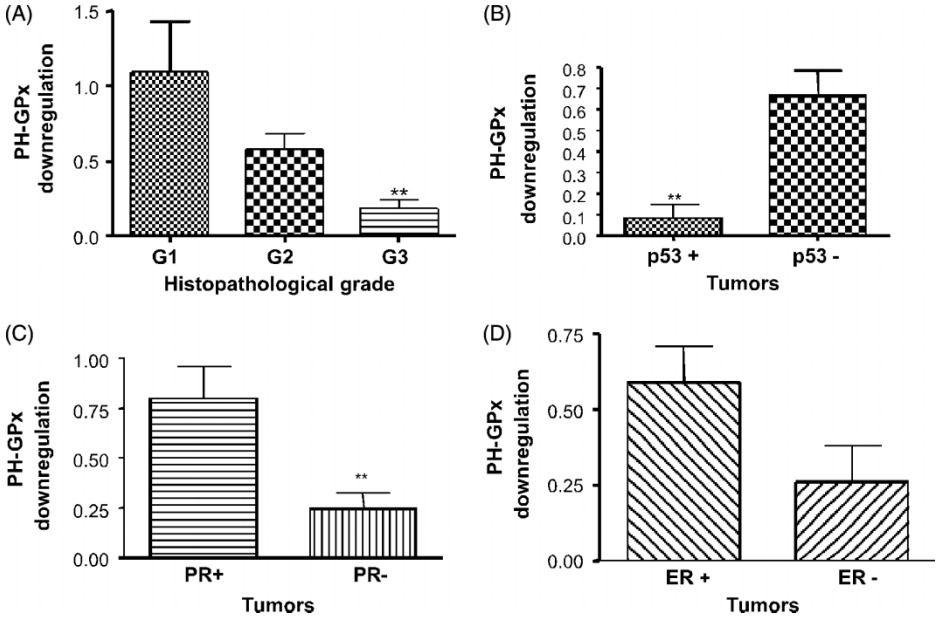

Figure 2. PHGPx relative quantification compared with histological grade, and p53 and hormonal receptor immunostaining. A, PHGPx is repressed in poorly differentiated (G3) invasive ductal carcinoma cases when compared with well differentiated (G1) invasive ductal carcinoma cases ($P = 0.0043$). B, PHGPx is repressed in cases showing p53 immunostaining when compared with cases not showing p53 immunostaining ($P = 0.0011$). C, PHGPx is repressed in cases not showing PR immunostaining when compared with cases showing PR immunostaining ($P = 0.0165$). D, PHGPx is repressed in cases not showing ER immunostaining when compared with cases showing ER immunostaining.

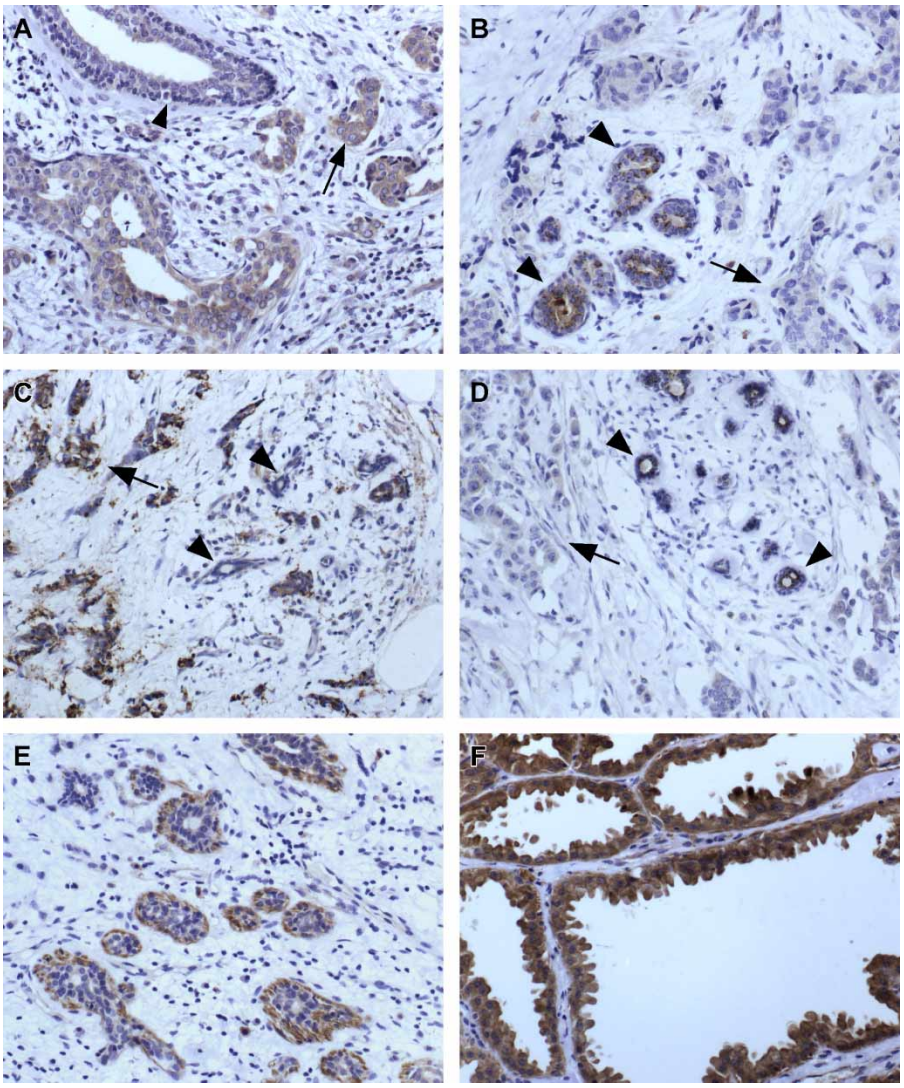

Figure 3. Immunohistochemical study of PHGPx. A, PHGPx immunostaining in well-differentiated (G1) breast invasive ductal carcinoma and in a benign epithelial duct. B, PHGPx immunostaining in poorly differentiated (G3) breast invasive ductal carcinoma and in benign epithelial lobules. C, PHGPx immunostaining in breast invasive ductal carcinoma and in benign epithelial ducts in a case showing PR and ER immunostaining. D, PHGPx immunostaining in breast invasive ductal carcinoma and in benign epithelial duct in a case that did not show PR and ER immunostaining. E, PHGPx immunostaining in myoepithelial cells in benign epithelial ducts and lobules. F, PHGPx immunostaining in epithelial cells showing apocrine metaplasia. Arrows point tumor cells and arrowheads point benign cells. A–F: 20 ×.

breast cancer tissues compared with their corresponding control of non-tumoral breast tissues. The PHGPx downregulation significantly correlates with some of the clinical and pathological data that give information about the prognosis of each patient at the moment of the diagnosis like tumor grade, p53 immunostaining, and PR and ER immunostaining.

PHGPx is significantly downregulated in less differentiated (G3) breast tumors. Moreover, PHGPx expression decreased gradually from G1 to G3 tumors. The most common histological grading system for IDC uses three parameters: tubule formation, nuclear pleomorphism and mitotic rate. Poorly differentiated G3 tumors show less tubules, marked variation in nuclear size and a higher mitotic rate than well differentiated G1 and moderately differentiated G2 tumors [21]. These observations suggest a possible link between PHGPx downregulation and a high proliferative and more dedifferentiated cancer phenotype. Heirman et al. have reported that PHGPx inhibitis tumor growth and malignancy through its downregulatory effects on tumor cell eicosanoids synthesis [4]. According to this, PHGPx downregulation found in G3 IDC cases could be related with an increase in eicosanoids synthesis that could promote the high mitotic rate of these tumors.

We also found a significant association between downregulation of PHGPx expression levels and p53 accumulation. Immunostaining of p53 has been a popular surrogate marker for p53 mutational status, as p53 accumulation occurs when p53 is mutated [22–24]. Mutations of the p53 gene or overexpression of its protein product have been identified in 14–52% of primary breast tumor specimens, and these alterations

were found to be associated with poor prognosis in an analysis of more than 3000 patients with primary breast cancer [25]. A p53 response element in the promoter region of cytosolic GPx has been demonstrated [26]. On the contrary, no p53 response elements have been found in the PHGPx promotor [27–29]. However, transcriptional PHGPx regulation is still not well understood.

We also found a downregulation of PHGPx expression levels in cases without PR and ER immunostaining compared to those with PR and ER immunostaining. Estrogens could activate PHGPx expression in cases with PR and ER positive expression. Accordingly, PHGPx seems to be regulated by hormone responsive elements. A dependence of PHGPx on gonadotrophic hormone has been shown [30]. Also, an upregulation of PHGPx by 17β estradiol in bovine oviducts has been demonstrated [31]. Interestingly, the first intron of the PHGPx gene, where promoter activity is supposed to exist, contains a sterol regulatory element-binding protein responding element [27].

Breast IDC cases showing G3 grade, presence of p53 immunostaining and absence of PR and ER immunostaining have a poorer prognosis than breast IDC cases showing G1 or 2, absence of p53 immunostaining and presence of PR and ER immunostaining. These suggest that PHGPx downregulation could be itself a poor prognostic indicator in breast IDC.

Finally, no significant association was found between PHGPx expression levels and the remaining clinical and pathological features of the patients.

We have also detected PHGPx protein by immunohistochemistry in IDC and in benign breast ductal epithelium. We found a similar intensity of immunostaining between G1 tumor cells and benign ductal epithelium. G3 cells immunostaining, on the contrary, was lower compared with benign ductal epithelium. These data seem to be consistent with those obtained by QRT-PCR. However, due to the fact that immunohistochemistry is not a quantitative method we have not enough information to suggest any parallelism between PHGPx mRNA and protein expression.

In summary, our results show a downregulation of PHGPx expression levels in breast IDC cases that show poor prognostic factors. This is a noteworthy observation that suggests a potential lack of antioxidant control in aggressive breast cancer tumors. However, further studies are needed to understand the implication of PHGPx in breast cancer behavior and prognosis.

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