Role of Glutathione Peroxidase 1 in Breast Cancer: Loss of Heterozygosity and Allelic Differences in the Response to Selenium[1]

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ABSTRACT

A role for allelic variation within the gene for the antioxidant seleno-protein glutathione peroxidase 1 (GPx-1) in the risk or etiology of breast cancer was investigated. By analyzing the frequency of a polymorphism within the GPx-1 gene resulting in a leucine or proline at codon 198, it was determined that the leucine-containing allele was more frequently associated with breast cancer than the proline-containing allele (odds ratio = 1.9; $P < 0.05$). However, the heterozygosity index for this polymorphism was lower in the breast cancer samples. To determine whether this was because of the loss of heterozygosity (LOH) during tumor development, another polymorphic marker within GPx-1, which is frequently heterozygous in the human population, was analyzed. These studies indicated that LOH at this locus is a frequent event, occurring in approximately 36% of the breast tumor DNAs analyzed. The consequences of the identity of the amino acid at position 198 were investigated by engineering breast carcinoma cells that exclusively express either the leucine- or proline-containing GPx-1 allele and studying the response to increasing concentrations of selenium. These studies indicated that the leucine-containing allele was less responsive to the stimulation of GPx-1 enzyme activity observed during selenium supplementation than the allele differing only by a proline at that position. These studies support a role for GPx-1 allelic identity and LOH as factors of significance to breast cancer development.

INTRODUCTION

Research spanning the last 35 years has established that selenium is effective in the reduction of cancer incidence when provided to animals at nontoxic doses only 5–10-fold above the nutritional requirement (1, 2). Chemoprevention studies with selenium in animal model systems have indicated that dietary supplementation with this element is effective for most, if not all, organ systems and is protective against the carcinogenic effects of a wide variety of insults (1). Numerous studies have indicated the efficacy of selenium in the significant reduction of mammary tumor incidence after exposure to carcinogens, including 2-acetylaminofluorene, methylnitrosourea, and 7,12-dimethylbenz(*a*)anthracene, and selenium has also been shown to be effective against the development of spontaneous mammary tumors in the C3H mouse model (1). In humans, selenium supplementation has been shown to reduce the incidence of liver, colon, prostate, and lung cancer (3, 4). Epidemiological data have also supported a protective effect for selenium in humans with regard to the prevention of both prostate cancer (5, 6) and lung cancer (7). Less clear is the relationship between selenium status and the incidence of breast cancer, with one study indicating a protective role (8), whereas other studies have not (9–12).

The cytosolic form of GPx-1[3] is the first and best-characterized selenoprotein (13). This selenium-dependent enzyme is ubiquitously expressed and detoxifies both hydrogen and lipid peroxides (13). Mice that are null for GPx-1 develop normally and are without overt phenotype unless challenged by oxidative stress (14). Given the antioxidant function of GPx-1, it was often considered a potential mediator of the anticarcinogenic properties of selenium, but the enthusiasm for this hypothesis was reduced because of the observations that the level of GPx-1 in mouse erythrocytes is not typically affected by pharmacological doses of selenium. However, more recent data have provided compelling evidence for the reconsideration of the role of this enzyme in chemoprevention by selenium. In 1994, Moscow *et al.* (15) reported genetic variants of GPx-1 in the human population and provided data demonstrating LOH in lung cancer DNA, using microsatellite markers that flank this gene at the 3p21 locus. These data were recently extended to demonstrate reduced GPx-1 enzyme activity in lung cancers exhibiting GPx-1 LOH (16). GPx-1 was also implicated in cancer risk when another group examined the frequency of GPx-1 alleles containing either leucine or proline at codon 198 in a case-control study involving lung cancer patients and controls (17). The allele with leucine at that position was present in 58% of controls and 71% of individuals with lung cancer ($P < 0.01$). In addition, the ORs were 1.8 for heterozygotes and 2.3 for homozygotes of the leucine-containing allele compared with the allele encoding proline at that position (17).

In the present study, a role of GPx-1 in breast cancer was explored. Genotyping DNA from cancer-free individuals, as well as breast cancer specimens, indicated that the leucine-containing allele was more likely to be associated with breast cancer and that LOH occurs at that locus during tumor development. In addition, we demonstrate that the GPx-1 activity associated with the protein encoded by the leucine-containing allele is less responsive to added selenium than the same protein containing a proline at that position, thus assigning a functional consequence to this allelic variation. These studies therefore provide support for a role of GPx-1 in cancer risk and/or etiology.

MATERIALS AND METHODS

Selection of Studied Population. Tissue from breast tumors was obtained from the Department of Surgical Oncology Tissue and Sera Bank at the University of Illinois at Chicago, under an Institutional Review Board-approved protocol. The tissues were collected from women with confirmed histopathological diagnosis of breast cancer who were undergoing lumpectomy or mastectomy. A section of the tissue obtained as part of that required for diagnostic purposes, and that would have otherwise been discarded, was provided to the Department of Pathology, University of Illinois at Chicago. Paraffin materials were cut into sections, mounted on microscopic slides, and stained with H&E. A pathologist identified areas containing tumor tissue and those containing normal breast tissue, which were then microdissected and immediately frozen in liquid nitrogen and then stored at −70°C. DNAs from peripheral blood of cancer-free women asked to participate in a study of diet and heart disease under an Institutional Review Board-approved protocol were obtained from Loyola University (Maywood, IL) and have been used in our previous studies (18). All subjects were >18 years of age and provided written consent; exclusion criteria included any history of cancer or non-insulin-dependent diabetes mellitus.

DNA Isolation and Analysis. DNA from samples was isolated with the Puragene DNA Isolation Kit (Gentra), following the manufacturer's instruc-

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tions, and was used as a template for PCR amplification at the GPx-1 locus by a modification of the procedure reported by Ratnasinghe *et al.* (17). PCR primers based on the human GPx-1 gene sequence (19) flanking the 198 polymorphism (GPx-1 forward primer, 5′-TGTGCCCCTACGGTACA-3′; GPx-1 reverse primer, 5′-CCAAATGACAATGACACAGG-3′) were used to generate a 337-bp amplification product. To genotype the GPx-1 locus at position 198, amplified DNA was digested with the *Apa*1 restriction endonuclease that recognizes the sequence GGGCCC. A proline codon (CCC) at position 198 was cleaved by that enzyme, but a leucine (CTC) was not. PCR was performed by denaturing at 95°C for 3 min; followed by 35 cycles of 95°C for 30 s, 58°C for 60 s, and 72°C for 90 s; with final extension at 72°C for 10 min. PCR amplifications omitting template DNA were included in every experiment as a control for contaminating DNA. The PCR products were digested with 5 units of *Apa*1 overnight at 37°C and electrophoresed on 2% agarose gels, and samples of known genotypes were always included in experiments to control for enzyme activity. Bands were visualized with ethidium bromide to distinguish the uncut 337-bp fragment indicative of the leucine allele from the 258-bp fragment (the 79-bp fragment is typically not visible on the gels) indicative of a proline at position 198.

The number of alanine repeat codons in the GPx-1 gene was determined by a modification of the procedure used by Moscow *et al.* (15). Sequences, including the polyalanine polymorphism in GPx-1 exon 1, were amplified by PCR using the forward primer (5′-ATGTGTGCTGCTCGGCTA-3′) and the reverse primer (5′-AGAAGGCATACACCGACTGG-3′). PCR was performed at 95°C for 3 min, 61°C for 1 min, and 72°C for 1 min for 45 cycles, with a final extension at 72°C for 10 min. As in the procedures noted above, PCR amplifications omitting template DNA were included in every experiment as a control for contaminating DNA. The PCR product was labeled with [^{32}P]ATP by T4 polynucleotide kinase. Alleles containing 5, 6, or 7 alanine codons in GPx-1 resulted in PCR products of 52-, 55-, or 58-bp in length, respectively. The sizes of the PCR products were assessed by 11% PAGE with 10% spreadex (Elchrom Scientific) and detected by autoradiography. Samples with known genotypes were always included in each experiment to control for artifacts associated with the procedures.

Allelic-specific GPx-1 Expression Constructs. A GPx-1 expression construct (designated hGPX198pro) was generated by RT-PCR amplification with RNA obtained from the MDR321 human breast carcinoma cell line, using a *Hin*dIII-containing forward primer (5′-pATAAAGCTTCGCTGGCTTCTTG-GACAAT-3′) and a *Cla*1-containing reverse primer (5′-ATAATCGATTGA-CACCCGGCA-3′) by one-step RT-PCR (Roche Biochemicals). The RT-PCR product was digested with *Hin*dIII and *Cla*1 and directionally inserted into pLNCX retroviral vector (20). Using the resulting construct as a template, a derivative plasmid (hGPX198leu) was generated by mutation of codon 198 from that encoding proline (C**T**C) to one encoding leucine (C**C**C) with a forward primer (5′-pCCTGCTGTCTCAAGGGC**T**CAGCTGTGCCTAGG-3′) and a reverse primer (5′-CCTAGGCACAGCTG**A**GCCCTTGAGACAG-CAGG-3′) by using the QuickChange Site Directed Mutagenesis Kit (Invitrogen). Successful mutagenesis was confirmed by digestion of the plasmid with *Apa*1, and the inserts from both the hGPX198pro and hGPX198leu plasmids were confirmed by sequencing, using services provided by the Research Resources Center at the University of Illinois at Chicago.

Assessment of GPx-1 Activity and Expression. GPx-1 activity in MCF-7 transfectants was measured using the standard coupled spectrophotometric method as described by Samuels *et al.* (21). Briefly, cells were washed with ice-cold PBS, harvested by scraping, and maintained on ice. Cells were resuspended in ice-cold sodium phosphate buffer (0.1 M; pH 7.0) and lysed by sonication. Determination of GPx-1 activity was calculated based on the consumption of reduced NADPH measured spectrophotometrically by absorbance at 339 nm in a reaction containing reduced glutathione, glutathione reductase, cell lysate, and hydrogen peroxide. Units of GPx-1 activity are expressed as nmol NADPH oxidized/min/mg protein. Assays were done in triplicate, using three independently generated lysates from separate cultures.

Northern Blot Analysis. Total RNA was extracted from tissue culture cells using the RNeasy total RNA preparation kit, according to the manufacturer's suggested protocol (Qiagen). RNA was electrophoresed in 1% agarose/formaldehyde denaturing gels and transferred to Gene Screen Plus hybridization membranes (DuPont). Bovine GPx-1 cDNA was used as a hybridization probe after labeling with [^{32}P]dCTP by the random primer method. Hybridization was performed at 65°C for 16 h, after which time the filters were washed in 0.5% SSC and 1% SDS at 65°C three times for 2 h each time. Autoradiographs were obtained by exposure to BioMax film using an intensifying screen at −70°C.

Statistical Evaluation. The statistical difference in the distributions of the GPx-1–198 polymorphisms were calculated by χ^2. The ORs and 95% confidence intervals were calculated with an unconditional logistic regression model by using the SAS statistical analysis program. *P*s were two-sided.

RESULTS

Genotyping DNA from Breast Cancers at the GPx-1 Locus. Previous data have been published describing allelic variants of GPx-1 in the human population and LOH at that allele in lung cancer (15, 17). More recently, Hardie *et al.* (16) reported reduced GPx-1 activity in lung tumor samples that have undergone loss of that allele. That, along with the studies of Ratnasinghe *et al.* (17) indicating an increased risk of lung cancer in individuals with a leucine at position 198 of the GPx-1 allele compared with individuals with a proline at that position, led us to compare GPx-1 allele frequencies in breast tumors and the germ line of cancer-free individuals. Because our previous studies have indicated dramatic variation in allele frequencies of another selenoprotein, Sep15, in different ethnic backgrounds (18), these studies were limited to samples obtained from African-American women to ensure that such variations were not a confounding variable in the GPx-1 genetic analyses. DNA was analyzed by PCR amplification and differential restriction enzyme digestion, and the data are summarized in Table 1. The allelic distribution observed among African Americans in this study is similar to that reported for the Finnish population examined by Ratnisinghe *et al.* (17), indicating the lack of ethnicity-specific variations at this position, at least in the populations examined in these two studies. The analysis presented in Table 1 indicates that there is an increased representation of the *Leu/Leu* genotype in the DNA obtained from breast cancers, as observed for lung cancer. The OR of the *Leu/Leu* genotype is 1.9, when compared with the *Pro/Pro* genotype. As can also be seen in Table 1, the *Pro/Leu* genotype is underrepresented in DNA obtained from breast tumors. One possible reason for this observation is the LOH at the GPx-1 locus during breast cancer development.

Genetic Analysis of Alanine Repeats in the GPx-1 Gene. The GPx-1 gene has been shown to be polymorphic in the number of alanine codons present in exon 1, with variants of 5, 6, or 7 being described (15). To examine whether LOH was occurring at the GPx-1 locus in breast cancer DNAs, the frequency of these polymorphic alleles was determined in DNA from cancer-free individuals and from breast cancer samples (Table 2). We examined a representative sample of DNA obtained from cancer-free African-American women, and the heterozygosity frequency obtained (75%) was similar to that from the Moscow *et al.* study (70%; Ref. 15) described above. The heterozygosity frequency observed in breast cancers was considerably lower (39%), suggesting allelic loss of approximately 36% during tumor development.

Table 1 *Genotype analysis of the pro/leu polymorphism at codon 198 of the human GPx-1 gene obtained from breast cancer samples and cancer-free individuals*

DNAs from individuals were genotyped at codon 198 of the GPx-1 gene as described in the text from either tumor samples or lymphocytes from cancer-free individuals and are presented in the table. Statistical significance for the difference in allele distribution was $P < 0.05$. The OR of the *Leu/Leu* subgroup is 1.906 compared to the *Pro/Pro* subgroup ($P = 0.045$).

GPx-1 (codon 198)	Cancer-free ($n = 517$)	Breast cancer ($n = 79$)	OR (95% confidence limits)
Pro/Pro	244 (47.2%)	36 (45.6%)	1.00
Pro/Leu	209 (40.4%)	25 (31.6%)	0.81 (0.471–1.395)
Leu/Leu	64 (12.4%)	18 (22.7%)	1.906 (1.016–3.576)

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DNAs were genotyped to determine the number of alanine codon repeats in the gene for human GPx-1 as described in the text, and the data presented in the table represent the determined genotypes for individuals analyzed.

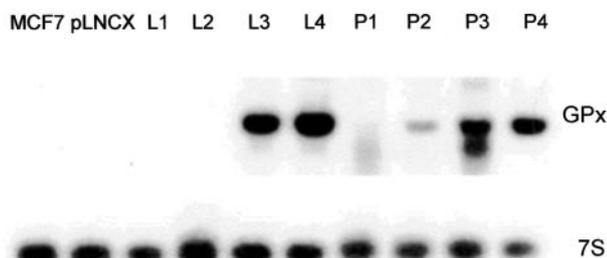| GPx-1 (no. of Ala repeats)	Cancer-free ($n = 109$)	Breast cancer ($n = 74$)
5/5	20	23
6/6	6	17
7/7	1	5
Homozygosity	24.8%	60.8%
5/6	38	12
5/7	33	9
6/7	11	8
Heterozygosity	75.2%	39.2%

Fig. 1. GPx-1 allelic-specific expression in transfected MCF-7 cells. Total RNA from MCF-7 cells (*MCF7*), MCF-7 cells transfected with the pLNCX vector only (*pLNCX*), MCF-7 cells transfected with a GPx-1 expression construct representing the leucine-containing allele (*L1, L2, L3,* and *L4*), and MCF-7 cells transfected with a GPx-1 expression construct representing the proline-containing allele (*P1, P2, P3,* and *P4*) were hybridized with a ^{32}P-labeled probe representing the bovine GPx-1 cDNA. The clones *L3* and *P4* were chosen for further study (see Fig. 2).

Functional Differences between the Leucine- and Proline-containing GPx-1 Alleles. Given the genetic data described above indicating differences in allele frequencies at the GPx-1 locus in cancers *versus* control samples, we investigated whether there was a functional difference between the two proteins differing at codon 198. GPx-1 expression constructs that differ only in the resulting GPx-1 protein having a leucine or proline at position 198 were generated for this purpose. Using PCR amplification and differential restriction enzyme digestion with *Apa*1, it was determined that the GPx-1 gene of the MDR321 breast carcinoma cell line was homozygous for the proline-containing allele. Total RNA from these cells was therefore used as a template for RT-PCR, and the amplification product was inserted into the pLNCX expression vector (20). The resulting plasmid was also used for *in vitro* mutagenesis to generate a derivative expression construct representing the leucine-containing allele. Each plasmid was independently transfected into MCF-7 cells, which were chosen because they produce very low levels of endogenous GPx-1 activity and undetectable levels of GPx-1 mRNA. Transfectants were selected for drug resistance with the antibiotic G418, and individual colonies were expanded in culture. To assess transcription from the transfected GPx-1 expression constructs, total RNA was isolated and analyzed by Northern blotting using either a ^{32}P-labeled probe for bovine GPx-1 or 7S RNA, with the latter included as a control for equal loading of the agarose gel. A representative autoradiogram of such a Northern analysis is presented in Fig. 1. As seen in Fig. 1, it is apparent that endogenous GPx-1 mRNA is undetectable in either native MCF-7 or MCF-7 cells transfected with vector only, even when longer exposures of the autoradiogram were examined (data not shown). A GPx-1 hybridizing band (approximately 1.8 kb) derived from the construct and containing additional vector sequences is clearly visible in MCF-7 cells transfected with the expression construct, in contrast to a band of 0.8 kb that would have been predicted

for the endogenous GPx-1 transcript (22). All transfectants were analyzed for GPx enzyme activity using a coupled spectrophotometric assay. This assay is generally regarded as being quantitative for protein levels because all components except GPx enzymes present in cellular extracts and NADPH are in excess. The GPx activity in untransfected MCF-7 cells was 6.7 ± 1.1 units, which did not change when cells were transfected with the empty vector (7.7 ± 0.7 units). The GPx activity of GPx-1 transfectants ranged between 180 and 300 units.

Two transfectants with similar levels of GPx-1 mRNA and representing either the leucine or proline variant were selected for further study: P4 expressed the proline-containing allele and exhibits GPx activity of 266.9 ± 60.2 units; and L3 expressed the leucine-containing allele and exhibits GPx activity of 353.4 ± 76.8 units. However, when the cells were evaluated for changes in GPx-1 activity with increasing selenium supplementation of the culture media, there was a clear difference, with the leucine-containing allele being less responsive to added sodium selenite (Fig. 2). The data are presented as the relative increase in GPx-1 activity and illustrate that the leucine-containing variant is less responsive to increasing selenium supplementation at each level tested.

To establish whether the difference in GPx activity seen during selenium supplementation was because of an effect on steady-state levels of GPx-1 mRNA, total RNA was prepared from each transfectant incubated at the indicated selenium concentrations, and GPx-1 mRNA levels were assessed by Northern blotting. As seen in Fig. 3, steady-state levels of GPx-1 mRNA were unchanged by selenium supplementation relative to actin RNA constitutive expression. These results are therefore the first to identify functional differences between GPx-1 proteins derived from alleles differing only at position 198.
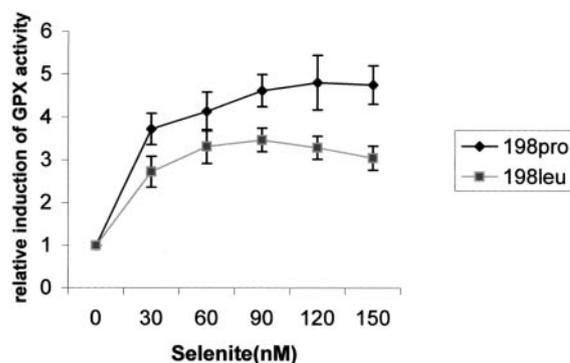

Fig. 2. Selenium induction of GPx activity in MCF-7 cells expressing either the proline-containing or leucine-containing allele. GPx activity was measured after supplementation of the culture media with the indicated concentration of selenium in the form of sodium selenite for 5 days. The GPx activity is expressed as the relative increase in activity compared with the baseline value of each transfectant.
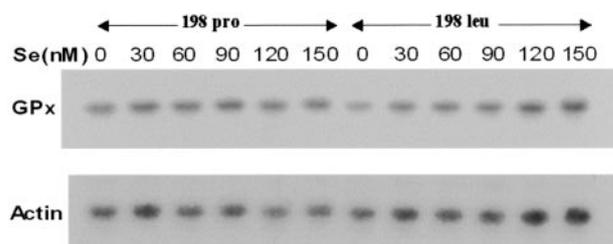

Fig. 3. GPx-1 transcript levels in MCF-7 cells transfected with either the proline or leucine expression constructs as a function of increasing selenium supplementation. Total RNA was prepared from the transfectants presented in Fig. 2 and incubated in the indicated levels of sodium selenite, and GPx-1 mRNA levels were assessed by Northern blotting. The same filters were rehybridized with a ^{32}P-labeled probe for actin as a measure of equal loading of RNA onto the gel.

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DISCUSSION

Selenium has been shown to be effective in reducing carcinogenesis in animal model systems, and human studies supporting a protective role of this element have been reported. Selenoproteins that have ultimate levels that can be influenced by selenium offer one mechanism for how this element reduces cancer incidence, although data in support of this hypothesis remain limited. Previous work has established a putative role for GPx-1 in both the risk (17) and progression (15) of lung cancer, and the data presented in this article extend that data to breast cancers as well. The allele frequency for GPx-1 *Pro* and *Leu* variants derived from tumor DNA from African-American breast cancer patients differed from that determined for peripheral lymphocyte DNA from cancer-free women of the same ethnicity. As seen in Table 1, the *Leu/Leu* genotype is represented at a significantly higher frequency in breast cancer samples, with an OR of 1.9 ($P < 0.05$), similar to that observed for lung cancer, for which an OR of 2.3 was reported (17). If this polymorphism were directly involved with the risk of developing cancer, one would anticipate detecting a functional consequence of the amino acid at that position. This was directly assessed by generating two GPx-1 expression constructs that differed only at the position of the codon 198 polymorphism and expressing each allele in the MCF-7 human breast carcinoma cell line in which GPx-1 activity is virtually undetectable. By assessing the production of GPx-1 enzyme in breast cancer cells that exclusively expressed these alleles, it was apparent that GPx-1 activity derived from the leucine-containing allele was less responsive to increasing selenium supplementation as compared with the proline-containing allele. It should be pointed out that tissue culture media is generally regarded as being selenium deficient, and the levels of selenium obtained because of supplementation and at which the differences in GPx activity are apparent are those doses that best approximate what would be anticipated in human serum. To determine whether changes in GPx-1 mRNA levels were involved in the differential response to selenium induction, steady-state levels of GPx-1 mRNA in the different transfectants at increasing selenium concentrations were assessed by Northern analysis. The results of these studies indicated that the stimulation in GPx activity determined by enzymatic assay was a consequence of either the selenium-mediated stimulation of GPx-1 translation or protein function. The translation of selenoproteins is a complicated process involving the recognition of in-frame UGA codons as the selenium-containing amino acid selenocysteine, a process requiring a specific recognition element in the 3'-untranslated region of selenoprotein mRNAs (23). It remains undetermined how the proline *versus* leucine amino acid ultimately influences selenium stimulation of GPx-1 activity. However, because segregation analyses performed previously indicated that GPx-1 alleles with 5 or 7 alanine repeats are linked to a proline at position 198 and alleles with 6 alanine repeats are associated with leucine (15), our data indicating a functional consequence to the amino acid identity at codon 198 argue that position may be the significant polymorphism with regard to cancer risk. A similar argument has been presented based on the likely structural consequences of the codon 198 polymorphism (17).

It is worth noting, however, that the genotype that is associated with cancer risk was that shown to be less responsive to selenium simulation. The analysis of GPx-1 activity in erythrocytes of individuals with the distinct codon 198 genotypes has been investigated, and no differences as a function of genotype were reported (24). It is unclear whether the cellular environment (erythrocyte *versus* breast carcinoma), other polymorphisms within the GPx-1 gene, or the plasma selenium levels account for the differences between that study and the data presented here.

The observation that fewer Pro/Leu heterozygotes were present in tumor samples raised the possibility that LOH was occurring during cancer development. To address this, we analyzed the frequency of a polymorphic track of polyalanine residues that has been reported to be heterozygous in 70% of the population (15), this frequency being confirmed for the African-American population used in this study. These results, presented in Table 2, indicated an approximately 35% reduction in heterozygosity at this marker in tumor DNA. Given a 36% LOH at the GPx-1 locus in DNA obtained from breast tumors, it would be expected that heterozygosity for the tumor-derived samples would decline by 14% [36% LOH × 39.2% (heterozygosity frequency obtained from cancer-free samples)]. This value is very close to our observed result of a reduction in heterozygosity at the Pro/Leu polymorphism in breast cancer DNA as compared with the control group (40.4% − 31.6% = 8.8%). LOH at the GPx-1 locus has been shown to occur frequently in lung cancers as well (15).

The GPx-1 gene is located at chromosome 3p21, which has been found to be a frequent LOH locus in cancers of the lung, breast, and ovary (25). In the case of breast cancer, the 3p21.3p region has been shown to exhibit the highest frequency of LOH (26). Breast cancer risk is also associated with prolonged ovarian function that results in elevated circulating levels of estrogen (27). Recently, MCF-10F cells exposed to 17β-estradiol exhibited transformation and LOH in chromosome 3p21 (28), raising the possibility that impaired responses to estrogen-mediated oxidative stress, occurring as a result of reduced GPx-1 levels, are contributing to transformation in this system. In support of this concept, lung tumors exhibiting 3p LOH have been shown to have compromised oxidative defense mechanism and reduced GPx-1 enzyme activity, as well as elevated levels of the DNA oxidation product 8-hydroxydeoxyguanosine (16). LOH at this position is also associated with a higher number of relapses and shorter disease-free survival for lung cancer patients (29).

In summary, the studies presented above implicate allelic variants of GPx-1 in breast cancer risk and LOH at this locus as a contributing factor in breast cancer development, as had been described for lung cancer. Previous work has implicated LOH of another selenoprotein, Sep15, in the development of both breast cancer and cancer of the head and neck, and polymorphic variants of this gene that are less responsive to selenium stimulation, albeit by a different mechanism, were described (18). Collectively, these studies raise the possibility that reduced levels of certain selenoproteins, including Sep15 and GPx-1, may increase the risk or promote the development of cancer. It is conceivable that deficiency in protective selenoproteins may exist as a consequence of reduced dietary intake of selenium, possession of a genotype that requires elevated levels of selenium to retain the basal level of protection, or gene dosage effects that are the consequences of LOH.

ACKNOWLEDGMENTS

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