A Loss-of-Function Polymorphism in the Propeptide Domain of the LOX Gene and Breast Cancer

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Abstract

The lysyl oxidase (LOX) gene reverted Ras transformation of NIH 3T3 fibroblasts and tumor formation by gastric cancer cells, which frequently carry mutant RAS genes. The secreted lysyl oxidase proenzyme is processed to a propeptide (LOX-PP) and a functional enzyme (LOX). Unexpectedly, the tumor suppressor activity mapped to the LOX-PP domain, which inhibited tumor formation and the invasive phenotype of NF639 breast cancer cells driven by human epidermal growth factor receptor-2/neu, which signals via Ras. A single-nucleotide polymorphism, G473A (rs1800449), resulting in an Arg158Gln substitution in a highly conserved region within LOX-PP, occurs with an average 473A allele carrier frequency of 24.6% in the HapMap database, but was present in many breast cancer cell lines examined. Here, we show that the Arg-to-Gln substitution profoundly impairs the ability of LOX-PP to inhibit the invasive phenotype and tumor formation of NF639 cells in a xenograft model. LOX-PP Gln displayed attenuated ability to oppose the effects of LOX, which promoted a more invasive phenotype. In a case-control study of African American women, a potential association of the Gln allele was observed with increased risk of estrogen receptor (ER)–negative invasive breast cancer in African American women. Consistently, LOX gene expression was higher in ER-negative versus ER-positive primary breast cancers, and LOX-PP Gln was unable to inhibit invasion by ER-negative cell lines. Thus, these findings identify for the first time genetic polymorphism as a mechanism of impaired tumor suppressor function of LOX-PP and suggest that it may play an etiologic role in ER-negative breast cancer. [Cancer Res 2009;69(16):6685–93]

Introduction

The copper amine oxidase lysyl oxidase is required for the maturation of collagen and elastin precursors in the biosynthesis of a functional extracellular matrix (1, 2). The lysyl oxidase (LOX) gene inhibited the transforming activity of the Ras oncogene in NIH 3T3 fibroblasts and was named the ras recision gene (rrg; refs. 3, 4). Reduced LOX expression has been reported in many carcinomas (5–10). Ectopic LOX gene expression in gastric cancer cells reduced tumor formation in nude mice (7). LOX gene expression in Ras-transformed NIH 3T3 fibroblasts inhibited the activities of the Akt and extracellular signal–regulated kinase (Erk)-1/2 kinases and nuclear factor-κB transcription factors (11). Lysyl oxidase is secreted as a 50-kDa inactive proenzyme (Pro-LOX), which is processed by proteolytic cleavage to a functional 32-kDa enzyme (LOX) and an 18-kDa propeptide (LOX-PP; ref. 12). The LOX-PP domain was identified as the inhibitor of the transformed phenotype of Ras-NIH 3T3 fibroblasts (13), lung and pancreatic cancer cells with mutant RAS genes (10), and NF639 breast cancer cells, driven by human epidermal growth factor receptor-2 (Her-2)/neu, which signals via Ras (14). Specifically, LOX-PP decreased Her-2/neu–mediated signaling and mesenchymal phenotype in vitro and NF639-derived tumor xenograft formation in a nude mouse model (14). Furthermore, LOX-PP attenuated fibronectin-stimulated integrin signaling and migration in breast cancer cells (15). Thus, LOX-PP can inhibit the invasive phenotype of carcinomas.

High-penetrance germ-line mutations account for less than 25% of the familial risk of breast cancer. It has been hypothesized that the remaining susceptibility to breast cancer is polygenic in nature, involving a relatively large number of germ-line, genetic variations with low to moderate penetrance (16). We observed a single-nucleotide polymorphism (SNP) accompanied by a nonsynonymous amino acid substitution, G473A/Arg158Gln (rs1800449), in a highly conserved region within LOX-PP (Fig. 1) in six of nine breast cancer cell lines examined (Supplementary Fig. S1). The frequency of the 473A allele of the LOX gene in European, Asian, Sub-Saharan African, and African American populations in the International HapMap Project averaged 24.6%. Here, the Gln variant, encoded by this minor A allele, was shown to display impaired tumor suppressor ability compared with LOX-PP wild type (WT) encoded by the major G allele. As LOX-PP WT seemed to prevent a more invasive phenotype, the association of the LOX rs1800449 polymorphism with breast cancer risk was examined in a study of African American women who tend to have more aggressive breast cancer and higher mortality rates compared with Caucasian women (17, 18). In a nested case-control study within a cohort of participants of the Black Women’s Health Study (BWHS), the LOX G473A polymorphism seemed to be associated with increased risk of estrogen receptor (ER)–negative invasive breast cancer. Thus, our findings identify genetic polymorphism as a mechanism of impaired tumor suppressor function of LOX-PP and suggest that further analysis of the potential association of the LOX
rs1800449 polymorphism with increased risk of ER-negative breast cancer is warranted.

**Materials and Methods**

**Plasmids.** V5/His-tagged WT murine Pro-LOX and LOX-PP vectors were described (14). The amino acid Arg152 corresponding to the rs1800449 polymorphism was mutated to Gln152 in the murine LOX-PP and Pro-LOX constructs. These DNA fragments were cloned into the retroviral vector pC4bsrR(TO) containing a doxycycline-inducible promoter and vector pCMXter under the control of a constitutive cytomegalovirus (CMV) promoter. Wild-type human LOX-PP was amplified by PCR and cloned into pcDNA4-V5/His vector. Human LOX-PP Gln was generated by site-directed mutagenesis.

**Cell culture conditions.** Mouse NF639 cells and human breast cancer and epithelial cells were cultured as described previously (14,19). Retrovirus stocks were made as described (14). Stable NF639 infectants carrying the doxycycline-inducible constructs of empty vector (EV), Pro-LOX WT, LOX-PP WT, Pro-LOX Gln, and LOX-PP Gln were generated by retroviral infection (14). Recombinant rat LOX-PP WT and LOX-PP Gln proteins were expressed and purified as published (20).

**Immunoblotting.** Whole-cell extracts were prepared and subjected to immunoblotting (14). Antibodies were as follows: phospho-Akt (Ser 473), Akt, phospho-Erk1/2, and Erk1/2 (Cell Signaling); vimentin (NeoMarker); fibronectin and E-cadherin (BD Transduction Laboratories); cyclin D1 (Santa Cruz Biotechnology); β-actin (Sigma); and V5 epitope (Invitrogen). The results from a minimum of three independent experiments were subjected to densitometry and, after normalizing to the lower side of the filter were quantified as described (15). All invasion values relative to control EV cells (set to 1.0) were subjected to densitometry and, after normalizing to the lower side of the filter were quantified as described (15). The detection of recombinant proteins in conditioned medium and whole-cell extracts has no effect on protein expression, secretion, or processing. A sequence alignment spanning the COOH terminus of LOX-PP and the NH2 terminus of the LOX enzyme. Identical amino acids in the propeptide region are in gray; arrow marks the cleavage site; amino acid numbers correspond to the last amino acid of LOX-PP shown for each species. B and C, expression of the proteins was induced in stable pools of NF639-EV (EV), -Pro-LOX WT, -Pro-LOX Gln, -LOX-PP WT (PP WT), or -LOX-PP Gln (PP Gln) cells with 2 μg/mL doxycycline for 48 h. Medium (MED) was subjected to immunoprecipitation as described. Immunoblot analysis was done on whole-cell extracts (WCE; 20 μg) and immunoprecipitates using anti-V5 antibody followed by horseradish peroxidase–labeled protein A. All lanes were from the same blots and were cut to contiguously align the relevant samples, where indicated by the solid vertical line.

**Xenograft mouse model.** NCr nude mice, purchased from Taconic Laboratories at 7 to 9 wk of age, received 2% sucrose plus 2 mg/mL doxycycline solution in lieu of water 3 d before tumor cell inoculation. Doxycycline-inducible stable cell lines were pretreated with 2 μg/mL doxycycline for 24 h, and then 4 x 10^6 cells were injected s.c. in both flanks of the same mouse (n = 6) with NF639-EV (EV, -Pro-LOX WT, -Pro-LOX Gln, -LOX-PP WT (PP WT), or -LOX-PP Gln (PP Gln) cells with 2 μg/mL doxycycline for 48 h. Medium (MED) was subjected to immunoprecipitation as described. Immunoblot analysis was done on whole-cell extracts (WCE; 20 μg) and immunoprecipitates using anti-V5 antibody followed by horseradish peroxidase–labeled protein A. All lanes were from the same blots and were cut to contiguously align the relevant samples, where indicated by the solid vertical line.

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geographic region, family history of breast cancer, age at menarche, body mass index at age 18, parity, age at first birth, oral contraceptive use, menopausal female hormone use, and years of education. There was little evidence of confounding, as OR estimates from these multivariable models were closely similar to estimates from models adjusting for age only. We carried out a test for trend by the inclusion of a term in the logistic regression, in which the number of variant alleles was entered as 0, 1, or 2 (23). We combined the GA and AA genotypes in a second model, assuming a dominant genetic model comparing the presence of either one or two variant alleles (GA or AA) with none (GG). Analyses were conducted separately for all invasive, ER-positive, ER-negative, and HER2-positive breast cancers.

Results

The Gln variant of LOX-PP displayed reduced ability to suppress Ras signaling. We first asked whether the G473A/Arg158Gln polymorphism is present in human breast cancer cells. The results indicated that the untransformed MCF-10A line and breast cancer lines MDA-MB-231, MCF7, and ZR75 were homozygous for the major G allele. Five lines (BT474, BT549, SKBR3, T47D, and BT20) were heterozygous and one line (Hs578T) was homozygous for the minor A allele (Supplementary Fig. S1). Thus, of the nine human breast cancer cell lines examined, six carry the
minor 473A allele, substantially above the 24.6% seen in the HapMap database.

This finding led us to question whether the Gln variant has altered ability to impair Ras signaling in breast cancer cells. NF639 breast cancer cells express low levels of ER and E-cadherin and have a highly invasive phenotype (19). Given that the substitution falls within the COOH-terminal region of the propeptide domain, which is required for Pro-LOX secretion (14, 24), we first compared the expression, secretion, and proteolytic cleavage of Pro-LOX WT and Pro-LOX Gln in stable populations of NF639 cells expressing doxycycline-inducible DNAs. No substantial differences were seen in expression or secretion of Pro-LOX Gln relative to Pro-LOX WT (Fig. 1B). Furthermore, a comparable or even higher level of LOX-PP Gln versus LOX-PP WT was detected in the culture medium (Fig. 1C). Similar data have been obtained in Hs578T human breast cancer cells (not shown). Thus, synthesis and secretion are not reduced by the Gln variant.

The ability of LOX-PP Gln versus LOX-PP WT to inhibit Her-2/neu signaling was then compared. The LOX-PP Gln variant showed a markedly reduced ability to inhibit cyclin D1 (14). The Gln variant showed a markedly reduced ability to inhibit cyclin D1 expression (see Fig. 2A legend). Next, we compared the effects of LOX-PP Gln versus LOX-PP WT on NF639 cell numbers under serum deprivation conditions using a GFP expression vector as a marker of transfected cells. Inhibitory effects of LOX-PP WT were much more robust than those of the Gln variant at both 24 and 72 hours after transfection (Fig. 2B). Interestingly, we failed to detect the induction of poly(ADP-ribose) polymerase cleavage by LOX-PP WT (not shown), suggesting that it did not induce substantial levels of apoptosis of NF639 cells. Together, these findings suggest that LOX-PP reduces proliferation of NF639 cells under serum deprivation conditions, whereas LOX-PP Gln has lost this ability.

Low expression of E-cadherin is frequently associated with a more migratory, invasive phenotype, and the induction of its levels can lead to enhanced cell-cell contacts. The undetectable levels of E-cadherin normally found in the highly invasive NF639 cells were robustly induced by LOX-PP WT, whereas a much more modest effect was seen with LOX-PP Gln (Fig. 2A). Quantification of three independent experiments indicated that E-cadherin induction by LOX-PP WT was 4.8 ± 1.9-fold higher than that by LOX-PP Gln. Consistently, the Gln variant also displayed a profoundly reduced ability to inhibit formation of branching structures in Matrigel (Fig. 2C) as well as invasion through Matrigel (Fig. 2D) compared with LOX-PP WT. Thus, the LOX-PP Gln variant has a substantially reduced ability to suppress Her-2/neu tumor formation. Doxycycline-inducible cells were pretreated with 2 μg/mL doxycycline and injected s.c. in the flanks of NCr\textsuperscript{nu/nu} nude mice (n = 6). A and C, tumor volumes were measured and tumor growth curves plotted. Bars, SE. B and D, average tumor weights at day 30. Bars, SE. The average tumor weight for NF639-LOX-PP WT xenografts was 52% of that for the NF639-EV group (P = 0.03; B). There is no significant difference between NF639-LOX-PP Gln xenografts compared with NF639-EV control (P = 0.38; D).
impaired ability to inhibit the transformed phenotype in these Her-2-high/ER-low breast cancer cells.

**The LOX polymorphism reduced the ability of LOX-PP to suppress tumor formation in nude mice.** Previously, we showed that LOX-PP WT expression, driven constitutively by the CMV promoter, reduced the average weight of tumors formed by NF639 cells in nude mice by ~60% (14). We next compared the effects of the two genotypes on xenograft tumor formation by NF639 cells using the inducible vector system. Tumors resulting from NF639-LOX-PP WT cells began to grow at a noticeably slower rate than tumors from NF639-EV cells by day 17, and a significant difference was reached on days 27 and 30 (Fig. 3A). Consistently, the average tumor weight for LOX-PP WT cell xenografts was 52% of that for the EV group on day 30 (P = 0.03; Fig. 3B). Expression of LOX-PP Gln in NF639 cells had no significant effect on either tumor growth rate or tumor weight (Fig. 3C and D). The average tumor weight for NF639 LOX-PP Gln xenografts was 126% of that for the EV group (P = 0.38; Fig. 3D). Thus, the LOX-PP Gln variant has substantially reduced tumor suppressor activity.

**The LOX rs1800449 SNP impaired the ability of Pro-LOX to inhibit the transformed phenotype.** Whereas LOX-PP suppresses Ras-mediated transformation, the LOX enzyme has recently been implicated in promoting tumor progression (14, 15, 26–28). The effects of the two genotypes within the full precursor protein (Pro-LOX Gln versus Pro-LOX WT) on signaling and transformed phenotype were next compared. Previously, we observed that Pro-LOX WT attenuated activation of Akt and Erk1/2 and had some modest effects, reducing cyclin D1 while leaving vimentin unaffected (14). Expression of the Pro-LOX Gln variant increased Erk1/2 phosphorylation, whereas a small decrease was noted with Pro-LOX WT (Fig. 4A). Furthermore, the Pro-LOX Gln variant was unable to substantially reduce phosphorylation of Akt, in contrast to Pro-LOX WT (see Fig. 4A legend for quantitation). A slight decrease in cyclin D1 was seen with Pro-LOX WT, whereas no substantial changes were seen in vimentin or fibronectin (Fig. 4A). Interestingly, Pro-LOX Gln caused a slight induction in the levels of vimentin and fibronectin while having little effect on cyclin D1 (Fig. 4A). As E-cadherin expression was not induced by Pro-LOX (14), the effects of the Pro-LOX Gln variant on this gene were not pursued. Notably, whereas Pro-LOX WT decreased branching structure formation and invasion through Matrigel, Pro-LOX Gln robustly increased branching formation (Fig. 4B) and Matrigel invasion (Fig. 4C). These findings are consistent with a combined effect of loss of functional tumor suppressor activity of LOX-PP Gln and maintenance of the protumorigenic role of LOX (see below).

**The LOX rs1800449 polymorphism was associated with an increased risk of ER-negative breast cancer in a cohort of women in the BWHS.** African American women tend to have more aggressive breast cancer compared with Caucasian women, such as ER-negative tumors (17, 18). We next tested the hypothesis that the LOX rs1800449 SNP is associated with risk of breast cancer in participants in the BWHS, DNAs from 311 incident cases of invasive breast cancer and 446 controls, matched to the cases on age and geographic region, were assayed for the rs1800449 SNP in the LOX-PD domain. No association of LOX rs1800449 polymorphism with overall invasive breast cancer risk was seen (OR, 1.14; 95% CI, 0.82–1.58; Table 1). The OR for the homozygous variant (AA) relative to the homozygous WT (GG) was 1.99 (95% CI, 0.86–4.61), and this increase was largely accounted for by ER-negative breast cancer cases. When we analyzed ER-negative cases

**Figure 4.** The G473A polymorphism impairs the ability of Pro-LOX to inhibit the transformed phenotype. A, NF639-EV, -Pro-LOX WT, and -Pro-LOX Gln cells were starved, stimulated, and analyzed as in Fig. 2A. Quantification of this and two duplicate experiments indicates that Pro-LOX WT and Pro-LOX Gln compared with EV DNA (set to 1.0) gave values for phospho-Erk1/2 of 0.91 ± 0.03 and 2.41 ± 1.20, for phospho-Akt of 0.68 ± 0.10 and 0.98 ± 0.07, for cyclin D1 of 0.71 ± 0.32 and 0.97 ± 0.27, for vimentin of 1.01 ± 0.02 and 1.61 ± 0.54, and for fibronectin (FN) of 0.91 ± 0.16 and 1.44 ± 0.36, respectively. B, cells were subjected to Matrigel outgrowth assay as described in Fig. 2C. Total colonies and colonies with branching structures were counted across nine images per sample at ×50 magnification. Numbers below represent percentage of branching colonies/total colonies from two independent experiments. C, cells were subjected to a Matrigel invasion assay as in Fig. 2D. Pro-LOX WT significantly reduced cell invasion (*, P = 5.0e⁻⁵), whereas Pro-LOX Gln significantly increased invasion of these cells (*, P = 0.002). Columns, average invasion from three independent experiments relative to vehicle control (set at 100%); bars, SD.
specifically, there was a significant dose-dependent association ($P_{\text{trend}} = 0.045$) of the A allele with increased risk of ER-negative breast cancer in African American women, with the OR increasing from 1.40 (95% CI, 0.89–2.19) for the heterozygous GA genotype to 2.34 (95% CI, 0.81–6.74) for the homozygous AA genotype (Table 1). In the dominant model, the OR was 1.48 (95% CI, 0.96–2.28). Associations of LOX rs1800449 with risk of ER-positive breast cancer were weaker than those with risk of ER-negative breast cancer, with no significant trend ($P_{\text{trend}} = 0.44$). In addition, there was no significant association of LOX rs1800449 with risk of HER2-positive breast cancer, but there were only 56 HER2-positive cases (Table 1). A small proportion (4%) of the women had parents who were born outside of the United States. After controlling for parental birthplace, the results were unchanged. Thus, a dose-dependent association of the Gln-encoding A allele was seen with increased risk of ER-negative breast cancer in African American women in this hypothesis-generating initial study.

**ER-negative breast cancers display higher LOX gene expression compared with ER-positive cancers.** Based on the preliminary findings that LOX rs1800449 polymorphism has a stronger association with risk of ER-negative than ER-positive breast cancers, we hypothesized that LOX gene expression is higher in these ER-negative breast cancers. Microarray gene expression data sets available at Oncomine$^5$ were analyzed. Figure 5A shows the box plots of three data sets [van de Vijver (29), Sotiriou (30), and Hess (31)]. Levels of LOX mRNA were significantly higher in ER-negative versus ER-positive breast cancers in all three studies (see Fig. 5A legend), consistent with the findings of Erler and colleagues (26). Similar data were seen with eight other data sets at Oncomine$^5$ (Supplementary Fig. S2). Thus, ER-negative breast cancers express significantly higher LOX levels than the ER-positive ones.

**The Gln variant of LOX-PP has impaired ability to suppress the invasive phenotype of ER-negative breast cancer cells.** ER-negative breast cancers have a highly invasive phenotype. The functional consequences of Arg-to-Gln substitution on the ability of ER-negative human breast cancer cell lines MDA-MB-231 and HS578T to invade through Matrigel were next examined (Fig. 5B–D). Consistent with the assays of the invasive phenotype of NF639 cells above, purified LOX-PP WT protein significantly reduced invasion of MDA-MB-231 and HS578T cells at 55.5 and 111 nmol/L, whereas LOX-PP Gln had no significant effect at either dose (Fig. 5B and C). Furthermore, invasion of HS578T cells was significantly reduced by ectopic expression of LOX-PP WT but not of LOX-PP Gln (Fig. 5D). Together, these data indicate that compared with LOX-PP WT, LOX-PP Gln has impaired ability to suppress the invasive phenotype of ER-negative cells.

### Discussion

Here for the first time, the minor A allele of the rs1800449 SNP within the LOX gene region encoding the propeptide domain is shown to profoundly impair its ability to inhibit growth, reverse the invasive phenotype of breast cancer cells in culture, and function as a tumor suppressor in a mouse xenograft model. Importantly, a dose-dependent association of the Gln-encoding A allele with increased risk of ER-negative breast cancer was suggested in a nested case-control study within a cohort of participants in the BWHS. Although our case-control study has a relatively small sample size, the association between the LOX Gln-encoding allele and ER-negative breast cancer risk in African American women is strongly supported by functional evidence and microarray analysis. The Arg-to-Gln substitution in LOX-PP impaired its ability to reduce the transformed phenotype or the invasive properties of ER-negative or ER-low breast cancer cells or to effectively oppose the protumorigenic effects of LOX. ER-negative tumors expressed significantly higher levels of LOX mRNA than did the ER-positive ones, further supporting the finding given that a polymorphism within a gene tends to have stronger association with risk of the disease displaying higher levels of its expression. Although we cannot rule out selection bias due to lack of control for population stratification because cases and controls came from the same cohort of women being followed, it is unlikely that population stratification would have influenced the results. Overall, this first epidemiologic study of the LOX 473A allele and breast cancer

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**Table 1. Association of the LOX rs1800449 polymorphism with risk of invasive breast cancer**

<table>
<thead>
<tr>
<th></th>
<th>GG (%)</th>
<th>GA (%)</th>
<th>AA (%)</th>
<th>$P_{\text{trend}}$</th>
<th>GA + AA (%)</th>
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<tr>
<td>Controls, $n$</td>
<td>313 (70.2)</td>
<td>122 (27.3)</td>
<td>11 (2.5)</td>
<td>133 (29.8)</td>
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<tr>
<td>All cases, $n$</td>
<td>211 (67.9)</td>
<td>86 (27.6)</td>
<td>14 (4.5)</td>
<td>100 (32.1)</td>
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<tr>
<td>OR* (95% CI)</td>
<td>1.00 (reference)</td>
<td>1.06 (0.75–1.49)</td>
<td>1.99 (0.86–4.61)</td>
<td>0.24</td>
<td>1.44 (0.82–2.58)</td>
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<tr>
<td>ER+ cases, $n$</td>
<td>84 (68.9)</td>
<td>33 (27.0)</td>
<td>5 (4.1)</td>
<td>38 (31.1)</td>
<td></td>
</tr>
<tr>
<td>OR (95% CI)</td>
<td>1.00 (reference)</td>
<td>1.13 (0.70–1.83)</td>
<td>1.47 (0.47–4.59)</td>
<td>0.44</td>
<td>1.17 (0.74–1.85)</td>
</tr>
<tr>
<td>ER− cases, $n$</td>
<td>84 (61.2)</td>
<td>46 (33.6)</td>
<td>7 (5.1)</td>
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<tr>
<td>OR (95% CI)</td>
<td>1.00 (reference)</td>
<td>1.40 (0.89–2.19)</td>
<td>2.34 (0.81–6.74)</td>
<td>0.045</td>
<td>1.48 (0.96–2.28)</td>
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<tr>
<td>HER2+ cases, $n$</td>
<td>34 (60.7)</td>
<td>20 (35.7)</td>
<td>2 (3.6)</td>
<td>22 (39.3)</td>
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<tr>
<td>OR (95% CI)</td>
<td>1.00 (reference)</td>
<td>1.50 (0.78–2.87)</td>
<td>1.70 (0.83–3.85)</td>
<td>0.19</td>
<td>1.52 (0.81–2.84)</td>
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NOTE: DNA samples were assayed from the 336 incident cases of breast cancer and 465 controls for the G473A/Arg158Gln (rs1800449) SNP in the LOX-PP domain. A TaqMan genotyping assay run on an ABI PRISM 7900HT Real Time PCR System was used. A total of 311 (93%) invasive breast cancer cases and 446 (96%) controls were successfully genotyped. The distribution of the variants among the controls was in Hardy-Weinberg equilibrium.

*ORs (with 95% CI) are adjusted for age, geographic region, family history of breast cancer, age at menarche, body mass index at age 18, parity, age at first birth, oral contraceptive use, female hormone use, and years of education.

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5 http://www.oncomine.org
suggests that this variant is a potential risk allele for ER-negative breast cancer in African American women, but further studies are needed to confirm this conclusion.

Tumor suppressor genes are frequently subjected to genetic or epigenetic variations that reduce their activity (32, 33). The human LOX gene maps to chromosome 5q23.2 (34, 35), which is a frequent site of loss of heterozygosity (LOH) in many cancers (e.g., colorectal and gastric cancers; refs. 34, 36). In breast cancers, LOH within chromosomal region 5q21–32 marked “basal-like” breast cancers (37). Of note, LOH has been shown to coincide with promoter methylation resulting in a profound suppression of gene expression (9, 38). Consistently, LOX promoter methylation has been reported in several cancers (7, 9); for example, LOX was one of the most frequently and specifically methylated genes in non–small-cell lung cancers (95%) and breast cancer. Here, polymorphic genetic variation is identified as a new mechanism whereby the tumor suppressor activity of LOX-PP can be attenuated.

Figure 5. ER-negative breast cancers display elevated LOX gene expression and sensitivity to LOX-PP WT but not to the Gln variant. A, box plots of data from the van de Vijver (set 1), Sotiriou (set 2), and Hess (set 3) breast carcinoma microarray analyses, accessed using the Oncomine (http://www.oncomine.org) database, are plotted on a log scale (n, sample number). Student’s t test, done through the Oncomine 3.0 software, showed that the difference in LOX expression between the two ER groups was significant \(P = 3.7 \times 10^{-4}\) (set 1), \(P = 2.1 \times 10^{-4}\) (set 2), and \(P = 1.6 \times 10^{-4}\) (set 3), respectively. B and C, MDA-MB-231 (B) and Hs578T (C) cells were pretreated with recombinant LOX-PP WT or LOX-PP Gln proteins at 55.5 or 111 nmol/L or water vehicle control for 24 h in DMEM-10% FBS or DMEM-5% FBS, respectively. Cells were then subjected, in triplicate, to a Matrigel invasion assay for 24 h (B) or 16 h (C) in the presence of the same doses of proteins. Columns, average invasion from three independent experiments relative to vehicle control (set at 100%); bars, SD. LOX-PP WT significantly suppressed invasion of MDA-MB-231 cells at 55.5 nmol/L \((P = 3.2 \times 10^{-6})\) and 111 nmol/L \((P = 0.0033)\), whereas LOX-PP Gln was not effective at 55.5 nmol/L \((P = 0.08)\) or 111 nmol/L \((P = 0.57)\), as determined by Student’s t test. Similarly, LOX-PP WT (WT) significantly reduced invasion of Hs578T cells at 55.5 nmol/L \((P = 0.0046)\) and 111 nmol/L \((P = 0.0004)\), whereas LOX-PP Gln \((Gln)\) was ineffective \((55.5 \text{ nmol/L, } P = 0.8; 111 \text{ nmol/L, } P = 0.9)\). D, Hs578T cells were transfected with human pcDNA4-V5/His-LOX-PP WT or pcDNA4-V5/His-LOX-PP Gln or EV control for 24 h in DMEM-5% FBS. Cells were subjected to an invasion assay for 16 h. Only expression of LOX-PP WT significantly reduced cell invasion \((*, P = 9.5 \times 10^{-5})\; \text{LOX-PP Gln, } P = 0.57)\).
The biology of lysyl oxidase in tumor progression is complex. Studies from several laboratories have shown that the LOX gene, and specifically LOX-PP, functions as a Ras tumor suppressor (3, 4, 10, 11, 13–15, 39). Notably, we observed that LOX-PP reverts the highly mesenchymal breast cancer cells to a more epithelial phenotype. Additional work is needed to determine whether LOX-PP can functionally activate a process of mesenchymal-to-epithelial transition. In contrast, the LOX enzyme was found to facilitate a more migratory and invasive phenotype during breast cancer progression (14, 15, 26–28). Here, LOX-PP Gln was observed to have impaired ability to inhibit the transformed phenotype of breast cancer cells, whereas Pro-LOX Gln induced Erk activity, levels of fibronectin and vimentin, and more invasive properties in Matrigel. These observations suggest that the facilitating effect of the Pro-LOX Gln variant on the invasive phenotype is due to a profound reduction in the tumor suppressor function of LOX-PP, while the ability of the LOX enzyme to promote the transformed phenotype is retained. The functional consequences of the Arg-to-Gln substitution provide an important biological rationale for the potential association of the LOX Gln-encoding allele with increased risk of ER-negative breast cancer observed in our hypothesis-raising study of African American women.

An estimated 10% to 25% of breast cancer cases cluster in families and are believed to have a genetic component or basis (40). High-penetration germ-line mutations have been found in the BRCA1, BRCA2, TP53, PTEN, and CDH1 genes. These mutations account for ~25% of the familial risk (41). The remaining factors fall into two categories: very rare, moderate-penetration and common low-penetrance breast cancer susceptibility genes. Notably, the very rare, moderate-penetration susceptibility genes ATM, BRIP1, CHEK2, and PALB2 interact or intersect with the BRCA1 and BRCA2 protein DNA repair pathways, suggesting their involvement in common mechanisms. Genome-wide association studies have identified a small number of common low-penetrance susceptibility genes, including MAP3K1 (42), FGFR2 (42, 43), and TWIST1 (44). Interestingly, the rs1800449 polymorphism, which would fit in the low-penetrance, high-abundance category, can be connected via involvement in Ras signaling and regulation of nuclear factor κB with the previously identified MAP3K1, PTEN, and TWIST1 genes.

Breast cancer is a highly heterogeneous disease. Accumulating epidemiologic data suggest that different subtypes of breast cancers have different risk factor profiles (45, 46). Associations between breast cancer risk and common genetic variants are often modified by tumor characteristics such as ER status (47). Here, LOX rs1800449 polymorphism has a stronger association with the risk of ER-negative versus ER-positive breast cancer, supporting the recent hypothesis that ER-negative and ER-positive tumors arise from different etiologic pathways, rather than representing different stages of tumor progression (47, 48).

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References

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