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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 pro-enzyme 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 invasive phenotype of NF639 breast cancer cells driven by Her-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 invasive phenotype and tumor formation by 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-encoding A allele was seen with increased risk of estrogen receptor α (ER) negative invasive breast cancer in African-American women. Consistently, *LOX* gene expression was higher in ER negative vs 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 it may play an etiological role in ER negative breast cancer.

Keywords

lysyl oxidase; breast cancer; estrogen receptor; tumor suppressor; polymorphism; NF- κ B

Introduction

The copper amine enzyme lysyl oxidase is required for the maturation of collagen and elastin precursors in the biosynthesis of a functional extracellular matrix (1,2). The *LOX* gene inhibited the transforming activity of the *Ras* oncogene in NIH 3T3 fibroblasts and was named the

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“*ras* recision” gene (*rrg*) (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 Erk1/2 kinases and NF- κ B transcription factors (11). Lysyl oxidase is secreted as a 50 kDa inactive pro-enzyme (Pro-*LOX*), which is processed by proteolytic cleavage to a functional 32 kDa enzyme (*LOX*) and an 18 kDa propeptide (*LOX*-PP) (12). The *LOX*-PP domain was identified as the inhibitor of transformed phenotype of *Ras*-NIH 3T3 fibroblasts (13), lung and pancreatic cancer cells with mutant *RAS* genes (10), and of NF639 breast cancer cells, driven by 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 germline 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 germline, genetic variations with low to moderate penetrance (16). We observed a single nucleotide polymorphism (SNP) accompanied by a non-synonymous amino acid substitution G473A/Arg158Gln (rs1800449) in a highly conserved region within *LOX*-PP (Fig. 1A) in 6 of 9 breast cancer cell lines examined (Supplemental Fig. 1). 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 appeared 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 than 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 appeared associated with increased risk of 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 wild type 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 pC4_{bsr}R(TO) containing a doxycycline (Dox) inducible promoter and vector pCX_{bsr} under the control of 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 Dox 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 (WCEs) were prepared and subjected to immunoblotting (14). Antibodies were as follows: phospho-Akt (Ser⁴⁷³), 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; V5 epitope, Invitrogen. The results from a minimum of three independent experiments were subjected to densitometry and, after normalizing to β -actin, the mean values relative to control EV cells (set to 1.0) $\pm$ SD given. The detection of recombinant proteins in conditioned medium and WCEs were performed as described (14).

Matrigel outgrowth and invasion assays

Matrigel outgrowth and invasion assays were performed as published (14,19). Cells that had migrated to the lower side of the filter were quantified as described (15). All invasion assays were performed three times in triplicate.

Cell growth assay

Cells (6×10^4 cells/well) were plated in 6-well plates. After overnight incubation, cells were co-transfected with 2 μ g of the appropriate cDNA in pCX_{bsr} vector and 1 μ g of a green fluorescent protein (GFP) expression vector in DMEM-0.5% FBS using Eugene 6 reagent. The number of GFP positive cells was determined 24 h and 72 h post-transfection by fluorescence microscopy, as described in the legend.

Xenograft mouse model

NCR^{nu/nu} nude mice, purchased from Taconic Laboratories at 7 to 9 weeks of age, received 2% sucrose plus 2 mg/mL Dox solution in lieu of water 3 days before tumor cell inoculation. Dox inducible stable cells were pre-treated with 2 μ g/mL Dox for 24 h and then 4×10^6 cells injected subcutaneously in both flanks of the same mouse ($n = 6$) with NF639-EV, left vs -LOX-PP WT, right or NF639-EV, left vs -LOX-PP Gln, right. Tumor size was measured, as described (14). Mice were sacrificed 30 days after inoculation and tumors dissected and weighed. For statistical analysis, tumor volumes and tumor weights of NF639-LOX-PP WT vs -EV cells and NF639-LOX-PP Gln vs -EV cells were compared using paired Student's *t* test.

BWHS population and DNA collection

The BWHS is an ongoing prospective follow-up study of cancer and others illnesses among black women in the United States, involving 59,000 African-American women 21-69 years of age from across the U.S at baseline in 1995. In follow-up questionnaires, sent every two years, participants report the occurrence of incident cancer and information on risk factors such as age at first birth and family history of breast cancer. Information has been abstracted from medical records on tumor characteristics such as ER and HER2 status. To date, among all self-reported cases for which records have been obtained, 99% have been confirmed as breast cancer.

DNA was isolated from mouthwash/saliva samples, obtained from participants (21), using the QIAAMP DNA Mini Kit (Qiagen) (21) and quality tested by a real-time PCR SNP-genotyping assay for the Paraionase gene (rs854565). A >97% call rate was achieved and the resulting data met Hardy-Weinberg expectations for genotype distribution. Whole genome amplification of the DNA samples was performed using the Qiagen REPLI-g Kit. Study protocols were approved by the Institutional Review Board of Boston University.

Study sample

For the present analyses, 336 cases of incident invasive breast cancer identified during follow-up were matched on age and geographic region (Northeast, South, Midwest, West) with 465 women unaffected by breast cancer (controls), selected at random from among unaffected women. Because of our interest in ER negative breast cancer, which is more common in African-American women than in other ethnic groups, we included all ER negative cases and a sample of ER positive cases.

Statistical analysis of the case-control study

Using multivariable logistic regression (22), we calculated odds ratio (OR) and 95% confidence interval (CI) for the heterozygous (GA) and homozygous (AA) genotypes in one model, relative to the wildtype (GG) genotype, 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, 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) to 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 (Supplemental Fig. 1). Thus, of the 9 human breast cancer cell lines examined, 6 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 carboxy-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 Dox 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 vs 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 vs LOX-PP WT to inhibit Her-2/neu signaling was then compared. The LOX-PP Gln variant appeared unable to suppress phosphorylation of Akt in contrast to LOX-PP WT, while it retained partial ability to suppress Erk1/2 activation (Fig. 2A). Quantification of data from this and two duplicate experiments, given in the legend for Fig. 2, confirmed these observations. Akt activation in breast cancer cells with high levels of Her-2/neu is required for D-type cyclin expression (25), which promotes their proliferation. Previously, we demonstrated that LOX-PP reduced 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 *vs* LOX-PP WT on NF639 cell numbers under serum deprivation condition 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 h and 72 h post-transfection (Fig. 2B). Interestingly, we failed to detect the induction of PARP cleavage by LOX-PP WT (not shown), suggesting 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 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 to LOX-PP WT. Thus, the LOX-PP Gln variant has a substantially impaired ability to inhibit 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 demonstrated 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 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 those 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 3D). The average tumor weight for NF639 LOX-PP Gln xenografts was 126% of those 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 transformed phenotype

While 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 *vs* Pro-LOX WT) on signaling and transformed phenotype were next compared. Previously, we observed 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, while 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 the 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, as well as of 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, while 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 pro-tumorigenic 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 than 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. DNA 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-PP 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 wild type (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 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 heterozygous GA genotype to 2.34 (95% CI, 0.81-6.74) for 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 ER negative breast cancer with no significant trend ($P_{\text{trend}} = 0.44$). Also, 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 than ER positive ones

Based on the preliminary findings that *LOX* rs1800449 polymorphism has a stronger association with risk of ER negative than positive breast cancers, we hypothesized that *LOX* gene expression is higher in these ER negative breast cancers. Microarray gene expression datasets available at Oncomine.org were analyzed. Figure 5A shows the box plots of three datasets [Van de Vijver (29), Sotiriou (30), and Hess (31)]. Levels of *LOX* mRNA were significantly higher in ER negative vs ER positive breast cancers in all 3 studies (see Fig. 5A legend), consistent with the findings of Erler et al. (26). Similar data were seen with 8 other datasets at Oncomine.org (Supplemental Fig. 2). Thus, ER negative breast cancers express significantly higher *LOX* levels than ER positive ones.

The Gln variant of LOX-PP has impaired ability to suppress 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 invasive phenotype of NF639 cells above, purified LOX-PP WT protein significantly reduced invasion of MDA-MB-231 and Hs578T cells at 55.5 nM and 111 nM, whereas the LOX-PP Gln had no significant effect at either dose (Fig. 5B and 5C). Furthermore, invasion of Hs578T cells was significantly reduced by ectopic expression of LOX-PP WT, but not LOX-PP Gln (Fig. 5D). Together, these data indicate that compared to LOX-PP WT, LOX-PP Gln has impaired ability to suppress 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 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. While our case-control study has a relatively small sample size, the association between *LOX* Gln-encoding allele with ER negative breast cancer risk in African-American women is strongly supported by functional evidence and the microarray analysis. The Arg-to-Gln substitution in LOX-PP impaired its ability to reduce transformed phenotype or invasive properties of ER negative or low breast cancer cells or to effectively oppose the pro-tumorigenic effects of LOX. ER negative tumors expressed significantly higher levels of *LOX* mRNA than ER positive ones further supporting the findings as a polymorphism within a gene tends to have stronger association with risk of the disease displaying higher levels of its expression. Although we can not 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 *LOX* 473A allele and breast cancer 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 (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), e.g., *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.

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 (MET). 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 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 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 transformed phenotype is retained. The functional consequences of the Arg-to-Gln substitution provide an important biological rationale for the potential association of *LOX* Gln-encoding allele with increased risk of ER negative breast cancer observed in our hypothesis-raising study of African-American women.

An estimated 10-25% of breast cancer cases cluster in families and are believed to have a genetic component or basis (40). High-penetrance germline mutations have been found in the *BRCA1*, *BRCA2*, *TP53*, *PTEN*, and *CDH1* genes. These mutations account for approximately 25% of the familial risk (41). The remaining factors fall into two categories: very rare,

moderate-penetrance and common low-penetrance breast cancer susceptibility genes. Notably, the very rare, moderate-penetrance 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 NF- κ B 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 than ER positive breast cancer, supporting the recent hypothesis that ER negative and positive tumors arise from different etiologic pathways, rather than representing different stages of tumor progression (47,48).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. The Arg-to-Gln substitution has no effect on protein expression, secretion or processing. *A*, Sequence alignment spanning the carboxyl terminus of LOX-PP and the amino terminus of the LOX enzyme; identical amino acids in the propeptide region are in grey, 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, -Pro-LOX WT, -Pro-LOX Gln, -LOX-PP WT (PP WT) or -LOX-PP Gln (PP Gln) cells with 2 µg/mL Dox for 48 h. Medium (MED) was subjected to immunoprecipitation as described. Immunoblot analysis was performed on WCEs (20 µg) and immunoprecipitates using anti-V5 Ab followed by HRP-labeled protein A. All lanes in the panels were from the same blots, and cut to contiguously align the relevant samples.

Figure 2.

LOX-PP Gln variant displays reduced ability to attenuate Her-2/neu-mediated signaling and malignant phenotype. **A**, The indicated NF639 cells were treated with Dox for 4 h, serum starved in DMEM-0.5% FBS with Dox for 48 h and stimulated with 10% FBS for 90 min for phospho-Akt (P-Akt) and phospho-Erk1/2 (P-Erk1/2), or 15 h for cyclin D1 and E-cadherin (E-cad). WCEs (50 μ g) were subjected to immunoblotting as indicated. Quantification of this and two duplicate experiments indicate that the PP WT and PP Gln compared to EV DNA (set to 1.0) gave values for P-Akt of 0.67 ± 0.23 and 1.07 ± 0.12 , respectively and for P-Erk1/2 of 0.60 ± 0.11 and 0.77 ± 0.12 , respectively and for cyclin D1 of 0.51 ± 0.17 and 0.99 ± 0.11 , respectively. E-cadherin was undetectable in the NF639-EV cells, precluding analysis of fold

changes compared to this sample. *B*, NF639 cells were co-transfected, in triplicate, with pCX_{bsr} vector carrying the indicated cDNAs and a GFP expression vector in DMEM-0.5% FBS. The numbers of GFP positive cells were determined 24 h and 72 h post-transfection by fluorescence microscopy (50 × magnification, 9 fields/well). Only LOX-PP WT significantly reduced numbers of GFP positive cells (*, $P = 0.009$) (LOX-PP Gln, $P = 0.97$) at 72 h post-transfection as determined by Student's *t* test. Data are presented as average GFP positive cells/image relative to EV control at 24 h (set at 100%) from three independent experiments ± SD. *C*, After induction with Dox for 24 h, NF639-EV, -PP WT, and -PP Gln cells were subjected to Matrigel outgrowth assay. After 5 days, cultures were photographed at 50 × magnification and representative images are shown. *D*, After induction with Dox for 24 h in DMEM-5% FBS, cells were subjected to Matrigel invasion assay for 20 h. LOX-PP WT significantly reduced cell invasion (*, $P = 0.0005$) whereas LOX-PP Gln was not effective ($P = 0.48$). Data for the average invasion from three independent experiments relative to vehicle control (set at 100%) ± SD are presented.

Figure 3. LOX-PP Gln variant has reduced ability to suppress Her-2/neu tumor formation. Dox inducible cells were pre-treated with 2 μ g/mL Dox and injected subcutaneously in the flanks of NCr^{nu/nu} nude mice (n = 6). A and C, Tumor volumes were measured and tumor growth curves plotted. Bars represent SEM. B and D, Average tumor weights at day 30. Bars represent SEM. The average tumor weight for NF639-LOX-PP WT xenografts were 52% of those for the NF639-EV group ($P = 0.03$) (B). There is no significant difference between NF639-LOX-PP Gln xenografts compared to NF639-EV control ($P = 0.38$) (D).

Figure 4.

The G473A polymorphism impairs the ability of Pro-LOX to inhibit transformed phenotype. **A**, NF639-EV, -Pro-LOX WT, -Pro-LOX Gln cells were starved, stimulated and analyzed as in Fig. 2A. Quantification of this and two duplicate experiments indicate that the Pro-LOX WT and Pro-LOX Gln compared to EV DNA (set to 1.0), respectively gave values for phospho-Erk1/2 of 0.91 ± 0.03 and 2.41 ± 1.20 ; phospho-Akt of 0.68 ± 0.10 and 0.98 ± 0.07 ; cyclin D1 of 0.71 ± 0.32 and 0.97 ± 0.27 ; vimentin of 1.01 ± 0.02 and 1.61 ± 0.54 ; and fibronectin (FN) of 0.91 ± 0.16 and 1.44 ± 0.36 . **B**, Cells were subjected to Matrigel outgrowth assay as described in Fig. 2C. Total colonies and colonies with branching structures were counted across 9 images/sample at $50\times$ magnification. Numbers below represent percentage of branching colonies/total colonies from two independent experiments. **C**, Cells were subjected to Matrigel invasion assay as in Fig. 2D. Pro-LOX WT significantly reduced cell invasion (*, $P = 2.0 \times 10^{-5}$) whereas Pro-LOX Gln significantly increased invasion of these cells (*, $P = 0.002$). Data for the average invasion from three independent experiments relative to vehicle control (set at 100%) $\pm$ SD are presented.

Figure 5.

ER negative breast cancers display elevated *LOX* gene expression and sensitivity to LOX-PP WT but not 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 www.oncomine.org database, are plotted on a log scale (n = sample number). Student's *t* test, performed through the Oncomine 3.0 software, showed the difference in *LOX* expression between the two ER groups was significant ($P = 3.7 \times 10^{-4}$ (set 1), 2.1×10^{-4} (set 2), 1.6×10^{-4} (set 3), respectively). **B** and **C**, MDA-MB-231 (**B**) and Hs578T (**C**) cells were pre-treated with recombinant LOX-PP WT or LOX-PP Gln proteins at 55.5 nM or 111 nM 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. Data for the average invasion from three independent experiments relative to vehicle control (set at 100%) $\pm$ SD are presented. LOX-PP WT significantly suppressed invasion of MDA-MB-231 cells at 55.5 nM ($P = 3.2 \times 10^{-6}$) and 111 nM ($P = 0.0033$), whereas LOX-PP Gln was not effective at 55.5 nM ($P = 0.08$) or 111 nM ($P = 0.57$) as determined by Student's *t* test. Similarly, LOX-PP WT (WT) significantly reduced invasion of Hs578T cells at 55.5 nM ($P = 0.0046$) and 111 nM ($P = 0.0004$) while LOX-PP Gln (Gln) was ineffective [55.5 nM ($P = 0.8$), 111 nM ($P = 0.9$)]. 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 invasion assay for 16 h. Only expression of LOX-PP WT significantly reduced cell invasion (*, $P = 9.5 \times 10^{-8}$) (LOX-PP Gln, $P = 0.57$).

Table 1
Association of the LOX rs 1800449 polymorphism with risk of invasive breast cancer[§]

	GG	GA	AA	P for trend	GA+AA
Controls	313 (70.2%)	122 (27.3%)	11 (2.5%)		133 (29.8%)
All cases	211 (67.9%)	86 (27.6%)	14 (4.5%)		100 (32.1%)
OR [‡] (95% CI)	1.00 (ref)	1.06 (0.75-1.49)	1.99 (0.86-4.61)	0.24	1.14 (0.82-1.58)
ER+ cases	84 (68.9%)	33 (27.0%)	5 (4.1%)		38 (31.1%)
OR (95% CI)	1.00 (ref)	1.13 (0.70-1.83)	1.47 (0.47-4.59)	0.44	1.17 (0.74-1.85)
ER- cases	84 (61.2%)	46 (33.6%)	7 (5.1%)		53 (38.7%)
OR (95% CI)	1.00 (ref)	1.40 (0.89-2.19)	2.34 (0.81-6.74)	0.045	1.48 (0.96-2.28)
HER2+ cases	34 (60.7%)	20 (35.7%)	2 (3.6%)		22 (39.3%)
OR (95% CI)	1.00 (ref)	1.50 (0.78-2.87)	1.70 (0.33-8.75)	0.19	1.52 (0.81-2.84)

[§]DNA samples were assayed from the 336 incident cases of breast cancer and the 465 controls for the G473A/Arg158Gln (rs1800449) SNP in the LOX-PP domain. A TaqMan genotyping assay run on ABI PRISM 7900HT Time PCR System was used. A total of 311 invasive breast cancer cases (93%) and 446 controls (96%) 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.