RESEARCH ARTICLE

Silencing of Lysyl Oxidase Gene Expression by RNA Interference Suppresses Metastasis of Breast Cancer

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Abstract

Objective: The aim of this study was to investigate possible mechanisms of LOX gene effects on invasion and metastasis of breast cancer cells by RNA interference. Methods: LOX-RNAi-LV was designed, synthesized, and then transfected into a breast cancer cell line (MDA-MB-231). Expression of LOX, MMP-2 and MMP-9 was determined by real-time PCR, and protein expression of LOX by Western blotting. Cell migration and invasiveness were assessed with Transwell chambers. A total of 111 cases of breast cancer tissues, cancer-adjacent normal breast tissues, and 20 cases of benign lesion tissues were assessed by immunohistochemistry. Results: Expression of LOX mRNA and protein was suppressed, and the expression of MMP-2 and MMP-9 was significantly lower in the RNAi group than the control group (P<0.05), after LOX-RNAi-LV was transfection into MDA-MB-231 cells. Migration and invasion abilities were obviously inhibited. The expression of LOX protein in breast cancer, cancer-adjacent normal breast tissues and benign breast tumor were 48.6% (54/111), 26.1% (29/111), 20.0% (4/20), respectively, associations being noted with clinical stage, lymph node metastasis, tumor size and ER, PR, HER2, but not age. LOX protein was positively correlated with MMP-2 and MMP-9. Conclusion: LOX displayed an important role in invasion and metastasis of breast cancer by regulating MMP-2 and MMP-9 expression which probably exerted synergistic effects on the extracellular matrix (ECM).

Keywords: Breast cancer - lysyl oxidase - RNA interference (RNAi) - metastasis - matrix metalloproteinases

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Introduction

Breast cancer is one of the malignant tumors that occur most commonly in women. The incidence shows an increasing tendency every year that maybe associates with some wrong modern lifestyle (Loh et al., 2011; Sulaiman et al., 2011). The main reason for the treatment failure is the metastasis and invasiveness of the cancer. Therefore, it is very important to find the possible mechanism of the recurrence and the metastasis of breast cancer.

Lysyl oxidase (LOX) is an amine oxidase that cross-links collagens and elastins in the extracellular matrix. LOX controls both the structure and the tensile strength of ECM, and thus acts to preserve tissue integrity. Recent studies indicated that LOX promotes malignant progression in cancer (Peyrol et al., 1997; Woznick et al., 2005; Fong et al., 2007). However, the specific mechanism of LOX-mediated promotion of breast cancer metastasis is unclear. In our research, we investigated the possible mechanism of LOX gene affecting on the invasion and metastasis of breast cancer cells by RNA interference (RNAi). LOX-RNAi-LV was constructed to lower the LOX gene expression level in the MDA-MB-231 breast cancer cells, so that we could observe how LOX gene regulates metastatic colonization and growth of breast cancer in vitro and in vivo. Using the result of Hafez's research (Hafez et al., 2012) for reference that the breast cancer patients showed an up-regulation in RNAs with an upregulation in MMP2 and MMP-9 genes, we also investigated the expression of the invasion associating factors, MMP-2 and MMP-9. Therefore, the role of LOX in the invasion of breast cancer was studied.

Materials and Methods

Clinical information

Samples, including breast cancer tissue (N=111), cancer-adjacent normal breast tissues (N=111), benign lesion tissues (N=20), were collected from patients who underwent surgical resection at Guangxi Cancer Hospital from April 2006 to July 2009. Breast cancer tissue and corresponding cancer-adjacent normal breast tissues came from the same case. All the tumor samples came from female patients. The median age is 47 (from 19 to 78). Cases were assessed by a clinical TNM stage according to the sixth edition AJCC: stage I (N=10), stage II (N=58), stage III (N=35), stage IV (N=8). There were 76 cases with positive axillary lymph node, and 35 cases with negative axillary lymph node. No patients received chemotherapy or radiotherapy before the surgery.

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Constructing LOX-RNAi-LV

Ambition Company designed and synthesized siRNA against human LOX gene. The sequences were as follows: forward, GGA ACU UUA GUG AAA CAU AAU; reverse, UAU GUU UCA CUA AAG UUC CAG. The retrieval of gene database confirmed that siRNA had no homology with the human gene sequence except for LOX. Double-stranded DNA oligo interfering sequence was constructed, and then inserted into pGCL-GFP vector (including U6 promoter). The product was switched to bacterial competent cells. PCR was used to identify the clone. The standard for successfully construction of LOX-RNAi-LV was positive clone. We then packaged the virus and examined the titer and multiplicity of infection index (MOI Value: the ratio of the number of infectious virus particles to the number of target cells) before transfection.

Cell culture and transfection

MDA-MB-231 cells grew in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS) at 37 °C with 5% CO_2 humidified atmosphere. Cells were treated with 0.25% trypsin for passage. We took logarithm growth period cells to perform experiments. Three groups were set up. They were interference group (RNAi group) that was transfected with LOX-RNAi-LV, negative control group (Mock group) that was transfected with GFP-NC-LV and blank control group (Con group) that was transfected with empty vector. 3×10^5 Cells were seed onto the plastic of six-well plates and incubated for 12-24 hours. When the rate of cells covering was 60% under microscope, we did the transfection. Then, 1 ml of culture solution (supplemented with serum less than 10%) was added to each. RNAi group was mixed with 30 μl of 1.5×10^8 TU/ml LOX-RNAi-LV, and Mock group was mixed with 30 μl of 1.5×10^8 TU/ml GFP-NC-LV. 100 μl diluent of polybrene was added to each of RNAi group and Mock group, and the plates were incubated at 37 °C with 5% CO_2 humidified atmosphere.

Real-time fluorescence quantitative PCR

Real-time fluorescence quantitative PCR was used to study LOX, MMP-2 and MMP-9 mRNA expression. Trizol assay was used to purify RNA. Then we examine the total RNA concentration. 2 μg RNA was used to perform on reverse-transcribed complementary DNA (cDNA).

The primer sequences are showed in Table 1. Real-time PCR was operated according to the instruction of Quant SYBR Green PCR detection kits. Reactions for each sample were performed in triplicate, and amplified products were visualized in a real-time quantitative fluorescence gene amplification device (America, Bio-Rad, iQ™ 5 Multicolor). The PCR conditions were 94 °C for 3 minutes, 94 °C for 30 seconds, 59 °C for 30 seconds, ansd 72°C for 30 seconds, followed by 40 cycles. After reading the cycle threshold (Ct) value, we used the equation-- ΔCT=mean value of CT (LOX)–mean value of CT (β-actin) --to calculate the control group and RNAi group ΔCT. Then the equations--inhibition ratio=$(1–2^{-\Delta\Delta CT})\times100\%$(ΔΔCT=ΔCT of RNAi group–ΔCT of control group)-- was administered to calculate the inhibition ratio of LOX gene expression (Livak and Schmittgen, 2001).

Western Blot

Western Blot Analysis was used to examine LOX protein level. Three groups of cells were collected after transfection. Cells were lysed in buffer. The samples were then centrifuged at 12,000 rpm at 4°C for 30 min. Protein concentration was quantified using BCA, followed by the separation with 10% sodium dodecyl sulfate polyacrylamide gel electropheresis (SDS-PAGE) by 60 μg total protein per pore. They were then transferred to polyvinylidene difluoride (PVDF) membrane. Membranes were blocked with 5% nonfat dry milk, and were then added with antibody (LOX at 1:1000 and β-actin at 1:3000) at room temperature for 30 min, followed by incubation at 4°C overnight. The membranes were washed, added with secondary antibodies (1:10000), marked by HRP, and then incubated at room temperature for 30 min. Chemiluminescence reagent, ECL, was used to develop darkroom exposure after the wash of the membrane. LOX and β-actin protein band grayscale ratio was used to normalize the LOX protein expression.

Transwell assay

Transwell assay was used to determine the ability of cell invasion and migration. Three groups of cells were grown in serum-free DMEM medium for 12-24 hours. After digested by pancreatic enzymes, cells were centrifuged. Phosphate buffered saline (PBS) was used to wash them 1-2 times. Then we abandoned the liquid. DMEM was used to dilute the residue to 1×10^6/ml cell suspension, which was plated in the upper chamber (Matrigel-coated Transwell chamber was used in the invasion assay and the chamber without Matrigel was used in the migration assay). Lower chamber medium containing 10% fetal bovine serum (FBS) incubated at 37 °C for 18-24 hours with 5% CO_2. Transwell chamber was taken out of the double chambers. Then we abandoned the liquid and washed it with PBS. Cells were fixed with formaldehyde for 30min and stained using Giemsa for 30min. Then ddH2O was added to wash cells, followed by being counted under the microscope. We randomly selected 8 fields of view (every group was repeated for three times), and took photos of them.

Immunohistochemical Analysis

Immunohistochemistry was used to detect LOX and MMP-2, MMP-9 protein expression. Marked

Table 1. The Primer Sequences Used in This Study

Gene name	Forward sequence (5'-3')	Reverse sequence(5'-3')	PCR product length (bp)
LOX	CAGGCACCGACCTGGATATGG	CGTACGTGGATGCCTGGATGTAGT	193
β-actin	AGTTGCGTTACACCCTTTCTTG	CACCTTCACCGTTCCAGTTTT	148
MMP-2	TGCCCAAGAATAGATGCTGAC	GAAAGGAGAAGAGCCTGAAGTG	160
MMP-9	CTTCTGCCCGGACCAAGGATAC	TTCAGGGCGAGGACCATAGAGG	187

Table 2. LOX mRNA Relative Expression Before and After Transfection ($2^{-\triangle\triangle CT}$)

Group	LOX mRNA	LOX protein relative expression (LOX/β-actin gray level ratio)	nvasion ability	migration ability	MMP-2	MMP-9
RNAi group	0.108±0.013*	0.156±0.004*	47 ±2*	63 ±2*	0.496±0.021	0.571±0.099
Con group	1.000±0.000	0.916±0.007	100±1	118±2	1.000±0.000	1.000±0.000
Mock group	0.855±0.008	0.696±0.020	88 ±2	96 ±2	0.846±0.047	0.786±0.042

*Compare with Con group and Mock group, P=0.000

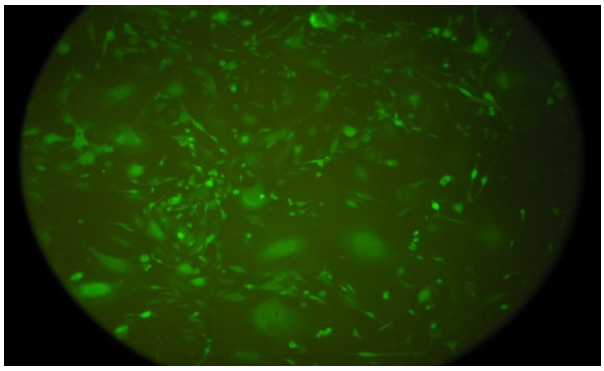

Figure 1. Transfection Efficiency of Breast Cancer Cells MDA-MB-231 Transfected by LOX-RNAi-LV

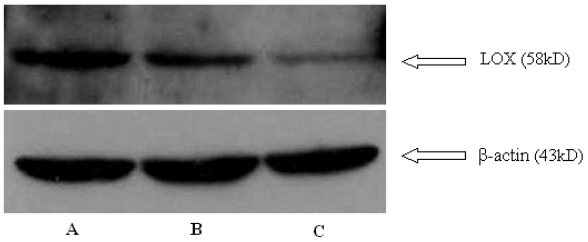

Figure 2. Western Blot Analysis of LOX Protein Expression. A: Control group; B: Mock group; C: RNAi group

Streptavidin-Peroxidase (S-P) had been assessed to the immunohistochemical analysis. Rabbit anti-human LOX polyclonal antibody was provided by the NOVUS Biotechnology Company. Mouse anti-human MMp-2 and MMP-9 monoclonal antibody was purchased from Fuzhou Wallace New Biotechnology Company. The experiment procedures followed instructions. The known positive section was used as positive staining control, and PBS was used as negative staining control.

Evaluated Result

The positive result was that the cytoplasm showed brown granules after LOX and MMP-2, MMP-9 was stained. We randomly selected 10 fields of view at ×400 magnification. According to the number of staining cells, the intensity of cytoplasmic staining was graded by semi quantitative analysis-- "negative" (-) when no staining was detected; "weak" (+) when cytoplasmic staining cells were < 25%; "moderate" (++) when cytoplasmic staining cells were 25%~50%, and "strong" (+++) when cytoplasmic staining cells were > 50%.

Statistical Analysis

Statistical analysis was performed using SPSS 13.0 Software. All data were plotted as ±S. Data were analyzed by Student's paired t tests, one-way ANOVA and spearman's rank correlation test. Significant level is α=s0.05. Values of P < 0.05 were considered statistically significant.

Results

Transfection efficiency

LOX-RNAi-LV was transfected into MDA-MB-231. Green fluorescent protein (GFP) had fluorescent expression after 60 hours. The expression was obviously strong after 72 hours, and was 90% (Figure 1). The data of real-time fluorescent quantitative PCR and Western blot assay demonstrated that LOX mRNA and protein expression were significantly inhibited after transfection. Compared with Con group, RNAi group suppression rate of LOX mRNA and protein is 89.2% and 82.97%, respectively (Table 2).

MMP-2 and MMP-9 mRNA expression

The results of real-time fluorescent quantitative PCR revealed that the MMP-2 and MMP-9 mRNA expression in the RNAi group obviously lower than Mock and Con group after 72 hours of transfection. It showed significantly difference on the statistical analysis (P=0.000) (Table 2).

Invasion and migration of MDA-MB-231

Transwell chamber assay indicated that the number of the cells in the RNAi group invading through filtration membrane was dramatically less than Con group and Mock group (P=0.000). Therefore knockdown of LOX expression could decrease the MDA-MB-231 invasion and migration ability (Table 2).

LOX protein expression

LOX protein was over-expressed in the breast cancer tissue. The positive expression rate was 48.64%, compared with those in the nomal breast tissue and in the benign breast tumor, which were 26.13% and 20.00%, respectively. It showed significantly difference on the statistical analysis (P < 0.05) (Figure 2).

The relationship between LOX protein and clinical pathology

According to Table 3, the larger diameter the tumor has, the higher positive rate the LOX protein expression (P=0.000). We also observed an increased level in staging III and IV compared with staging I and II (P=0.000). LOX expression was elevated (P=0.007) with the advent of lymph node metastasis. Furthermore ER and PR negative breast cancer selected more LOX protein than ER and PR positive (P < 0.05). Conversely HER-2 positive breast cancers selected more LOX protein than HER-2 negative.

The relationship between LOX protein and MMP-2, MMP-9

Statistical analysis results revealed that LOX protein expression had a positive correlation with MM-2(r=0.262,

Table 3. The Relationship Between LOX Protein and Clinical Pathology

Clinical data	N	LOX		Positive rate	χ^2	P
		negtive	positive	(%)		
Age						
<50y	64	20	44	68.75	1.367	0.242
>50y	47	10	37	78.72		
Tumor size						
≤2cm	7	5	2	28.57	18.289	0.000*
2-5cm	61	22	39	63.93		
≥5cm	43	3	40	93.02		
Clinical staging						
I	11	5	5	18.18	24.537	0.000*
II	62	21	35	35.48		
III	28	4	24	71.43		
IV	10	0	8	100		
Lymph node metastasis						
positive	77	15	62	80.52	7.259	0.007
negtive	34	15	19	55.88		
ER						
negtive	53	8	45	84.91	7.323	0.007
positive	58	22	36	62.07		
PR						
negtive	51	7	44	86.27	8.464	0.004
positive	60	23	37	61.67		
HER-2						
negtive	36	15	21	58.33	5.79	0.016
positive	75	15	60	80		

*Fisher exact test

P=0.005) and MMP-9(r=0.424, P=0.000) protein expression.

Discussion

Recent researches indicated that LOX might correlate positively with breast tumor metastatic potential. LOX expression level was elevated in metastatic tumors, associated with increasing stages of breast cancer progression (Payne et al., 2005; Erler et al., 2006). Kirschmann et al. (2002; 2005) demonstrated that up-regulation LOX was just detectable in the highly invasive breast cancer cells by comparing the activity of LOX in the MDA-MB-231 and MCF-7. Furthermore LOX had relationship with metastatic breast cancer cell line (MDA-MB-231). The LOX inhibitor, β-aminoproprionitrile (β-APN), diminished the metastatic colonization potential of highly invasive breast cancer cells. On the contrary, when the nonmetastatic breast cancer cell line (MCF-7) was transfected with LOX gene, the ability of invasiveness was doubled. And β-APN administration was able to reduce the frequency of invasiveness. It showed that up-regulation of LOX expression level was connected to motility and invasiveness of breast cancer. In the previous study, we observed LOX gene over-expressed in the MDA-MB-231cells. But when they were transfected with the siRNA using RNA interference technique, LOX gene expression was inhibited and motility and invasiveness of cells would be significantly reduced. In this study, LOX-RNAi-LV was stably transfected with LOX siRNA. LOX mRNA and protein expression levels were (0.108±0.013) and (0.156±0.004), respectively. Compared with Con group and Mock group, RNAi group was obviously decreased ($P < 0.05$). Therefore it indicated that, LOX protein and gene levels were substantial reduced, after MDA-MB-231 cells were transfected with LOX-RNAi-LV. We utilized Transwell chamber assay to determine the invasion and migration of the cells before and after transfection. It showed that in the RNAi group, the invasion and migration ability was (46.63±1.625) and (63.38±1.861), respectively. Compared with the Con group and Mock group, they obviously decreased ($P < 0.05$). These data demonstrated that the elevated LOX expression was positively-correlated with breast cancer invasiveness. The results of clinical study also confirmed this point (Kirschmann et al., 2005). Akiri et al. (2003) examined LOX gene and protein expression in the breast cancer tissues, cancer-adjacent normal breast tissues, lymph node tissues, and benign lesion tissues by RT-PCR and immunohistochemistry assay. They found an increased level of LOX in patient cancer tissues, compared with those in normal breast and benign tumor tissues. This level was further increased in metastatic tumors, associated with increasing stages of breast cancer disease progression ($P < 0.05$). With the advent of breast cancer growth and lymph node metastasis, LOX expression was elevated. We detected the LOX level of 10 cases breast cancer with distant metastasis which staged IV. The positive rate was 100.00%. So we proposed that LOX played an important role in cell proliferation and clinical progression, which means that LOX can promote breast cancer metastasis. It has long been known that tumor metastasis potentiality and lymph node metastasis are the major points to influence the prognosis. Therefore LOX up-regulation has been correlated with a poor prognosis in breast cancer. Erler and Giaccia (2006) proposed that LOX in cell-matrix adhesion and migration is certain to additionally affect later stages of metastasis, when cells must adhere to vessel walls, extravasate, and migrate to colonize secondary organs. However the mechanism of LOX affecting on invasion and metastasis of breast cancer cells remained unclear. In this study, RT-PCR and immunohistochemistry assay were performed to examine LOX, MMP-2 and MMP-9 in MDA-MB-231 and breast cancer tissues before and after transfection. RNAi group MMP-2 and MMP-9 gene levels are obviously reduced ($P < 0.05$). It indicated that LOX inhibition could decrease the MMP-2 and MMP-9 secretion. There was a positive correlation between LOX and MMP-2, MMP-9 secretion in the transplanted tumor and breast cancer tissues ($P < 0.05$). For breast malignant cancer biological behaviors, they had a strong association to cooperate to promote breast cancer invasion and metastasis.

LOX and MMPs genes had a deep relationship with extracellular matrix (ECM) and basement membrane (BM). ECM consists of a network of collagen and various kinds of protein, such as laminin and mucoprotein, which formed basement membrane. ECM degradation by MMPs accelerated the progress of the development of malignant tumor (Holtmeier et al., 2003; Kirschmann et al., 2005). MMP-2 and MMP-9 were known to cleave collagen IV into peptides. Based on it MMP-2 and MMP-9 played a role in epithelial–mesenchymal transitions (EMT) (Zucker et al., 2001; Jing et al., 2006). Many reports

suggest that MMP-2 and MMP-9 were closely associated with breast cancer, especially with tumor cell invasion and metastasis (Liotta et al., 1991; Yu and Stamenkovic, 2000; Nakopoulou et al., 2002; Talvensaari et al., 2003). We got the idea that MMPs were endopeptidases which degraded most of the components of the ECM, including dense collagenous structure, to enable the easy migration and spread of cancer cells. Elevated LOX activity was correlated with the collagen accumulation to increase ECM tension and stiffness, a reaction that caused the change of collagen fiber structure by activating particular signal. After that the tumor cells associated the matrix system was set up, which promoted tumor cells to adhere. We also proposed that LOX can change ECM structure and up-regulate expression of MMPs by stimulating some signal. MMPs contributed to invasion and metastasis of tumor cells by degrading the surrounding BM and ECM barriers, which increased microvascular permeability induced by destroying integrality of vascular wall. Then it was easy for endothelial cell motility and invasiveness to form new blood vessels. As a result, it enabled the easy migration and spread of cancer cells (Lhotak et al., 2000).

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