Original Article

Lysyl oxidase G473A promotes migration, invasion and metastasis of ovarian cancer cells through regulating p38/Akt signaling pathways

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Abstract: Objectives: Lysyl oxidase (LOX) always promotes cancer cell metastasis. This study aimed to investigate the influence of LOX G473A on ovarian cancer cells function and the related cell signaling pathways. Methods: Expression of LOX G473A in 3 ovarian cancer cell lines HO-8910 and A2780 as well as the siRNA-LOX G473A transfected SKOV3 cells were detected by western blotting analysis. Migration and invasion as well as mitosis ability of transfected cells were detected using in vitro transwell assay and flow cytometry, respectively. Cytokines related to cell migration, invasion and metastasis were detected to investigate the influence of siRNA-LOX G473A on their expression. Results: LOX G473A showed the highest expression level in SKOV3 cell line compared with the other two. SiRNA-LOX G473A significantly reduced the expression of LOX G473A protein as well as cell migration, invasion and mitosis abilities. Moreover, cytokines associated with cell migration, invasion and metastasis were dysregulated by siRNA-LOX G473A. Conclusions: LOX G473A was a crucial factor modulating cell migration, invasion and metastasis of ovarian cancer cell lines. The facts of LOX G473A silence could reduce cell migration, invasion and metastasis abilities demonstrated that LOX G473A might be served as a potential therapeutic target for ovarian cancer cells.

Keywords: Lysyl oxidase G473A, ovarian cancer cells, invasion, AKT

Introduction

Ovarian cancer is one top leading cause of cancer related death and most ovarian cancers were diagnosed in advanced stages followed by low overall survival rate [1]. The pathogenesis of ovarian cancer is identified to be involved in genetic alterations including lysyl oxidase (LOX) 473 G>A (rs1800449) polymorphism, E-cadherin [2], ERCC1 (excision repair cross-complementing 1) [3] and BRCA1 [4].

LOX is an enzyme located in extracellular matrix and crucial for hypoxia-induced metastasis [5]. LOX catalyzes collagens and elastin cross-linking in the extracellular compartment [1, 6]. Reportedly, polymorphism of LOX 473 G>A has been evidenced to be associated with metastasis of various diseases, including ovarian cancer [1, 6], breast cancer [7], gastric cancer [8] as well as coronary artery disease [9]. These researches displayed that the polymorphism of LOX G473A was significantly higher in patients with gastric cancer [8], breast cancer [7] and advanced ovarian cancer [1]. They demonstrated that LOX G473A polymorphism was a risk factor for these cancers.

It was reported that LOX G473A polymorphism affect ovarian cancer metastasis [1, 6]. Wang et al reported that overexpression of LOX suppressed the proliferation and invasion of ovarian cancer cells and LOX was a direct target of miR-29b [10]. Some reports showed that LOX was a tumor suppressor gene which could be inactivated by methylation [11]. However, there was no direct research focusing the effect of LOX G473A on cell function of ovarian cancer cells.

To investigate the effect of LOX G473A on cell function of ovarian cancer cells and verify the mechanisms related to the changes, we examined the expression of LOX G473A protein and
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regulated the expression of LOX G473A. The migration and invasion of ovarian cancer cells were determined and metastasis-associated cytokines or signaling pathway were detected as well. This study would provide us with more information on the effect of LOX G473A on cell functions of ovarian cells.

Materials and methods

Cell line and culture conditions

Three human ovarian cancer cell lines HO-8910, A2780 and SKOV3 were obtained from Shanghai Institute of Cellular Biology of Chinese Academy of Sciences. Cells were cultured in DMEM or RPMI-1640 (Dulbecco's modified Eagle's medium, HyClone, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Gibco Laboratories, Grand Island, NY, USA) and penicillin-streptomycin. Cells were cultured at 37°C with 5% CO2.

LOX G473A knockdown in ovarian cancer cell line

SiRNAs with sequence targeting LOX G473A were synthesized by GenePharma (Shanghai, China). SiRNA against LOX G473A were transfected into SKOV3 cells using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) following the manufactures' instruction. Cells plated into 24-well plate were transfected and cells transfected with siRNA-LOX G473A-NC was served as negative control. Cells were cultured at 37°C 5% CO2 after transfection.

In vitro migration and invasion assay

Migration of transfected SK-OV3 cells were detected using 24-well transwell chambers (Costar, Corning Incorporated, Corning, NY, USA) with polycarbonate membrane as described previously [12]. For cell migration assay, cells at a density of 50,000 were seeded into the upper chamber with DMEM medium without FBS. The lower chamber was filled with medium containing 10% FBS and incubated at 37°C 5% CO2 for 24 h. For cell invasion assay, the 24-well transwell were coated with 80 μL Matrigel (BD Biosciences, Franklin Lakes, NJ, USA). Cells at a density of 100,000 were seeded into the upper chamber medium. The lower chamber was filled with medium containing 10% FBS and incubated at 37°C 5% CO2 for 48 h. After incubation, cells on the upper surface filters were removed by cotton swab and filters were then fixed with paraformaldehyde for 10 min, followed with crystal violet staining. Stained cells were counted using a microscope and cells numbers at 5 randomly selected fields were averaged. All experiments were performed in triplicates and differences between groups were analyzed.

Cell cycle analysis

Cell cycle analysis for the transfected SKOV3 cells was performed using flow cytometry. Cells were harvested by trypsinization (Gibco Laboratories, Grand Island, NY, USA) and fixed using 70% ethanol for 24 h. DNA was stained with 50 μg/ml propidium iodide (PI, Biyuntian biological technology, Shanghai, China) and analyzed using a FACScalibur flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA). All experiments were performed in triplicates.

Western blot analysis

Cultured cells in 6-well plates at a density of 2.0×106 were harvested at 48 h post-transfection, washed in ice cold 1×PBS, and lysed in RIPA buffer (Thermo Scientific, Rockford, IL,
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USA). Protein concentrations were then determined using a BCA protein assay kit (Thermo Scientific, Rockford, IL, USA). An equal amount of 35 μg cell lysates were separated by 10% SDS-PAGE gels and were electrophoretically transferred to polyvinylidene difluoride (PVDF) membranes (Invitrogen Corp., Carlsbad, CA, USA). Then the PVDF membranes were blocked with 5% skimmed milk (BD Biosciences, Franklin Lakes, NJ, USA) and incubated with primary antibody against LOX G473A (dilution 1:1000), AKT (dilution 1:1000), phosphorylated (p)-AKT (dilution 1:1000), metastasis-associated protein (MTA) 1, E-cadherin (dilution 1:1000), RhoC (dilution 1:1000; Cell Signaling Technology, CST, Denvers, MA, USA), matrix metalloproteinase (MMP)9 (dilution 1:1000), and GAPDH (dilution 1:1500; Cell Signaling Technology, CST, Denvers, MA, USA) at 4°C overnight, and secondary antibodies for 1 h. The polypeptide bands were detected using an enhanced chemiluminescence (ECL) detection system (Pierce, Rockford, IL, USA) and quantified with AlphaEase software (Alpha, USA).

Statistical analysis

Data were expressed as mean ± standard deviation (SD) of 3 triplicates. Statistical analysis was performed using Student’s t test or a one-way analysis of variance (ANOVA) test. P<0.05 was considered to be statistically significant.

Results

LOX G473A expresses the highest in SKOV3

The expressions of LOX G473A protein levels in the 3 ovarian cancer cell lines of HO-8910, A2780 and SKOV3 before and after siRNA-LOX G473A transfection were detected using western blotting analysis. The results showed that LOX G473A expressed the highest in SKOV3 cell line compared with that in the other two cell lines (P<0.01, Figure 1A). Then we chose SKOV3 as the mediated cell in this study and successfully knocked down LOX G473A expression by 90% in SKOV3 cells using siRNA silencing methods (Figure 1B).

LOX G473A silence inhibits cell migration, invasion and mitosis

In order to detect the influence of LOX G473A on cell proliferation, we detected cell invasion, migration, and cell cycle of SKOV3 cells after siRNA-LOX G473A transfection. The results showed siRNA-LOX G473A transfected cells reduced migrated cells and invaded cells which determined that LOX G473A silence significantly inhibited cell ability of migration (P<0.01, Figure 2A) and invasion (P<0.01, Figure 2B). For cell cycle analysis, we confirmed that siRNA-LOX G473A inhibited cell proliferation by
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**LOX G473A silence effects expression of cell cytokines related to cell invasion and metastasis**

Given the above results of LOX G473A silence inhibited cell invasion and migration, we speculated that LOX G473A silence might affect the expression of some cytokines related to cell migration, invasion, and metastasis via signaling pathway. To verify this assumption, we detected the expression of MTA1, E-cadherin, MMP9, RhoC, p-and P38, p-and AKT which had been reported to be associated with cell migration, invasion and metastasis using western blotting analysis [13-15]. Analysis showed the expression of MMP9, MTA1, and RhoC were depressed by siRNA-LOX G473A in SKOV3 cells (P<0.01, Figure 4A). However, the expression of E-cadherin in SKOV3 cells was significantly upregulated by siRNA-LOX G473A (P<0.01, Figure 4A). To investigate the effect of LOX G473A silence on p38/AKT sig-

**Figure 3.** Effect of LOX G473A silence on cell mitosis. Cell cycle analysis was performed using flow cytometry. Fore flow cytometric analysis, DNA was stained with 50 μg/ml propidium iodide. SiRNA-LOX G473A inhibited cell proliferation by increasing cell percentage in the G1 and S phases, and decreasing cell percentage in the G2/M phase. SiRNA-LOX G473A got SKOV3 cells arrest in the S phase and promoted cell apoptosis. *indicates significant level at a p value of less than 0.05 comparing with the control.

increasing cell percentage in the G1 and S phases, and decreasing cell percentage in the G2/M phase (P<0.01, **Figure 3**). These showed that siRNA-LOX G473A got SKOV3 cells arrest in the S phase and promoted cell apoptosis. Taken together, the influence of siRNA-LOX G473A on SKOV3 cell function demonstrated that LOX G473A was positively correlated with cell growth and proliferation.
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expressed the highest in ovarian cancer cell line SKOV3 than HO-8910 and A2780 cell lines. After being transfected with siRNA-LOX G473A, the expression of LOX G473A as well as cell migration, invasion and mitosis ability were inhibited, and metastasis-related cytokines including E-cadherin, MMP9 and P38/AKT signaling pathway were dysregulated. This demonstrated that LOX G473A was associated with cell growth, proliferation and metastasis. As reported that LOX was a tumor suppressor and overexpression of it could suppress ovarian cancer cells proliferation and invasion [10, 11]. However, there is an opposite function of LOX in cancer cells acting as a promoter of cell metastasis [16], and polymorphism of LOX might associated with increased risk of cancers [7, 9, 17]. For example, patients with nonsmall cell lung cancer and cardiovascular diseases showed higher frequency of LOX 473 AA genotype and A allele, a novel polymorphism of LOX gene, compared with normal controls, showing LOX 473 G>A polymorphism is risk factor for cell metastasis and might be a prog-

Discussion

LOX G473A polymorphism is a new risk factor for cancers including ovarian cancer and LOX signaling affect the metastasis of ovarian cancer cells [1, 6]. This study was designed to investigate the expression of LOX G473A polymorphism in ovarian cancer cells and the effect of LOX G473A polymorphism on cell function of ovarian cancer cells. Results from the experiments showed ovarian LOX G473A

Figure 4. LOX G473A silence effects expression of cell cytokines related to cell invasion and metastasis. The expression of cytokines was performed using western blotting analysis. MTA1, E-cadherin, MMP9 and RhoC cytokines (A) and phosphorylated (p)-AKT, p-P38 and total AKT and P38 (B) were affected by siRNA-LOX G473A. ** and * indicates significant level at a $p$ value of less than 0.05 and 0.01 comparing with the control, respectively.
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Additionally, depression of E-cadherin in cancer was reported to be correlated with RhoC upregulation, which promotes epidermal growth factor receptor (EGFR) stimulated migration and invasion [19, 29]. RhoC is a Ras superfamily and has been shown to promote cancer metastasis by promoting AKT activity, a crucial factor for cell apoptosis and proliferation [18, 30]. Accordingly, knockdown of RhoC suppressed AKT signaling and inhibited tumor invasion [30, 31]. Moreover, EGFR is a suppressor of cell apoptosis and inhibition of EGFR enhanced cell apoptosis of head and neck squamous cell carcinoma [32]. EGFR mediates AKT and MAPK pathways to modulate cell migration, invasion and growth, as the inhibition of EGFR inhibits cell migration invasion and growth via suppressing PI3K/Akt and MAPK signaling pathways [33, 34]. Moreover, activated p38 MAPK signaling pathway is assistance to cell apoptosis, due to the G2/M arrest Mechanically, RhoC promotes the EGFR-activated PI3K/Akt and MAPK signaling pathways as well as inhibits cell apoptosis [35, 36]. In this study, we revealed that the inhibition of LOX G473A gene inhibited growth and promoted apoptosis by get an S-phase arrest of SKOV3 cells through modulated the expression and activity of p38/AKT pathway.

Conclusion

In this study, we demonstrated that LOX G473A gene was a tumor promoter of ovarian cancer cells. The knock down of LOX G473A gene by siRNA silencing suggested LOX G473A gene was a crucial factor for ovarian cancer cell migration, invasion, and metastasis. Furthermore, we demonstrated that the cell growth inhibition by LOX G473A gene was related to p38/AKT signaling pathway and the cytokines associated with this pathway, including MTA1, E-cadherin, MMP9, and RhoC. These demonstrated that LOX G473A gene polymorphism might be regarded as a potential therapeutic target for ovarian cancer cells.

Disclosure of conflict of interest

None.

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