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Original Article

Silencing of hypoxia-inducible tumor suppressor lysyl oxidase gene by promoter methylation activates carbonic anhydrase IX in nasopharyngeal carcinoma

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Abstract: Lysyl oxidase (LOX) is an oxidative enzyme known to initiate the cross-linking of collagens and elastin, and suggested recently as a tumor suppressor for several tumor types including lung, pancreatic and gastric cancers. Previously we showed that LOX is strongly induced upon hypoxia in nasopharyngeal carcinoma (NPC) cell lines CNE2 and HONE1 but only slightly in HK1 and not in C666-1. Here, we further studied the regulatory mechanism and functions of LOX in NPC. LOX is widely expressed in human normal tissues with variations in expression levels. LOX was expressed in most NPC cell lines except for C666-1, while HK1 and FaDu (laryngeal cancer) only expressed low level of LOX. Methylation analysis showed that the LOX promoter was methylated in C666-1 and partially methylated in HK1. After demethylation with 5-aza-2'-deoxycytidine, LOX expression was reactivated along with increased unmethylated alleles. LOX promoter methylation was detected in 42/49 (85.7%) of NPC primary tumors but only 3/16 (18.75%) of nose swab samples from NPC patients. LOX overexpression reduced the clonogenicity and cell growth of NPC cells, and also inhibited the migration and invasion of the NPC cells. Carbonic anhydrase IX (CA9) mRNA level was obviously decreased in HK1 cells after transfection with LOX. The elevation of CA9 protein upon hypoxia was inhibited in LOX-transfected HK1 cells. The protein levels of an apoptosis marker cPARP were increased in LOX-transfected HK1 cells upon hypoxia treatment. Our data showed that silencing or down-regulation of LOX in NPC was due to its promoter methylation and LOX acts as a tumor suppressor in NPC. LOX silencing would facilitate NPC cells to escape from hypoxia-induced apoptosis and maintains a malignant and metastatic phenotype.

Keywords: Lysyl oxidase, nasopharyngeal carcinoma, epigenetics, growth inhibition, apoptosis, metastasis, hypoxia, carbonic anhydrase IX

Introduction

Nasopharyngeal carcinoma (NPC) is prevalent in Southern Asia and Southern China including Hong Kong, ranked as the sixth common cancers in Hong Kong in men with an incident rate of 19.1 cases /100,000/year in 2011 [Hong Kong Cancer Registry. http://www3.ha.org.hk/cancereg/Summary%20of%20CanStat%202011.pdf]. Major etiological factors for NPC include genetic susceptibility, early-age exposure to chemical carcinogens and latent EBV infection [1]. Methylation of promoter CpG Islands is closely associated with gene silencing [2, 3]. Methylation of promoter regions of tumor suppressor genes (TSGs) has been found in multiple types of cancers [4]. Identification of epigenetic alternations including promoter methylation may have potential diagnostic and prognostic value in cancer patients [5, 6] and may also help to predict drug response in cancer patients [7, 8].

Tumor hypoxia frequently occurs in solid tumor and is associated with a more malignant phenotype, including increased invasiveness, metastases, and poor survival. Cancer cells adapt to hypoxic condition within tumors and show resistance to hypoxia-mediated apoptosis, while underlying mechanisms remain unclear. This adaptation may act through inactivation of several important hypoxia-inducible genes by genetic and epigenetic alterations including promoter methylation, thus resulting in an escape from the hypoxia-induced cell death.

Table 1. Sequences of LOX primers used in this study

PCR	Primers	Size (bp)	Annealing temperature (°C)	Cycles
RT-PCR for LOX	F2: CGA CCC TTA CAA CCC CTA CA	234	60	30
	R2: CTG GCC AGA CAG TTT TCC TC			
MSP for LOX promoter	M11: TTC GTT CGG GAT TGT CGC	170	62	40
	M2: C CAA AAA AAC GAA CGA AAC ACG			
	U11: TG TTT GTT TTT TGG GAT TGT TGT	169	58	40
	U2: CAA C CAA AAA AAC AAA CAA AAC ACA			
BGS for LOX promoter	BGS1: AGA TTA AGT TAG TGT GTT TT	541	58	40
	BGS2: TCA CTC CTT TTA CCA AAT TA			

Lysyl oxidase (LOX) as an extracellular copper-containing enzyme catalyzes the crosslinking of collagens and elastin, and plays a critical role in extracellular matrix organization. Previous studies on LOX were focused on its role in connective tissue disorders and fibrotic disorders such as atherosclerosis and liver fibrosis. Increasing evidence suggests that LOX is also involved in tumor progression and metastasis through the deregulation of tumor hypoxia and epithelial-mesenchymal transition (EMT), while its role is still contradictory in tumorigenesis. Both up-regulation and down-regulation of LOX has been reported in different types of cancer cell lines and primary tumors. Of note, LOX has been suggested as a TSG in various cancers including lung, pancreatic and gastric cancers [9, 10]. Inactivation of LOX by promoter methylation and loss of heterozygosity has been found in gastric cancer [11]. However, the expression and function of LOX in NPC has not been elucidated.

In this study, we investigated the expression of *LOX* in NPC cell lines, and further examined its promoter methylation in NPC cell lines and primary tumors. We also unveiled its tumor suppressor function in the context of hypoxia in NPC.

Materials and methods

Cell lines and tumor samples

NPC cell lines (C666-1, CNE1, CNE2, HNE1, HONE1 and HK1), laryngeal cancer cell line (FaDu) and immortalized normal nasopharyngeal epithelial cell line (NP69) were studied. NPC cell lines were routinely maintained in RMPI1640 medium and incubated at 37°C in a humid atmosphere of 5% carbon dioxide in air [12]. Hypoxia was created by culturing cells in a hypoxia chamber (Galaxy R CO_2 incubator, RS Biotech Laboratory Equipment Ltd., Ayrshire, Scotland) containing 0.1% O_2, 5% CO_2 and 94.9% N_2 [13]. For treatment combining 5-aza-2'-deoxycytidine (Aza) (Sigma, St. Louis, MO) and trichostatin A (TSA) (Cayman Chemical Co., Ann Arbor, MI), cells were treated with Aza (10 μmol/L) for 3 days and subsequently with TSA (100 ng/ mL) for 24 hours [14, 15]. All culture medium and reagents were purchased from GIBCO BRL (GIBCO BRL, Grand Island, NY, USA). Tumor biopsies were taken from treatment naïve NPC patients. All patients have been consented previously for tissue collection for research at the Tumor Bank, Department of Clinical Oncology, the Chinese University of Hong Kong, according to the approved Ethics Approval of Research Protocol.

RNA isolation and reverse transcription (RT)-PCR

Cells were lysed by TRIzol Reagent (Invitrogen, Carlsbad, CA) and total cellular RNA was extracted according to manufacturer's instruction. RT-PCR was performed as previously described [15]. Briefly, first-strand cDNA was prepared from 1 μg total cellular RNA using random hexamers primers and MuLV reverse transcriptase (Applied Biosystems, Foster City, USA). Primers are shown in **Table 1**. The PCR reaction was done with AmpliTaq Gold DNA polymerase (Applied Biosystems), using GAPDH as an internal control. The PCR program utilized initial denaturation at 95°C for 10 min, followed by 30 cycles (94°C for 30 s, 55-60°C for 30 s, and 72°C for 30 s), with final extension at 72°C for 10 min. Amplified products was electrophoresed through a 2% agarose gel pre-stained

with 1 μg/ml of ethidium bromide and visualized under UV light.

DNA isolation, bisulfite treatment and methylation analysis

Genomic DNA from NPC cell lines and biopsies were extracted by TRIzol Reagent (Invitrogen, Carlsbad, CA) according to manufacturer's instruction. Bisulfate modification of DNA, methylation specific-PCR (MSP), and bisulfite genomic sequencing (BGS) were carried out as previously described [14, 15]. Briefly, the genomic DNA was chemically modified with sodium metabisulfite. The bisulfite modified DNA was then amplified using MSP primer pairs that specifically amplify either methylated (M) or unmethylated (U) sequences of the LOX promoter. The MSP primers were tested for not amplifying any unconverted DNA. For BGS, the bisulfite modified DNA was amplified with BGS primers specific for a region of LOX promoter with 30 CpG sites. Four to six colonies were randomly chosen for analysis.

Plasmid construction

The LOX full-length cDNA was amplified using human larynx cDNA with high-fidelity enzyme. The PCR product was purified with Spin-X column and clone into pCR-BluntII-TOPO vector. The cDNA was further subcloned into pcDNA3.1 (+) vector and was sequenced using ABI Prism 3100 DNA sequencer.

Colony formation assay

Colony formation assays were carried out as described previously [14, 16]. Cells were plated in a 12-well plate and transfected with 1 μg *LOX* plasmid or empty vector using Lipofectamine LTX Reagent (Invitrogen). Cells were trypsinized and plated in a 6-well plate 48 hours post-transfection, and selected for 2 to 3 weeks with 300 μg/ml G418. Surviving colonies (> 50 cells per colony) were counted after Gentian Blue staining. All experiments were done in triplicate wells and repeated three times. The results were shown as values of mean ± SEM. Statistical analysis was carried out with Student's t-test and $P < 0.05$ was considered as statistically significant.

Cell proliferation assay

Cell proliferation assay was performed as previously described [14] with little modifications. HK1 cells were transfected with *LOX* plasmid or vector using Lipofectamine LTX Reagent (Invitrogen) and were then selected by culture medium containing 300 μg/ml G418 after transfection. Cells were plated at 1×10^5 per well in a 6-well plate and cells were counted at indicated time points using a hemocytometer based on trypan blue exclusion. The experiment was done in triplicate wells and repeated three times. Statistical analysis was carried out with Student's t-test and $P < 0.05$ was considered as statistically significant.

Migration and invasion assays

HK1 cells were transfected with empty vector or *LOX* plasmid. Cell migration and invasion were evaluated *in vitro* using Transwell Migration Chamber with a pore size of 8 μm (SPL Life Sciences, Korea) and MATRIGEL Invasion Chamber with a pore size of 8 μm (BD Biosciences, USA) respectively as previously described [17]. Cells were seeded onto the upper transwell chamber at a density of 1×10^5 cells/chamber and maintained in serum-free medium. The lower chamber contained complete medium. Cells were incubated for 27 h at 37°C in a 5% CO_2 incubator or a hypoxia chamber (0.1% O_2 and 5% CO_2). After incubation, non-migrated cells in the upper chamber were removed with a cotton swab. Cells migrated through the upper transwell chamber were stained with 1% Toluidine Blue O (Sigma, St. Louis, MO). Migrated cells were counted in ten random microscopic fields at 200× magnification. Triplicate experiments were performed. The migration or invasion of HK1-vector under normoxia was expressed as 100%. Statistical analysis was carried out with Student's t-test and $P < 0.05$ was considered as statistically significant.

Western blotting

After treatment, cells were lysed in Western lysis buffer (1% Nonidet-P40, 150 mM NaCl, 1 mM EDTA, 10 mM sodium phosphate buffer, pH 7.2, 0.25 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, and 10 μg/ml aprotinin) for 5 min at 4°C. Lysates were then centrifuged at 12,000 rpm, 4°C for 15 min, and supernatants were then collected for protein quantitation. Protein concentrations were measured by a RC DC Protein Assay (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Fifty

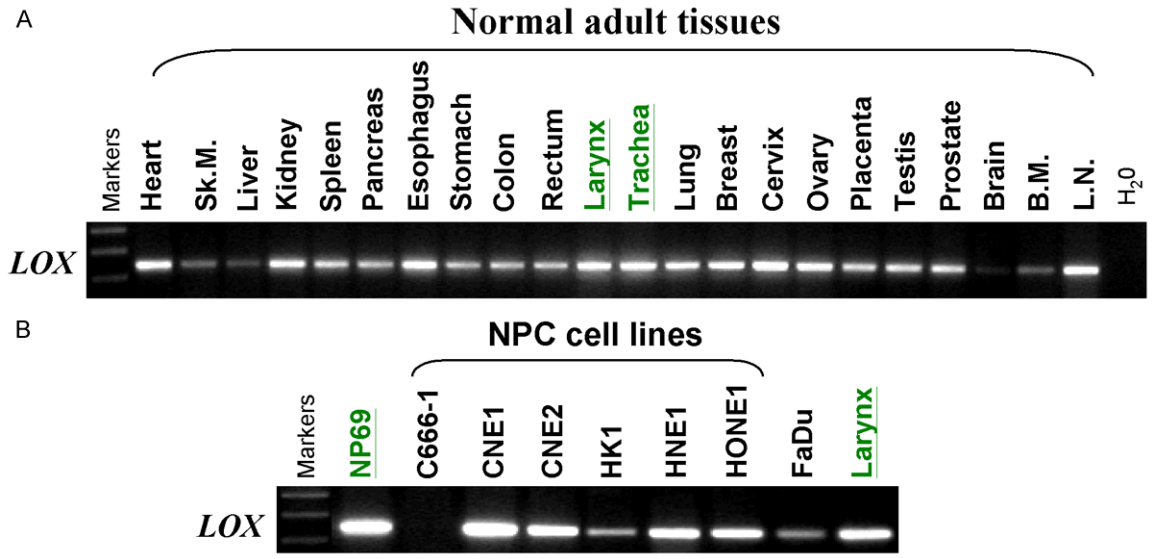

Figure 1. Analysis of *LOX* mRNA expression by semi-quantitative RT-PCR. A. Broad expression of *LOX* in human normal adult tissues. Sk.M., skeletal muscle; B.M., bone marrow; L.N., lymph node. B. Basal expression of *LOX* in NPC cell lines (C666-1, CNE1, CNE2, HK1, HNE1 and HONE1), an immortalized normal epithelial cell line (NP69), a laryngeal cancer cell line (FaDu) and normal adult larynx tissue.

μg of cellular proteins were separated by SDS-polyacrylamide gel. The separated proteins were electrophoretically transferred to Hybond ECL nitrocellulose membrane (Amersham Pharmacia Biotech Inc, Piscataway). Membranes were blocked with 5% nonfat milk in Tris-buffered saline containing 0.1% Tween-20 at room temperature for 1 hour and then incubated with primary antibody overnight at 4°C with gentle shaking. After incubation with horseradish peroxide conjugated secondary antibody, the enhanced chemiluminescence detection system ECL (Amersham Biosciences, Buckinghamshire, UK) was used to visualize the appropriate bands. Carbonic anhydrase IX (CA9) antibody and cleaved PARP antibody (human specific) were purchased from Novus Biologicals (Littleton, USA) and Cell Signaling Technology (Beverly, USA) respectively. Actin (Calbiochem, Merck KGaA, Darmstadt, Germany) was used as an internal control to verify equal protein loading in each NPC cell line during experiment. Secondary antibodies were from Invitrogen, CA. The immunoblots were analyzed by densitometry (Bio-Rad Quantity One 1-D Analysis Software Version 4.5.2). The value of band intensity from HK1-vector under normoxia was expressed as 100%. Statistical analysis was carried out with Student's t-test and $P < 0.05$ was considered as statistically significant.

Results

Expression of LOX in human normal tissues and NPC cells

We firstly examined the expression of *LOX* mRNA in human adult tissues and a panel of NPC cell lines. RT-PCR showed that *LOX* expression varied among different adult tissues. *LOX* was highly expressed in multiple human adult tissues including larynx and trachea, but lowly expressed in brain, liver, smooth muscle and bone marrow tissues (**Figure 1A**). We also found that *LOX* was downregulated or silenced in some NPC cell lines and Fadu cells, compared to the immortalized nasopharyngeal cell line NP69 and normal tissue larynx (**Figure 1B**). These data suggest that *LOX* is likely a TSG in NPC.

LOX promoter methylation was frequently detected in NPC

One of the mechanisms to silence gene expression is DNA methylation of the promoter region of the gene, thus MSP was performed to examine the promoter methylation status of *LOX* in NPC cell lines. It has been found that the *LOX* promoter was methylated in C666-1 and HK1 cell lines (**Figure 2A**). The *LOX* promoter methylation was further investigated in 49 NPC primary tumors and 16 nose swab samples from

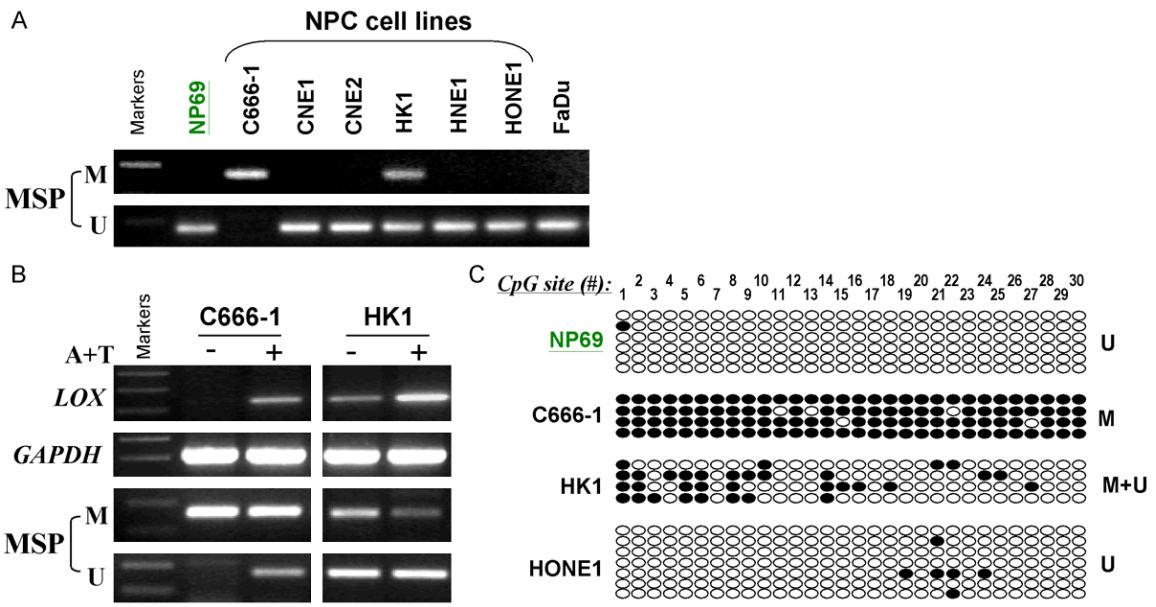

Figure 2. *LOX* promoter methylation analysis in NPC cell lines. A. Methylation–specific PCR (MSP) analysis of *LOX* promoter methylation status in NPC cell lines (C666-1, CNE1, CNE2, HK1, HNE1 and HONE1), NP69 and FaDu. Promoter region of *LOX* in C666-1 cells is methylated and in HK1 cells is partially methylated. M, methylated; U, unmethylated. B. Pharmacologic demethylation and reactivation of *LOX* with 5-aza-2'-deoxycytidine combined with trichostatin A (A+T) treatment in NPC cell lines. Semiquantitative RT-PCR analysis detected upregulated *LOX* expression in methylated and silenced NPC cell lines by A+T treatment (upper panel). MSP detected concomitant demethylation of the promoter in A+T-treated NPC cell lines (lower panel). C. High-resolution methylation analysis of the *LOX* promoter in an immortalized normal epithelial cell line (NP69) and NPC cell lines (C666-1, HK1 and HONE1). Each row indicates a promoter allele analyzed and each circle corresponds to a single CpG site. Dark circles (●) and empty circles (○) represent methylated and unmethylated CpG sites respectively.

NPC patients. Nose swab samples were non-tumor tissues taken from NPC patients. Results showed that 85.7% (42/49) of NPC primary tumors were methylated and only 18.75% (3/16) of the nose swab samples were methylated (**Figure 3A**, **3B**). High-resolution BGS was performed in C666-1, HK1, HONE1 and NP69 (**Figure 2C**) and 2 of the NPC primary tumors (**Figure 3C**). The BGS results showed that C666-1 was heavily methylated and HK1 was partly methylated.

LOX was silenced by methylation in NPC cells and could be activated by pharmacologic demethylation

To further confirm the silence of *LOX* gene was due to promoter methylation, cells were treated with 3-day Aza and 1-day TSA and then MSP and RT-PCR for *LOX* were performed. Results showed that the promoter of *LOX* gene in C666-1 was de-methylated and the *LOX* gene was re-expressed after the Aza/TSA treatment (**Figure 2B**). In HK1 cells, band intensity from the *LOX* MSP primers was decreased after de-methylation and the *LOX* mRNA level was also increased (**Figure 2B**).

Overexpression of LOX suppressed the growth and colony formation of NPC cells

Plasmid expressing full-length *LOX* cDNA (pcDNA3.1-LOX) was transfected to NPC cell lines HK1 and C666-1. HK1 cells with overexpression of *LOX* grew significantly lower than the empty vector-transfected cells (**Figure 4A**, 39.89% growth inhibition at day 7), indicating that *LOX* inhibited cell proliferation. The colony formation efficiency of HK1 cells and C666-1 cells were evaluated by monolayer culture with G418 selection. Overexpression of *LOX* in HK1 cells and C666-1 cells greatly reduced the number of colonies formed (**Figure 4B**, 30.3 ± 7.5% and 28.3 ± 4.9% compared to empty vector transfection respectively). This reduction of colony formation ability of the NPC cells suggesting *LOX* has a tumor suppressor function in these NPC cells.

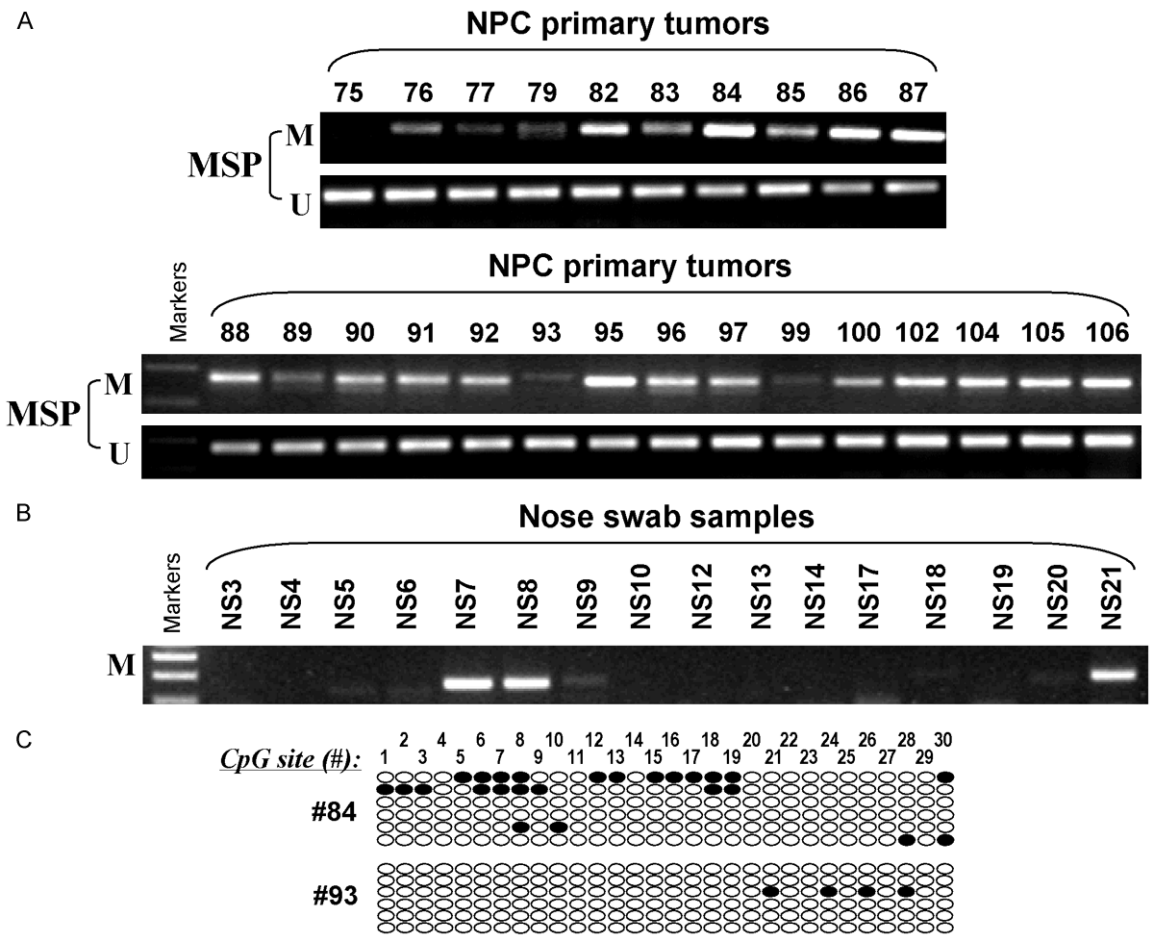

Figure 3. *LOX* promoter methylation analysis in NPC. Representative MSP results of (A) NPC primary tumors and (B) nose swab samples. (C) High-resolution methylation analysis of the *LOX* promoter in 2 NPC primary tumors. Each row indicates a promoter allele analyzed and each circle corresponds to a single CpG site. Dark circles (●) and empty circles (○) represent methylated and unmethylated CpG sites respectively.

Overexpression of LOX reduced migration and invasion of NPC cells under normoxia and hypoxia condition

The effect of *LOX* overexpression on the invasiveness of NPC cells was examined in HK1 cells. Cell migration and invasion were evaluated *in vitro* using Transwell Migration Chamber and MATRIGEL Invasion Chamber respectively. The results showed that *LOX* overexpression exhibited potent inhibition of *in vitro* migration and invasion in HK1 cells especially under hypoxia condition. As shown in **Figure 5A**, the migration of HK1 cells was reduced by *LOX* overexpression by 55.72% and 69.53% under normoxia and hypoxia condition respectively, when compared with vector control. Similarly, the invasion of HK1 was reduced by *LOX* overexpression by 78.01% and 81.36% under normoxia and hypoxia condition respectively (**Figure 5B**).

LOX overexpression affected carbonic anhydrase IX (CA9) and cPARP expression in NPC

The mRNA levels of *LOX*, *CA9* and vascular endothelial growth factor (*VEGF*) were examined in vector- or *LOX*-transfected HK1 cells. The *LOX* mRNA increased in *LOX*-transfected cells indicating that the transfections were successful. The levels of CA9 mRNA in HK1 cells were decreased after *LOX* overexpression while *VEGF* mRNA levels were not affected (**Figure 6A**). *CA9* is known to be upregulated by hypoxia and involved in tumor growth and progression. Here we examined the effect of hypoxia on CA9 protein levels in *LOX*-overexpressed HK1 cells. Upon hypoxia, the protein levels of CA9 were

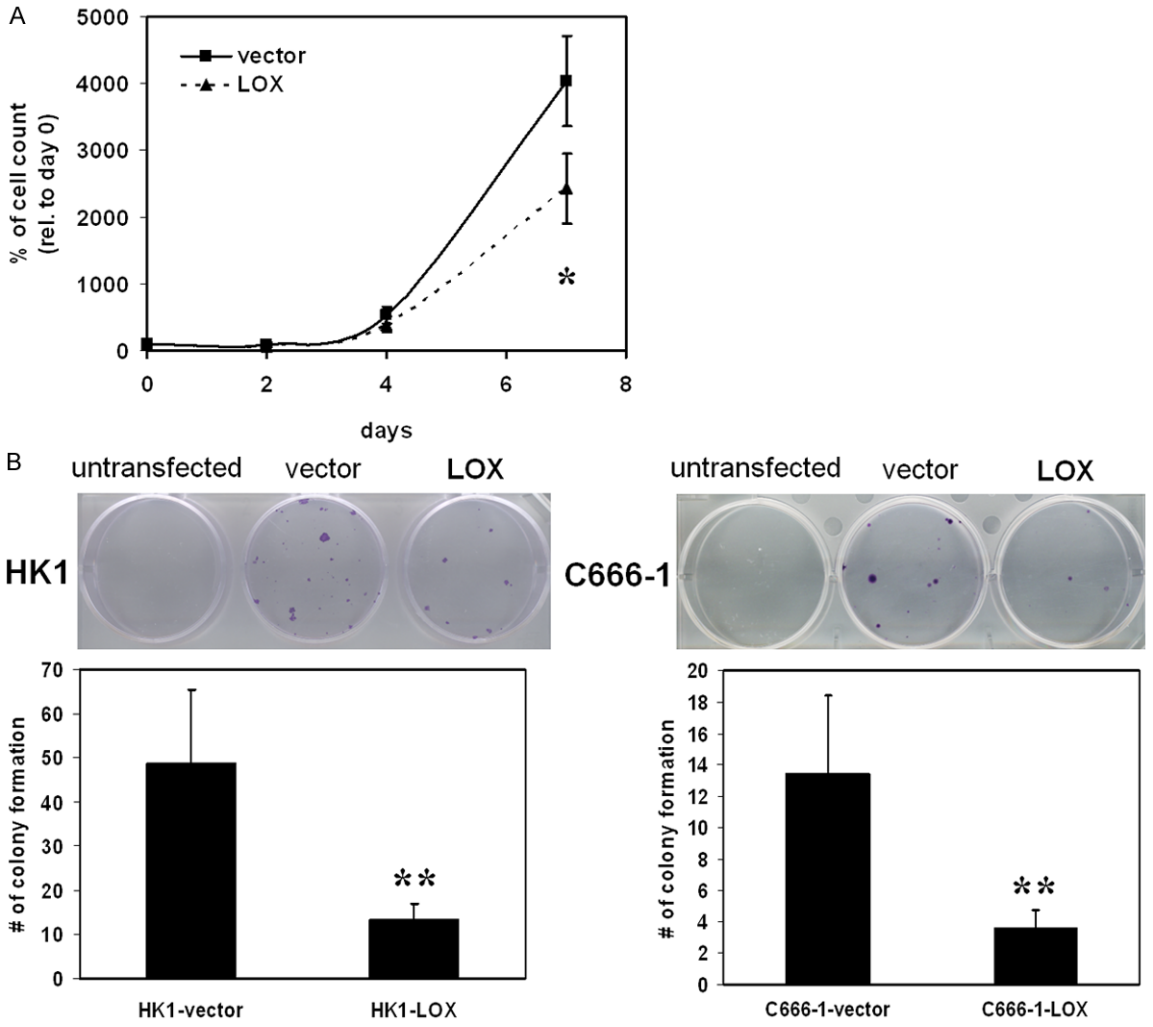

Figure 4. Overexpression of *LOX* suppressed growth and clonogenicity of NPC cells. A. Growth curves of HK1 cells after transfection with *LOX* plasmid or empty vector. HK1 cells were transfected with *LOX* plasmid (LOX) or empty vector (vector) and selected by culture medium containing G418. Cells were then plated at 1×10^5 per well in a 6-well plate. At day 2, 4 and 7, cell numbers were counted and plotted as values (% of cell number relative to day 0) of mean ± SEM of three independent experiments. B. Clonogenicity of NPC cells was analyzed by colony formation assays. Upper panels: HK1 or C666-1 cells were transfected with empty vector (vector) or *LOX*-plasmid (LOX) and selected with G418. Untransfected cells without vector or *LOX* plasmid would not survive G418 selection. Surviving colonies (≥ 50 cells per colony) were counted after Gentian Blue staining. Photos were taken from one of the representative experiments. Lower panels are quantitative analyses of colony numbers as values of mean ± SEM of three independent experiments. *P*-values were calculated using the Student's t test. The asterisk indicates statistical significant ($P < 0.05$). Double asterisk indicates statistical significant ($P < 0.01$).

increased in both vector- and *LOX*-transfected HK1 cells but the induction was significantly lower in *LOX*-transfected HK1 cells (**Figure 6B**, **6C**). The protein levels of cleaved poly (ADP-ribose) polymerase (cPARP), an apoptosis marker, were increased after hypoxia and the increase was significantly potentiated in *LOX*-transfected HK1 cells (**Figure 6B**, **6C**). However, the cPARP protein levels were not significantly changed by *LOX* overexpression alone without hypoxia treatment.

Discussion

In the present study, we demonstrated that *LOX* gene was epigenetically silenced by promoter methylation in some of the NPC cell lines and large numbers of primary tumors of NPC patients. Overexpression of *LOX* gene in NPC cell lines could significantly reduce the clonogenicity, the growth and the invasion of the cells indicating *LOX* gene may be a candidate tumor suppressor gene in NPC. Furthermore, we

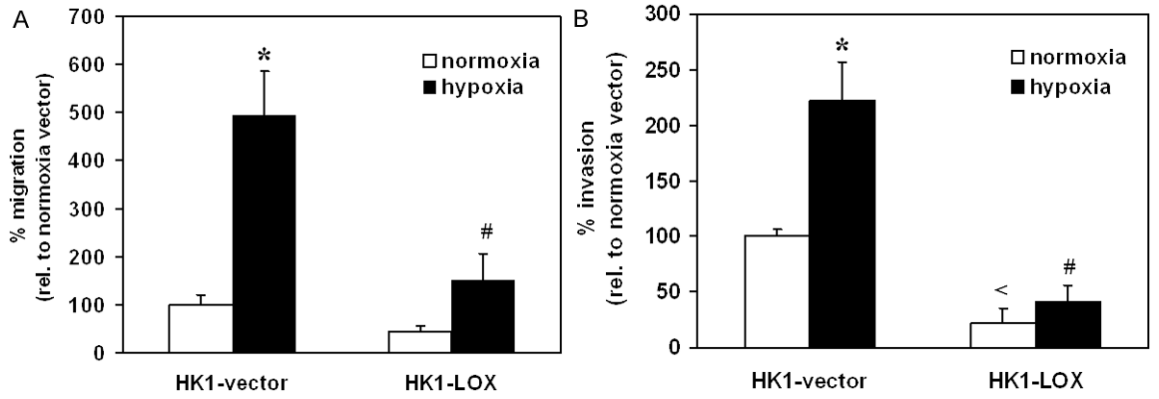

Figure 5. Overexpression of *LOX* inhibited (A) migration and (B) invasion of HK1 cells. Cells were seeded in serum-free medium in the inserts of Transwell chambers (A) or Matrigel invasion chambers (B). Medium with 10% FBS was added in the lower chambers. After 27 h, cells migrating or invading through the inserts were fixed, stained and counted (at 200× magnification). The migration or invasion of HK1-vector under normoxia was expressed as 100%. Results were depicted as means ± SEM of three independent experiments. The * indicates statistical significant when compared to same cells under normoxia condition. The # indicated statistical significant when compared to vector control during hypoxia conditions. The < indicates statistical significant when compared to vector control during normoxia conditions.

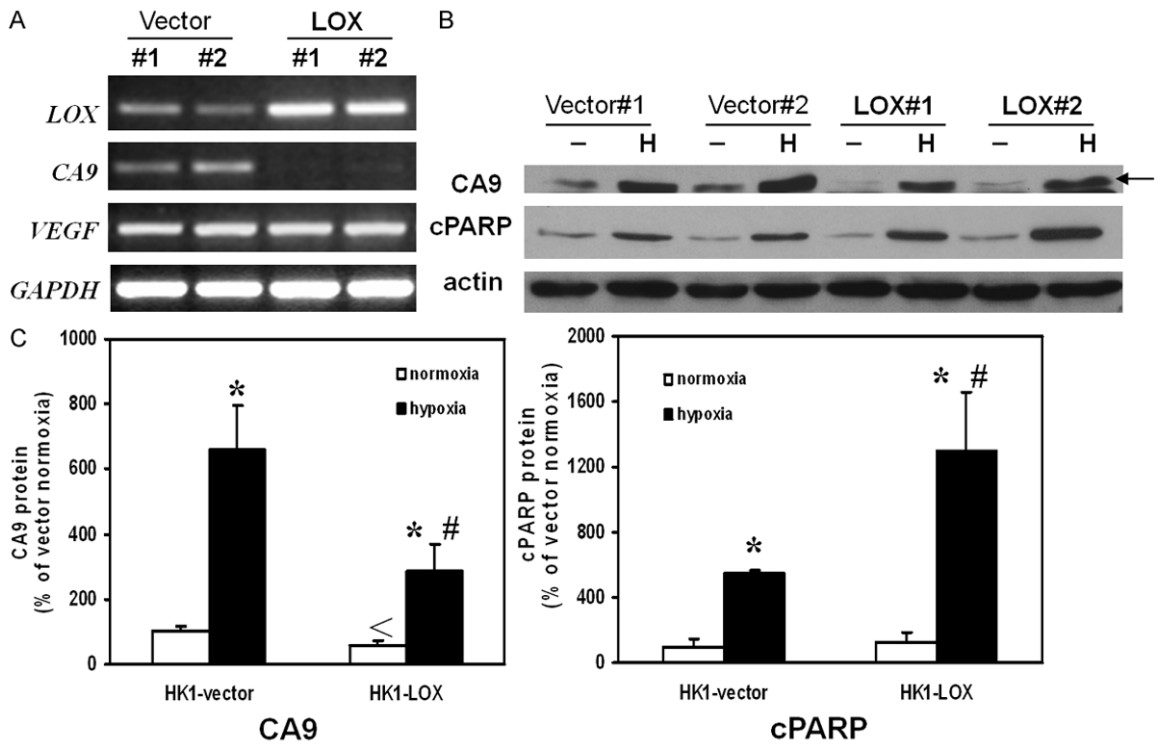

Figure 6. RT-PCR and Western Immunoblotting analysis. A. Expression of *LOX* in HK1 cells transfected with *LOX* plasmid was confirmed. Expression of *CA9* was down-regulated in *LOX* plasmid transfected HK1 cells while the expression of VEGF was not altered. B. Overexpression of *LOX* altered protein levels of CA9 and cPARP under hypoxia treatment. Representative photos were taken from three independent experiments. C. Quantitative analysis of CA9 protein and cPARP protein by densitometry. The value of band intensity from HK1-vector under normoxia was expressed as 100%. Results were depicted as means ± SEM of three independent experiments. The * indicates statistical significant when compared to same cells under same treatment condition. The # indicated statistical significant when compared to vector control during hypoxia conditions. The < indicates statistical significant when compared to vector control during normoxia conditions.

showed that *LOX* gene down-regulated the expression of carbonic anhydrase IX (*CA9*) in which this *CA9* down-regulation may contribute to the anti-tumor effects of *LOX* in NPC.

LOX is a hypoxia-inducible protein [18] and the *LOX* promoter contains a hypoxia-responsive element [19], so LOX is a transcriptional target of hypoxia-inducible factor (HIF). It is well-known that HIF-1α is the principal transcriptional factor involved in the regulation of the hypoxia-related responses in the level of transcription. Elevation of HIF-1α protein level is one of the major pathways that up-regulate the hypoxia-inducible genes upon hypoxia. Previously we have shown that upon hypoxia, all of the four NPC cell lines studied including CNE-2, HONE-1, HK1 and C666-1 demonstrated an increase in HIF-1α protein level and either VEGF or CA9 expression indicating that all these NPC cell lines have intact HIF-1-responsive machinery [13]. Upon hypoxia, the *LOX* mRNA levels were dramatically up-regulated in CNE2 and HONE1 cells, but only a low level of up-regulation in HK1 cells and no up-regulation in C666-1 cells were found [13]. The unresponsiveness of *LOX* induction in C666-1 cells is not due to the failure of HIF-1α protein function. It may be caused by epigenetic gene silencing of *LOX* in C666-1 cells.

Promoter methylation is one of the well-known mechanisms of gene silencing. Silencing of tumor suppressor gene by promoter methylation is one of the important factors for carcinogenesis in NPC and a number of tumor suppressor genes have been found to be methylated in NPC [1]. By the use of methylation-specific primers, the promoter of *LOX* gene has been shown to be methylated in C666-1 cells and HK1 cells (**Figure 2A**). The *LOX* gene is possibly silenced epigenetically by promoter methylation; therefore it cannot be up-regulated upon hypoxia in C666-1 cells. In HK1 cells, the promoter is partly methylated, so there is a weak elevation of *LOX* expression upon hypoxia. After pharmacological demethylation by 3-day Aza and 1-day TSA treatment, the *LOX* gene in C666-1 and HK1 was re-expressed, this result further confirmed that the gene silencing was due to promoter methylation (**Figure 2B**). After showing that the *LOX* gene is silenced by methylation in NPC cell lines, we elucidated the *LOX* methylation status in NPC patient samples. We found that promoter of *LOX* gene was methylated in 85.7% of primary tumors but only 18.75% in nose swab samples in NPC patients (**Figure 3**) suggesting that this gene is clinical relevant, not limited in NPC cell lines.

Next, the functional effects of *LOX* in NPC cell lines were investigated. The growth assay results showed that *LOX* overexpression could reduce cell growth in HK cells (**Figure 4A**). In addition, results from the colony formation assay suggested that overexpression of *LOX* in HK1 and C666-1 cells significantly reduced the clonogenicity in these cell lines (**Figure 4B**). Furthermore, the results from the migration assay and invasion assay demonstrated the *LOX* overexpression could reduce the invasiveness of the HK1 cells (**Figure 5**). It is probable that *LOX* may exert its tumor suppressor effects on NPC through inhibition of growth and metastasis.

In order to elucidate the possible mechanism of *LOX*-inhibited cell growth and invasion, the mRNA and protein levels were investigated in *LOX*-overexpressed cells. Carbonic anhydrase IX (CA9) is a hypoxia-inducible protein and has been suggested as an endogenous marker for tumor hypoxia [20, 21]. *CA9* expression is associated with the prognosis of multiple cancers including carcinomas of breast [22], head and neck [23], cervix [21], etc. *CA9* has been suggested to be associated with tumor progression [24, 25] and metastasis [26, 27] and is a potential target for antitumor therapy [28, 29]. *CA9* is frequently overexpressed in NPC and its expression as well as that of HIF-1α is associated with a worse progression-free survival in NPC patients [30]. It has been shown that inhibition of CA9 by selective inhibitors decreases cell proliferation and induces apoptosis in human renal carcinoma cell lines and cervical cancer cell lines [31]. The exact mechanisms of the oncogenic effects of *CA9* are not fully resolved. As CA9 is a transmembrane glycoprotein involved in physiologic acid/base regulation, one of the possible mechanisms is that during hypoxia CA9 contributes to a more acidic extracellular environment while helps to maintain a more alkaline intracellular pH [32, 33]. These changes in extra- and intra-cellular pH may facilitate the invasive properties and growth of tumor cells. In the present study, basal mRNA levels of *CA9* in *LOX*-overexpressed cells were decreased while those of another

hypoxia-inducible gene *VEGF* were unchanged (**Figure 6A**). Since both *LOX* and *CA9* are hypoxia-inducible genes, the changes of CA9 protein levels in *LOX*-overexpressed cells and empty vector-transfected cells were further investigated in hypoxia condition. Upon hypoxia treatment, CA9 protein levels were up-regulated in both *LOX*-overexpressed cells and empty vector-transfected cells. However, the induction was greatly inhibited in *LOX*-overexpressed cells (**Figure 6B**, **6C**). In addition, an apoptosis marker, cPARP, was also investigated under hypoxia condition in these cell lines. The cPARP protein levels were greatly up-regulated in *LOX*-overexpressed cells upon hypoxia (**Figure 6B**, **6C**), implying that *LOX* can enhance apoptosis of NPC during hypoxia condition.

LOX gene has recently been suggested as a tumor suppressor gene inactivated in various cancers including lung, pancreatic and gastric cancers [9, 10]. On the other hand, Giaccia's group has shown that *LOX* expression was negatively correlated with distant metastasis-free survival and overall survival in breast cancer patients with ER-negative tumors as well as in head and neck cancer patients [19]. *LOX* is also proposed to be oncogenic in several types of squamous cell carcinoma [34-36]. The functions of the *LOX* gene on tumors may depend on the tumor type and its functions on NPC remain unknown. The gene product of *LOX* gene is a 46 kDa preprotein. After removal of the signal peptide and glycosylation, a 50 kDa inactive proenzyme, i.e. proLOX, is produced. The proLOX is further processed to become an active LOX enzyme and a LOX propeptide (LOX-PP). LOX is well-known as its classic role as a collagen or elastin cross-linking enzyme. However, the functions of LOX-PP are largely unknown. Previously, LOX-PP has been shown to be not essential for the proper folding of the LOX protein as well as the generation of catalytic function and the secretion of the LOX protein [37]. Recently Trackman's group has demonstrated that the tumor suppressing effect of the *LOX* gene is mapped to the LOX-PP [38]. They also suggested that LOX enzyme alone may promote transformed phenotype of breast cancer cells but LOX-PP can reduce the transforming ability of LOX enzyme [39]. LOX-PP has recently been shown to suppressed Akt and ERK in Her-2/neu-driven breast cancer cells [40], H1299 lung cancer cells and PANC-1 pancreatic cancer cells [10]. It seems that the effects of *LOX* gene on specific cell types may be affected by the balance of the LOX enzyme and LOX-PP. Although not much information on the metabolism and cellular distribution of LOX-PP in different cell types is available from literature, data from our current study has strongly suggested that *LOX* gene has tumor suppressive effects on NPC.

In conclusion, our study suggests that *LOX* is a candidate TSG epigenetically silenced in NPC. Overexpression of *LOX* gene in NPC can reduce clonogenicity, cell growth as well as cell migration and invasion. *LOX* may also exert its tumor suppressive effects during hypoxia, a common condition in solid tumors including NPC, through reducing the CA9 induction and increasing apoptosis, leading to reduction in metastasis and growth of the tumor. This study helps to improve our understanding of the tumorigenesis of NPC, which may help to identify new therapeutic targets and provide new insights on treatment.

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Disclosure of conflict of interest

The authors declare no conflict of interest.

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