RESEARCH ARTICLE

Expression and Significance of Hypoxia Inducible Factor-1α and Lysyl Oxidase in Non-small Cell Lung Cancer

Wei Ping, Wen-Yang Jiang, Wen-Shu Chen, Wei Sun, Xiang-Ning Fu*

Abstract

Object: To detect expression of hypoxia inducible factor-1α (HIF-1α) and lysyl oxidase (LOX) in non-small cell lung cancer (NSCLC) and explore their roles in prognosis. Methods: The mRNA levels of HIF-1α and LOX were investigated by real-time reverse-transcriptase polymerase chain reaction in 40 cases of tumour and paired normal tissues. In addition, protein expression of HIF-1α and LOX was examined by immunohistochemistry in 82 cases of tumour and 45 paired normal tissues. The relationship between HIF-1α or LOX and clinicopathologic characteristics, as well as the correlation between HIF-1α and LOX, were also examined. Kaplan-Meier survival curves and the log-rank test were used to analyze progression-free survival. Results: HIF-1α or LOX mRNA levels in tumor tissues was significantly higher than those in paired normal tissues (p<0.01). Positive HIF-1α or LOX protein expression in tumor tissues was noted in 46/82 (56.1%) and 49/82 (59.8%) of the cases, respectively, being significantly higher than those in paired normal tissues (p<0.05). There was significant correlation between the expression of HIF-1α or LOX and tumor size, lymph node metastasis and pathological stage (p<0.05). The expression of HIF-1α and LOX had a significant inverse impact on survival of patients with NSCLC. Conclusion: HIF-1α and LOX may play a pivotal role in the development of NSCLC, and may act in synergy to promote the progression of NSCLC.

Keywords: HIF-1α - LOX - NSCLC - prognosis

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Introduction

Lung cancer is the leading cause of cancer death worldwide (Siegel et al., 2011). Non-small cell lung cancer (NSCLC) accounts for approximately 85% of all lung cancers. Despite diagnostic and therapeutic advances, the prognosis of lung cancer is still poor, with a 5-year survival rate of 17% (Siegel et al., 2012). Approximately half of radical resected NSCLC relapse with metastasis within 5 years, indicating that tumor cells invasion, migration and micrometastases may occur before surgical treatment and that the TNM classification alone may be insufficient to precisely predict which resected tumors are more likely to relapse with metastasis (Gu et al., 2002). Therefore, understanding the molecular biology of NSCLC is important for diagnosis, prevention and treatment of NSCLC.

A developing body of evidences has shown that hypoxia inducible factor-1α (HIF-1α) is involved in crucial aspects of cancer biology, including angiogenesis, proliferation, energy metabolism and invasion (Hiraki et al., 2012; Schito et al., 2012; Zhao et al., 2012; Cheng et al., 2013). Although the relevance of HIF-1α on the prognosis in NSCLC has been investigated, conflicting results have been reported from different laboratories (Volm et al., 2000; Hirami et al., 2004; Swinson et al., 2004; Hung et al., 2009; Park et al., 2011). This suggests that the application of HIF-1α alone as an independent predictor of prognosis might be invalid, and in combination with other tumor markers should be considered.

Recently, lysyl oxidase (LOX) has been identified as an important regulator of hypoxia induced tumor progression via an HIF-1α-dependent mechanism in a range of cancers, such as head and neck carcinomas (Le et al., 2007), colon (Tammali et al., 2011), breast (Wong et al., 2012) and prostate (Stewart et al., 2009). The primary function of LOX is the covalent cross-linking of collagens or elastin in extracellular matrix (ECM). The formation of collagens or elastin cross-links leads to an increase in structural integrity and tensile strength, which play an important role in normal connective tissue function and embryonic development (Payne et al., 2007). Therefore, aberrant LOX expression or enzymatic activity leads to a series of disease predominantly associated with the ECM, as well as in many human cancers. Increasing evidences have shown that LOX is linked to increased invasion and migration of hypoxic NSCLC cell lines in vitro (Sahlgren et al., 2008; Gao et al., 2010; Wei et al., 2012). However, whether there is some association between HIF-1α and LOX, and what role HIF-1α and LOX play in NSCLC has not previously been investigated.

In this study, we conducted an exploratory analysis...
to investigate the expression of HIF-1α and LOX in NSCLC patients by real-time polymerase chain reaction (RT-PCR) and immunohistochemical (IHC) methods. The association between HIF-1α and LOX, and impact of their expression on NSCLC patients’ survival were analyzed.

Materials and Methods

Patients and samples

In this study, samples of NSCLC tissues and paired normal tissues (5cm away from the malignant tissue) was obtained from 91 consecutive patients with NSCLC, who underwent anatomic resection at Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology, between March 2008 and February 2011. All samples were divided into two parts immediately after removed from patients, one was fixed in 4% buffered formaldehyde and embedded in paraffin wax for IHC, and the other was put into liquid nitrogen for RT-PCR. Of these patients, 4 were excluded due to death within 60 days of surgery to reduce the confounding variable of perioperative mortality. The 60-day cut-point was used in our study as it has been used to exclude postoperative mortality in several previous studies performed on this patient series and other IHC and surgical studies in NSCLC (Giatromanolaki et al., 2001; Swinson et al., 2004). 5 patients who had recurrent NSCLC or received chemoradiotherapy before resection were also excluded from the study. Of the 82 included patients, 48 were male and 34 were female. There were 47 adenocarcinoma and 35 squamous carcinoma. The median age at surgery was 58.5 years (range: 39-75). The final staging was based on the histopathology report and the findings at surgery. Among 82 patients, 28 were well differentiated, 28 were moderately differentiated, and 26 were poorly differentiated. The tumors were scored independently by two investigators (Chen WS and Jiang WY) who were blinded to the patient’s clinical data. The assessment of HIF-1α expression on NSCLC patients' survival were analyzed.

Immunohistochemistry

IHC was performed by the standard Streptavidin/ Peroxidase method (Streptavidin/Peroxidase immunohistochemical kit, Fuzhou Maixin Biotechnology Co., Ltd, Fujian, China). Each 4-µm-thick section was deparaffinized in xylene and rehydrated through a series of graded ethanol. Antigen retrieval was performed by microwaving the slides in citrate buffer (pH=9.0) for 15min at 95°C and then was cooled to room temperature. Endogenous peroxidase activity was blocked by incubation in 3% hydrogen peroxide for 20min. After washed in 0.1M phosphate-buffered saline (PBS, pH=7.4), non-specific binding sites were blocked with normal goat serum for 30min at room temperature. The sections were then exposed to the primary antibody (rabbit monoclonal anti-HIF-1α, 1:100, Epitomics, CA, USA; rabbit monoclonal anti-LOX, 1:400, Novus Biological, Inc., Littleton, CO, USA) overnight at 4°C. After washing in PBS, the slides were incubated with a biotinylated anti-rabbit secondary antibody for 60min at room temperature, and finally incubated in streptavidin-biotin peroxidase complex solution for 20min at room temperature. The slides were visualized with diamino benzidine-tetrahydrochloride (DAB kit, Zhonshan Goldenbridge Biological Technology Co., LTD, Beijing, China). After washed in water, the slides were lightly counterstained with hematoxylin. Negative controls were performed without the primary antibodies.

The tumors were scored independently by two investigators (Chen WS and Jiang WY) who were blinded to the patient’s clinical data. The assessment of HIF-1α and LOX expression was based on the percentage of stained tumor cells and the staining intensity. At least 3 different fields (×400) were examined. The staining intensity was rated as follows: 0=negative; 1=weak; 2=moderate; 3=strong staining intensity. The percentage of positive tumor cells was rated as follows: 1=1 to 10%; 2=11%-50%; 3=51%-80%; 4=81% to 100%. Points for staining intensity and percentage of positive tumor cells were added and the overall score were grouped into four categories: negative, ≤10% of tumor cells stained positive, regardless of intensity; weak expression=3; moderate expression=4 to 5; and strong expression=6 to 7. Moderate and strong expression was rated as positive, while weak expression was rated as negative for analysis. Specimens scored differently by the two investigators.

RNA isolation, reverse transcription and real-time PCR

The total RNA of NSCLC and paired normal tissues were extracted using Trizol (Invitrogen, USA) according to the manufacturer’s protocol. The concentration of RNA was measured by ultraviolet spectrophotometer. Three micrograms of RNA was used for reverse transcription. HIF-1α, LOX and β-actin genes were amplified in a fluorescence reader Roche LightCycler 480 system. The amplification was carried out in a total volume of 15µl containing 7.5µl TransStart Eco Green qPCR SuperMix (TransGen Biotech, China), 4.9µl sterile water, 2µl cDNA and 0.3µl of each primer. The primer sequences for β-actin gene were: 5'-CCTGGTTCCCTGAAACTCTGACT-3' (forward) and 5'-CCACACCCACACCCTCAATT-3' (reverse); the primer sequences for LOX gene were: 5'-TTGAATGTTAGACTTTCTATGA-3' (forward) and 5'-AGACATACTACCCTCTTT-3' (reverse); and the primer sequences for β-actin gene were: 5'-GCAAATGTTCAAAGGCGCAC-3' (forward) and 5'-GCTGTCACCTTACCCGTCC-3' (reverse). Cycling conditions were as follows: initial denaturation at 95°C for 10min, followed by 40 cycles at 95°C for 30s, at 60°C for 30s, at 72°C for 30s. The negative control was performed using normal NSCLC tissues taken far away from the tumor tissues. Each experiment was carried out in triplicate using β-actin as an internal standard. The relative expression of the mRNA was calculated with the following formula: \[ \text{Ratio} = 2^{-\Delta C_t} \], in which \( \Delta C_t = C_t \text{target gene} - C_t \beta\text{-actin} \).

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were reevaluated and then classified according to the best assessment of the observers. The criteria of evaluation were determined as described by previous reports (Albinger-Hegyi et al., 2010; Zhang et al., 2010).

**Statistical analysis**

Statistical analysis was performed using SPSS statistical software 11.0 for windows. Data were presented as mean ± standard deviation. Difference/correlations between two groups were assessed by student’s t test, χ² test, and Pearson’s correlation test. Survival curves were calculated using the Kaplan-Meier method and the statistical significance was assessed using the log-rank test. Differences at p<0.05 were considered to be statistically significant.

**Results**

**HIF-1α is overexpressed in NSCLC**

HIF-1α expression at the mRNA level was investigated in 40 NSCLC by RT-PCR. 34 out of 40 NSCLC (85%) showed increased HIF-1α mRNA in their tumor tissues than paired normal tissues. In tumor tissues, the mean level of HIF-1α mRNA was (1.37 ± 0.95) × 10⁻³, which was significantly higher than that observed in paired normal tissues [(0.59 ± 0.33) × 10⁻³, p<0.01, Figure 1a]. IHC result showed that the protein expression of HIF-1α in tumor tissues was localized in nuclear (Figure 2a), and is significantly higher than that in paired normal tissues (56.1% vs 28.9%, p<0.01, Table 1). Relationship between HIF-1α expression and clinicopathological characteristics were investigated at both the mRNA and protein levels.

### Table 1. Expression of LOX and HIF-1α Protein in NSCLC Tumor Tissues and Paired Normal Tissues

<table>
<thead>
<tr>
<th>n</th>
<th>HIF-1α protein</th>
<th>LOX protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tumor tissues</td>
<td>82</td>
<td>46</td>
</tr>
<tr>
<td>Normal tissues</td>
<td>45</td>
<td>13</td>
</tr>
</tbody>
</table>

### Table 2. The Relationship of Between LOX/ HIF-1α and Clinicopathological Characteristics

<table>
<thead>
<tr>
<th>Age</th>
<th>HIF-1α mRNA mean ± SD (×10⁻³)</th>
<th>p value</th>
<th>HIF-1α protein + - p value</th>
<th>LOX mRNA mean ± SD (×10⁻³)</th>
<th>p value</th>
<th>LOX protein + - p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt;60</td>
<td>1.48 ± 0.88</td>
<td>0.41</td>
<td>28 16</td>
<td>0.14</td>
<td>8.56 ± 5.42</td>
<td>0.42</td>
</tr>
<tr>
<td>≥60</td>
<td>1.23 ± 1.05</td>
<td></td>
<td>18 20</td>
<td></td>
<td>7.13 ± 5.42</td>
<td>0.42</td>
</tr>
<tr>
<td>Gender</td>
<td>Male</td>
<td>1.31 ± 0.89</td>
<td>0.59</td>
<td>27 21</td>
<td>0.97</td>
<td>8.54 ± 5.41</td>
</tr>
<tr>
<td></td>
<td>Female</td>
<td>1.48 ± 1.07</td>
<td>19 15</td>
<td></td>
<td></td>
<td>6.88 ± 5.40</td>
</tr>
<tr>
<td>Pathological type</td>
<td>Squamous</td>
<td>1.56 ± 0.82</td>
<td>0.33</td>
<td>20 15</td>
<td>0.87</td>
<td>9.58 ± 5.91</td>
</tr>
<tr>
<td></td>
<td>Adenocarcinoma</td>
<td>1.26 ± 1.02</td>
<td>26 21</td>
<td></td>
<td></td>
<td>6.98 ± 4.93</td>
</tr>
<tr>
<td>Differentiation</td>
<td>Well+Moderate</td>
<td>1.29 ± 0.93</td>
<td>0.50</td>
<td>30 26</td>
<td>0.50</td>
<td>7.31 ± 5.62</td>
</tr>
<tr>
<td></td>
<td>Poor</td>
<td>1.49 ± 0.98</td>
<td>16 10</td>
<td></td>
<td></td>
<td>8.83 ± 5.11</td>
</tr>
<tr>
<td>Tumor size (cm)</td>
<td>≤3</td>
<td>0.87 ± 0.66</td>
<td>0.02</td>
<td>11 18</td>
<td>0.01</td>
<td>5.29 ± 3.75</td>
</tr>
<tr>
<td></td>
<td>&gt;3</td>
<td>1.61 ± 0.98</td>
<td>35 18</td>
<td></td>
<td></td>
<td>9.24 ± 5.65</td>
</tr>
<tr>
<td>Lymph node metastasis</td>
<td>No</td>
<td>0.99 ± 0.63</td>
<td>0.02</td>
<td>18 23</td>
<td>0.03</td>
<td>5.93 ± 4.03</td>
</tr>
<tr>
<td></td>
<td>Yes</td>
<td>1.65 ± 1.06</td>
<td>28 13</td>
<td></td>
<td></td>
<td>9.46 ± 5.86</td>
</tr>
<tr>
<td>Pathological stage</td>
<td>I</td>
<td>0.78 ± 0.39</td>
<td>0.00</td>
<td>10 17</td>
<td>0.02</td>
<td>4.77 ± 4.17</td>
</tr>
<tr>
<td></td>
<td>II+III</td>
<td>1.63 ± 1.01</td>
<td>36 19</td>
<td></td>
<td></td>
<td>9.32 ± 5.34</td>
</tr>
</tbody>
</table>
High HIF-1α expression showed significant correlations with tumor size, lymph node metastasis and pathological stage (p<0.05, Table 2).

**LOX is overexpressed in NSCLC**

We continued to detect LOX mRNA level in NSCLC, and found that 32 out of 40 NSCLC (80%) showed higher LOX mRNA in their tumor tissues than its paired normal tissues. The mean level of LOX mRNA in tumor tissues was \((7.95 \pm 5.40) \times 10^{-3}\), and it was \((3.77 \pm 2.34) \times 10^{-3}\) in paired normal tissues. The LOX mRNA level in tumor tissues were significantly higher than that observed in paired normal tissues (p<0.01, Figure 1b). Expression of LOX in NSCLC at the protein level was investigated by IHC, and we found that LOX protein was localized in cytoplasm of tumor cells (Figure 2b). The result showed that the expression of LOX was 59.8% (49/82) in cancerous tissues, which was significant higher than that in paired normal tissues (59.8% vs 31.1%, p<0.01, Table 1). When we analyzed the relationship between LOX and clinicopathological characteristics, we found that the expression of LOX protein was significantly correlated with tumor size, lymph node involvement and pathological stage (p<0.05, Table 2).

**Correlation of HIF-1α and LOX expression in NSCLC**

In breast and colon cancers, hypoxia can raise LOX expression in a HIF-1α-dependent mechanism (Erler et al., 2006; Pez et al., 2011), but it remains unknown in tumor tissues of NSCLC. To study whether LOX is associated with HIF-1α in NSCLC, we performed the correlative analysis. The result showed that there was a significant correlation between HIF-1α mRNA and LOX mRNA (p_pearson=0.76, p<0.01, Figure 3). The similar result was also observed between HIF-1α protein and LOX protein (p_pearson=0.48, p<0.01, Table 3), which indicates that the increase in LOX expression is in keeping with the increase of HIF-1α expression in tumor tissues of NSCLC.

**Overexpression of HIF-1α and LOX in NSCLC has a negative impact on patient survival**

To investigate whether overexpression of HIF-1α and LOX protein in NSCLC has an impact on patient survival, we performed survival curves using the Kaplan-Meier method. The clinical end point used was the time to PFS, including relapse, metastasis and death. High HIF-1α expression was associated with a poor prognosis. The mean survival of negative expression of HIF-1α in tumor tissues was 22.0 mo (95% CI: 19.1-25.0), while it was 16.2 mo (95% CI: 13.5-19.0) with positive expression of HIF-1α. Log-rank analysis showed that there was a significant difference (p<0.05, Figure 4a). Similarly, the positive LOX expression showed an inverse impact on survival. The mean survival of patients with negative LOX expression in tumor tissues was 22.5 mo (95% CI: 19.4-25.7), being significant longer than patients with positive LOX expression, which was 16.3 mo (95% CI: 13.6-18.9, p=0.02, Figure 4b). Besides, the mean survival of patients with neither HIF-1α nor LOX positive expression was 24.6 mo (95% CI: 20.8-27.3), significantly longer than those with both positive expression, which was 15.6 mo (95% CI: 12.6-18.5, p=0.01, Figure 4c).

**Discussion**

Recently, various combined-modality therapies, including surgery, chemotherapy, and radiation therapy, have improved the outcome of patients with NSCLC. However, NSCLC is still one of the most common carcinoma characterized by a high incidence of early recurrence and poor prognosis (al-Kattan et al., 1997; Siegel et al., 2012). With the development of molecular biology, the identification of reliable biomarkers for the early diagnosis, treatment and prognostic assessment would represent an important step in the clinical management of NSCLC. In solid tumors, hypoxia is a common phenomenon, and it is associated with poor prognosis regardless of therapy strategy (Birner et al., 2000; Bachiari et al., 2003), suggesting that it might be an important therapeutic target.
As previously published, HIF-1α is overexpressed in many human malignancies and their metastases, compared with their paired normal tissues (Zhong et al., 1999; Zhang et al., 2010). However, association of HIF-1α with clinicopathological characteristics and prognosis is inconsistent. Previous studies reported that HIF-1α expression in NSCLC was marginally associated with histological types, T-stage and poor prognosis (Lee et al., 2003; Swinson et al., 2004). There are several possible reasons for the inconsistency. First, different laboratories used different kinds of antibodies. HIF-1α protein expression was shown in nuclear or cytoplasmic staining by different groups. Second, the scoring strategies and cutoff values in studies are variable. Third, diversity of therapy strategy for NSCLC makes the value of HIF-1α unclear.

In this study, we found that not only HIF-1α mRNA, but also HIF-1α protein was overexpressed in NSCLC compared with paired normal tissues. In addition, we found that overexpression of HIF-1α was related to tumor size, lymph node metastasis and pathological stage. Kaplan-Meier analysis showed that the mean survival of patients without HIF-1α protein expression in tumor tissues was significantly longer than those with HIF-1α expression. These data suggested that overexpression of HIF-1α protein in NSCLC may contribute to its progression. However, considering the inconsistent results, HIF-1α may not be an effective predictor of prognosis in NSCLC.

Several studies have shown that overexpression of LOX was significantly associated with poorly differentiated, high grade tumors, increased recurrence rates and decreased overall survival (Stassar et al., 2001; Lapointe et al., 2004; Albinger-Hegyi et al., 2010). Similar results were observed in our study, we found that the expression of LOX was significantly higher in NSCLC tumor tissues than paired normal tissue. Furthermore, overexpression of LOX protein was significantly correlated with tumor size, lymph node involvement and pathological stage. Kaplan-Meier analysis showed that the patients with overexpression of LOX had a poorer PFS. However, the impact of LOX in NSCLC is still unclear because decreased LOX expression has been shown to mediate an induction of Epithelial-mesenchymal transition, owing to LOX being the target of HIF-1α (Schietke et al., 2010). Moreover, in addition to establishing HIF-1α-dependent LOX action on tumor, LOX could upregulate HIF-1α protein expression in a manner requiring LOX-mediated hydrogen peroxide production in colorectal adenocarcinoma cells (Pez et al., 2011). Thus, HIF-1α signal pathway and LOX signal pathway may act in synergy to promote the progression of NSCLC.

Our analysis of HIF-1α and LOX in NSCLC patients may provide a valuable tool in elucidating tumor progression. However, our data still has week point that the conclusions of this study should be interpreted cautiously because of the small number included in our study. By taking into account the classical well-defined prognostic factors for NSCLC, our further studies will expand the number of patients with a longer follow-up to investigate the relationship between these biomarkers and aggressive and recurrent behavior of NSCLC.

In conclusion, our study demonstrates that HIF-1α and LOX play a significant role in NSCLC, and showed that HIF-1α and LOX were predictor factors of prognosis in NSCLC. Besides, there was positive correlation between HIF-1α and LOX expression. However, the exact mechanism of crosstalk between HIF-1α and LOX in NSCLC still needs to be further studied. A clearer interpretation of the mechanisms by which HIF-1α and LOX contribute synergistically to NSCLC progression has the potential for novel anti-metastasis therapeutics.

Acknowledgements

We declare that we have no conflict of interest.

References


of hypoxia-inducible factor 1alpha indicates diminished response to radiotherapy and unfavorable prognosis in patients receiving radical radiotherapy for cervical cancer. Clin Cancer Res, 9, 2234-40.


