Reports of Biochemistry & Molecular Biology
Vol.10, No.2, Jul 2021

Original article

www.RBMB.net

Association Between *NOX4* And *Nrf2* Genes in Non-Small-Cell Lung Carcinoma: A Case-Control Study

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Abstract

Background: Epithelial malignancy in lung cancer, which is initiated with myofibroblast differentiation and remodeling, promotes hypoxia and intracellular ROS generation most affected by the prototypical enzyme, NADPH oxidase 4 (*NOX4*). In addition, nuclear factor erythroid 2-related factor 2 (*Nrf2*) acts as a critical transcription factor by stimulating antioxidant proteins as redox homeostasis regulators. The aim of this study was to investigate a possible correlation between lung tissue *NOX4* and *Nrf2* genes (*NOX4* and *Nrf2*) mRNA expression and bronchoalveolar lavage fluid (BALF) protein expression in non-small-cell lung carcinoma (NSCLC) patients.

Methods: Samples from 25 patients with various NSCLC types and stages and 20 healthy controls were collected. *NOX4* and *Nrf2* mRNA were measured by qRT-PCR, and protein by western blot analysis.

Results: NOX4 mRNA and protein expression was significantly up-regulated in NSCLC patients' lung tissues and BALFs (p= 0.03 and 0.01, respectively). In addition, by adjusting for age, sex, and NSCLC types and stages, a significant and positive correlation was observed between *NOX4* and *Nrf2* mRNA expression (r= 0.927, p= 0.001). This was also true when not adjusted as above (r= 0.944, p< 0.001).

Conclusions: *NOX4* mRNA and protein expression is significantly up-regulated in NSCLC patients' lung tissues and BALFs, and *NOX4* and *Nrf2* mRNA expression is positively correlated in NSCLC tissues.

Keywords: Gene expression, Non-small-cell lung cancer, *NOX4*, *Nrf2*.

Introduction

Lung cancer, a major health problem affecting millions of people worldwide, is the leading cause of cancer-related mortality in humans (1). It is a complex and heterogeneous malignancy which, based on tumor histology, is classified into two main types; (I) non-small-cell, and (II) small cell, lung cancer (NSCLC and SCLC, respectively). NSCLC is the most common type with approximately 85% of all cases that contribute to metastatic lung cancers (2). Common NSCLC subtypes include squamous cell carcinoma, large cell carcinoma, and adenocarcinoma. Identifying the biological pathways and genetic abnormalities involved in lung cancer is essential for effective treatment (3). Hypoxia is a critical situation that influences the activity of important carcinogenic pathways. Hypoxia, tumor invasion, and metastasis contribute to the generation of TGF-β1, induced by the epithelial-mesenchymal transition (EMT) that is developed by losing cell-to-cell contact and transforming to mesenchymal properties. TGF-β1 is a multifunctional cytokine that induces its biological effects through both canonical and non-canonical pathways (4, 5).

Growing evidence suggests that the Smad-independent, non-canonical pathway, mediates p38 mitogen-activated protein kinase (P38

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Received: 14 May, 2021; Accepted: 23 May, 2021

[DOI: 10.52547/rbmb.10.2.327]

MAPK) and the phosphatidylinositol 3-kinase/Akt/mammalian target of rapamycin (PI3/AKT/mTOR) signaling pathway, and is thought to promote cancer progression through EMT involved in migration, invasion, and metastasis (5-7). İn this process, the hypoxia-induced EMT is involved so that TGF-β produces reactive oxygen species (ROS) mediated by *NOX4* upregulation (5). ROS play an important role in the early stage of hypoxia-induced EMT in various cancers, including lung cancer (8, 9). ROS contribute to cancer development by modulating transcription and signal transduction pathways, and increasing replication errors and genomic instabilities (10). NADPH oxidase (NOX) complexes are major sources of endogenous non-mitochondrial ROS production in normal and cancerous cells. *NOX4*, a member of the NOX family, is highly expressed and constitutively activated in airway and alveolar epithelial cells (11). *NOX4* is activated in response to pulmonary disorders including ischemia and hypoxia (12) and, in relation to TGF-β, produces ROS that are regulated via various transcription factors (TFs) including E2F, *Nrf2*, hypoxia-inducible factor 1-alpha (HIF-1a), NF-κB, and STAT3 through a mechanism that involves binding to the *NOX4* DNA promoter site (8, 13). One of these TFs is a basic leucine zipper called nuclear factor erythroid 2-related factor 2 (*Nrf2*), which, under special conditions, such as hyperoxia and oxidative stress, binds to antioxidant response elements on the *NOX4* promoter and regulates its expression (14).

Recent evidence suggested that *Nrf2* can up-regulate SOD and GST, which can deplete ROS and lipid peroxidation (15). On the other hand, PI3K/AKT mediated *Nrf2* up-regulation produces redox conditions, promoting cancer cell migration and survival (16). For example, using various NSCLC cell lines, Wu et al. reported that *NOX4*-derived H_2O_2 upregulates *Nrf2* activity and expression (17). Here, we aimed to analyze lung tissue for *Nrf2* and *NOX4* mRNA and BALF for *Nrf2* and *NOX4* proteins and their possible associations in patients who underwent lung bronchoscopies.

Materials and Methods

Study subjects, tissue, and lavage sampling

Patients with suspected cases of lung carcinoma who attended the five lung and respiratory disease offices in Tabriz, Iran, between Sept 2016 and Feb 2018 were subjected to diagnostic tests.

Patients with tuberculosis or hemoptysis, those with prior radiotherapy or chemotherapy, and those unwilling to participate were excluded from the study. Patient medical histories were reviewed and interpreted independently by both a pulmonary pathologist and a lung disease specialist. Those with NSCLC who agreed to participate were included in the study and their disease stages were determined.

Forty-five subjects underwent bronchoscopic needle biopsy and histopathological diagnosis at Imam Reza and Shahid Madani hospitals following previously described methods (18). The collected tissue samples were suspended immediately in RNAlater (Qiagen, Cat. No. 76106), and the BALF specimens were collected in 50 ml sterilized tubes and stored at -80 °C. The study protocol was approved by the Ethics Committee of the Tabriz University of Medical Sciences (IR.TBZMED.REC.1396.371) and all specimens were obtained from contributors with informed and signed consent.

RNA extraction and real-time PCR

Total RNA was extracted from tissue samples using the RNeasy Mini Kit (Qiagen, Cat. No. 74104) according to the manufacturer's instructions, and its quantity and quality were assessed on a Thermo Scientific™ NanoDrop™ One Spectrophotometer and by 1.5% agarose gel electrophoresis, respectively. Also, for cDNA synthesis a Thermo Scientific™ K1691 kit was used following the manufacturer's instruction. The specific primers (Table 1) for target genes *NOX4* and *Nrf2* were designed using Primer3, Gene Runner version 6.5.52, and NCBI-BLAST programs, and for internal control,

[DOI: 10.52547/rbmb.10.2.327]

the *GAPDH* gene primer sequences were obtained from a previous report (19-21). The real-time PCRs were performed at least in triplicate using a 48 well Step One™ Real-Time PCR System and RealQ Plus Master Mix Green kit (Ampliqon A/S, Denmark) with the following conditions: 94 °C for 15 min, 40 amplification cycles consisting of 94 °C for 15 sec, annealing temperature (Table 1) for 30 sec, and 72 °C for 35 sec. Melting curves were then determined with temperatures ranging from 60 to 95 °C. SYBR Green reagents were used for all real-time PCRs. Genes expression was analyzed based on the cycle threshold (Ct) and relative expression levels were determined as $2^{-[\Delta\Delta C(t)]}$. Each gene's expression was normalized to *GAPDH*.

Table 1. Primer sequences and Real-Time PCR conditions.

Gene	Primers	Band	Annealing temperature (°C)
NOX4	F: TTTAGATACCCACCCTCCCG R: AGCTTGGAATCTGGGCTCTT	181 bp	64
Nrf2	F: TCTCCACAGAAGACCCCAAC R: TGCTTTCAGGGTGGTTTTGG	234 bp	64
GAPDH	F: GACCCCTTCATTGACCTCAACTAC R: TCGCTCCTGGAAGATGGTGATGG	138 bp	62

Western-blot analysis

For immunoblotting, the BALF samples were lysed using the lysing buffer and the lysates were centrifuged at 13300 g. The total protein concentration in the supernatants was measured by Bradford assay. The proteins were separated by SDS-PAGE and transferred to PVDF membranes. The membranes were blocked with 2% low-fat milk in Tris-buffered saline and then incubated for 16-18 h with commercially available β-Actin (C4: sc-47778), *NOX4* (3H2G11: sc-517188), and *Nrf2* (A-10: sc-365949) primary antibodies. The membrane was washed in washing buffer, incubated with the appropriate HRP-conjugated secondary antibody (m-IgGκ BP-HRP: sc-516102), and bands were visualized by enhanced chemiluminescence. Densitometry was performed using NIH Image/Image J software. All antibodies were purchased from Santa Cruz Biotechnology Inc.

Statistics analysis

Kolmogorov-Smirnov's with kurtosis and skewness indices tests were applied to assess normality. Unpaired t-test was performed to compare expression of the target genes between the case and control groups. Results were expressed as means±SDs, and a two-tailed $p< 0.05$ indicated statistical significance. Additionally, the Pearson product-moment correlation test was used to test the correlation between the target genes' expression. Also, a partial correlation test was used to analyze the association of *NOX4* and *Nrf2* expression controlling for age, sex, and NSCLC types and stages. All analyses were performed using SPSS software, version 16.

Results

The participants' general characteristics are shown in Table 2a. Of the 45 participants, 25 were diagnosed as NSCLC cases including 8 adenocarcinoma, 10 squamous, and 7 large-cell cancerous patients, while 20 were diagnosed as noncancerous, and used as the control group (Table 2a). The frequency of each stage in the NSCLC-diagnosed patients is shown in Table 2b.

The quality and quantity of extracted RNA were confirmed by the A260/280 (1.8-2) and 230/260 (1.6-1.8) ratios, and the identification of sharp bands of the ribosomal RNAs on an agarose gel. *NOX4* expression was significantly greater in NSCLC patient tissue samples than in controls (p= 0.03) (Fig. 1A). Although the *Nrf2* expression was greater in NSCLC patients than in controls, this difference was not significant (p= 0.163) (Fig 1a).

[DOI: 10.52547/rbmb.10.2.327]

Table 2. General characteristic of study subjects (a) and frequency of cancer types and stages (b).

a				
Characteristic	**Case**		**Control**	
Age (Mean±SD)	58.76 (16.43)		58.05 (14.71)	
Sex	**number (%)**			
Male	18 (72%)			
Cancer type				
Adenocarcinoma	8 (32%)			
Squamous	10 (40%)			
Large Cell	7 (28%)			
Cancer stage n(%)				
I	1 (4%)			
II	9 (36%)			
III	8 (32%)			
IV	7 (28%)			
b				
Cancer type	**Stages**			
	I	**II**	**III**	**IV**
Adenocarcinoma	0	3	4	1
Squamous cells	0	4	3	3
Large cells	1	2	1	3

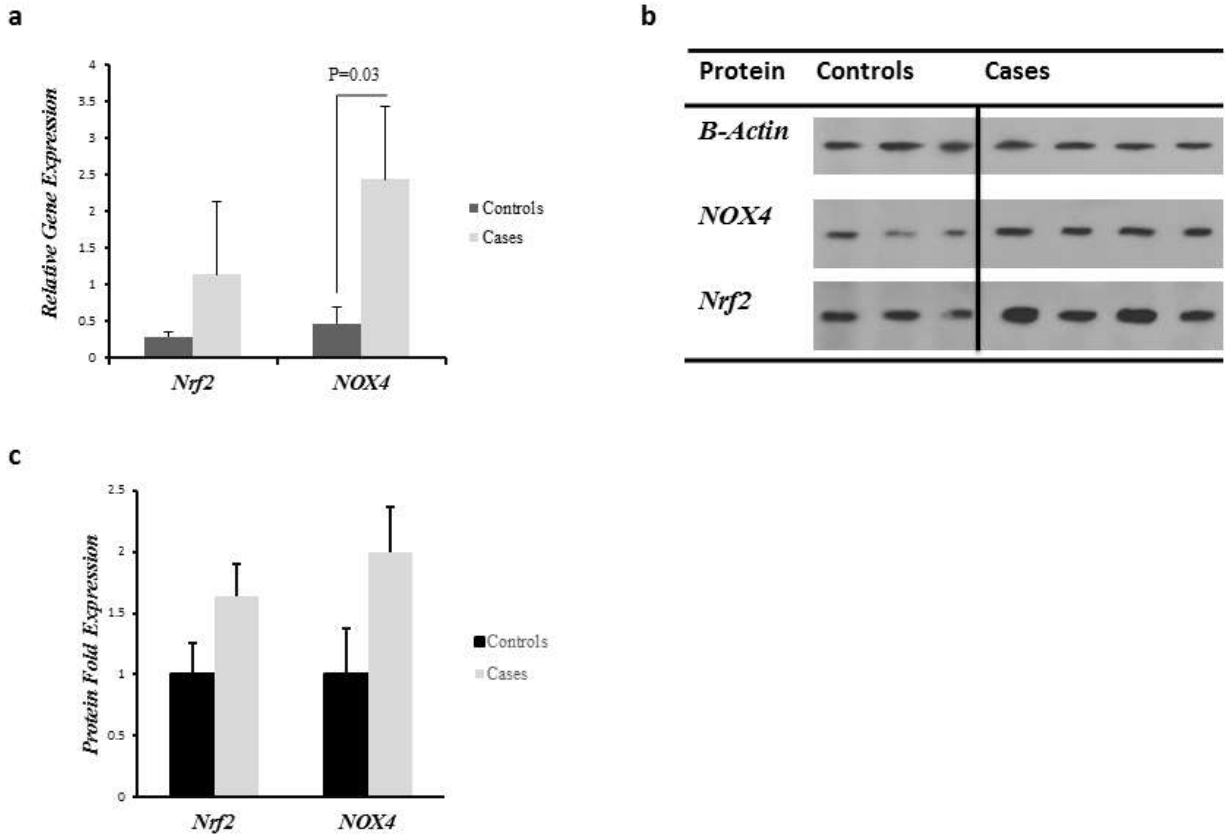

Fig. 1. *Nrf2* and *NOX4* expression in tissue samples and abundance of their associated proteins in the BALFs by RT-PCR and western blotting. (a) *Nrf2* and *NOX4* expression in the NSCLC patient tissue samples was greater than in controls, however, the increase was statistically significant only for *NOX4* (p= 0.03). (b) The protein levels, by western blotting. (c) Densitometry revealed greater abundances of both *Nrf2* and *NOX4* protein in patient BALFs than in controls, however as with the mRNAs, the difference was significant only for *NOX4* (p= 0.01).

[DOI: 10.52547/rbmb.10.2.327]

Western blot and densitometry showed that the *NOX4* protein level was significantly greater in the in the NSCLC patient BALF specimens than in controls (p= 0.01) (Figs. 1b and 1c). Although the *Nrf2* protein content was greater in NSCLC patients than in controls, the difference was not statistically significant.

The Pearson product-moment correlation test without adjusting for covariates showed a significant positive correlation between *Nrf2* and *NOX4* expression (r= 0.944, p< 0.001) in NSCLC tissue samples. A partial correlation test adjusted for age, sex, and NSCLC types and stages also showed a significant positive correlation between *Nrf2* and *NOX4* expression (r= 0.927, p= 0.001), and also when not adjusted for those factors (r= 0.944, p< 0.001).

Discussion

In this study, we found significantly greater *NOX4* expression and non-significantly greater *Nrf2* expression in NSCLC patient lung tissue than in that of controls. Moreover, similar results were observed for their respective proteins in the BALFs. In addition, we found a significant correlation between *Nrf2* and *NOX4* expression in the NSCLC patient lung tissues.

In response to hypoxia and inflammatory conditions in various cancer cells, including NSCLC, reactive oxygen and nitrogen species (RONS) are induced by the main ROS producer, *NOX4*, and nitric oxide synthases (22). RONS have detrimental effects on proteins, DNA, and organelles. Indeed, *NOX4*-induced oxidative stress promotes hypoxia-induced EMT through the driving force of TGFβ1 (22). Based on the physiological condition, concomitant with the accumulation of RONS, the enzymatic and non-enzymatic endogenous antioxidant systems are initiated to maintain cellular redox homeostasis. Recent evidence suggests that the redox-sensitive residues in regulatory proteins, including cysteine oxidation/S-sulfenylation/S-glutathionylation/S-nitrosylation and tyrosine nitration, activate intracellular signaling pathways and transcription factors that promote cell proliferation, migration, and survival (22). These oxidative/nitrosative modifications activate some intracellular transcription effectors, including *Nrf2* as a master of the antioxidant response regulator in PI3K/Akt-mediated signaling pathways (22). Recent studies indicate that *Nrf2* exhibits a dual role in cancer progression (23). During the early stage of cancer, under oxidative stress, *Nrf2* dissociates from Keap1, translocates into the nucleus, and binds to the antioxidant response element (ARE), which promotes downstream antioxidant protein expression. *Nrf2* overexpression also substantially reduces tumor cell sensitivity to chemotherapeutic agents (22).

Silva et al. reported that unbalanced levels of *Nrf2*/Keap1, in which *Nrf2* is increased, act as a biomarker for cisplatin resistance in LC cell lines (24). An *in vivo* study demonstrated that oncogenic KRAS activates the NOX complex, and subsequent downstream *Nrf2*-dependent antioxidant mechanisms, supporting tumor progression (25). Wu et al. found that *Nrf2* confers apoptosis resistance in *NOX4*-overexpressed NSCLC cells, and the enhancement effect of NOX4 on cell growth is reversible by Nrf2 inhibition (17).

As discussed above, oxidative/nitrosative modifications are a major factor in cancer initiation and progression. İn this line, *NOX4* and *Nrf2* play key roles in oxidative stress regulation and the EMT process via various signaling pathways. Based on our findings, increased *NOX4* and *Nrf2* mRNA and protein, and also their significant positive correlation, indicated that they are significant factors in lung cancer proliferation and progression.

Some limitations should be addressed regarding the present study. Because of the difficulty of sampling via biopsy from lung and BALF, we had limited tissue and BALF specimens. for that reason, we were unable to measure some of the agents implicated in *NOX4* and *Nrf2* function, for exampleoxidative stress and TGF-β1.

[DOI: 10.52547/rbmb.10.2.327]

Moreover, our subject number was relatively small.

In conclusion, we found upregulated *NOX4* and *Nrf2* mRNA and protein in lung tissue and BALF of NSCLC patients relative to controls. İn addition, a strongly positive association was observed between *NOX4* and *Nrf2*. Considering the implication of *NOX4* and *Nrf2* in key carcinogenic signaling pathways, our findings suggest that these biomarkers may be candidates as NSCLC diagnostic and treatment targets. Moreover, studying the molecular aspects of gene-gene interactions regarding *Nrf2* and *NOX4* could provide valuable information on the molecular regulation of the oxidative pathways in lung cancer pathobiology.

Acknowledgements

The help of staffs in the Bronchoscopy Room in Imam Reza Hospital, Tabriz, Iran is greatly appreciated. This study was funded by the Tuberculosis and Lung Diseases Research Center, Tabriz University of Medical Sciences, Tabriz, Iran (Grant No: 57908).

All authors declare they have no conflicts of interest.

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[DOI: 10.52547/rbmb.10.2.327]

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[DOI: 10.52547/rbmb.10.2.327]