

NAD(P)H:quinone oxidoreductase-1 overexpression predicts poor prognosis in small cell lung cancer

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Received June 4, 2014; Accepted August 6, 2014

DOI: 10.3892/or.2014.3494

Abstract. NAD(P)H:quinone oxidoreductase-1 (NQO1) is commonly elevated in various types of human cancers, including pancreatic, breast and thyroid cancer, as well as others. However, little is known concerning the status of NQO1 in small cell lung cancer (SCLC). To investigate the clinicopathological significance of NQO1 expression and evaluate its role as a potential prognostic marker in SCLC, protein and mRNA expression levels of NQO1 were determined in four fresh tissue samples of SCLC and paired adjacent non-cancerous tissues using western blotting and real-time qRT-PCR, respectively, and 115 cases of SCLC with strict follow-up were selected for immunohistochemical (IHC) staining of NQO1 protein. The correlation between NQO1 expression and the clinicopathological features of SCLC was evaluated using the Chi-square (χ^2) and Fisher's exact tests, survival rates were calculated using the Kaplan-Meier method, and univariate and multivariate analyses were performed using the Cox proportional hazards regression model. In regards to the results, levels of NQO1 protein and mRNA were significantly elevated in the four fresh tissue samples of SCLC compared with levels in the paired adjacent non-cancerous tissues, respectively. IHC analysis showed that the rate of strong positive NQO1 protein expression was significantly higher (48.70%) in SCLC when compared with the rate in either adjacent non-cancerous or normal lung tissues (both $P < 0.001$). The rate of strong positive NQO1 protein expression was correlated with large tumor size ($P = 0.019$), late pathologic stage ($P = 0.001$) and the presence of lymph node metastasis ($P = 0.001$). Moreover, high-level expression of NQO1 protein was significantly correlated with

lower disease-free survival ($P = 0.001$) and 5-year survival rates ($P < 0.001$) in SCLC patients, particularly early-stage patients ($P = 0.045$ and $P = 0.033$, respectively). Further Cox analysis revealed that NQO1 expression emerged as a significantly independent hazard factor for the 5-year survival rate of patients with SCLC ($P = 0.042$). In conclusion, NQO1 plays an important role in the progression of SCLC, and it may potentially be used as a biomarker and therapeutic target of SCLC.

Introduction

Small cell lung cancer (SCLC), which represents 12.95% of all lung cancer diagnoses and continues to be a major clinical issue worldwide (1), is an aggressive neuroendocrine subtype of lung cancer for which there is no effective treatment (2). Patients with limited disease have a median survival time of 16-24 months when treated with chemotherapy and concurrent thoracic radiation (3). The mechanism of carcinogenesis, similar to other cancers, is still not fully understood. Therefore, there is an urgent need for a better understanding of SCLC pathogenesis that may lead to more effective treatment strategies.

NAD(P)H:quinone oxidoreductase-1 (NQO1), which was discovered by Professor Ernster in 1958 (4) and originally called DT-diaphorase (5), is located on chromosome 16q22 (6). Several functions of NQO1 have been proposed, including xenobiotic detoxification, superoxide scavenging, maintenance of endogenous antioxidants, modulation of p53 and proteasomal degradation (7). It is conceivable that NQO1 functions primarily to protect normal cells from oxidant stress and electrophilic attack. These functions have also led to the suggestion that NQO1 plays an important role in cancer chemoprevention. On the other hand, NQO1 is expressed at high levels in most human solid tumors including those of the colon, breast, pancreas, ovaries and thyroid (8-10), and has also been detected following the induction of cell cycle progression and proliferation of melanoma cells (11). However, to date, the expression status of NQO1 in SCLC and its relationship with clinicopathological features/prognosis is unknown.

The critical role of NQO1 in numerous cancers compelled us to study the function of NQO1 in SCLC. Therefore, we performed western blotting, real-time RT-PCR (qRT-PCR) and immunohistochemical (IHC) staining of NQO1 in SCLC and normal lung tissues and found that NQO1 may be an independent prognostic biomarker for SCLC.

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Key words: NAD(P)H:quinone oxidoreductase-1, small cell lung cancer, prognosis, survival analysis

Materials and methods

Ethics statement. The present study complied with the Helsinki Declaration and was approved by the Human Ethics and Research Ethics Committees of Yanbian University Medical College in China. Through surgery consent form, patients were informed that the resected specimens would be stored by the hospital and would potentially be used for scientific research, and that their privacy would be maintained. Follow-up survival data were collected retrospectively through analysis of medical records.

Clinical samples. Fresh samples from four cases of SCLC were paired with adjacent non-cancerous tissues, and 115 cases of routinely processed and paraffin-embedded SCLC specimens with strict follow-up for survival status were selected randomly from patients who underwent surgery between 2003 and 2011 at Jilin University Hospital and the Tumor Tissue Bank of Yanbian University Medical College. The pathological parameters, including age, gender, smoking status, tumor size, location subtype, clinical stage, lymph node metastasis, neuron-specific enolase (NSE) level, disease-free and 5-year survival data, were carefully reviewed for all the above cases. The patient ages ranged from 26-76 years with a mean age of 49.6 years. The male to female ratio was 69:46. Tumors were staged according to the 7th edition of the American Joint Committee on Cancer. Of the 115 SCLCs, 70 were early-stage (I-II), and 45 cases were late-stage (III). Forty-four cases were localized in the central bronchus, and 71 cases in the peripheral bronchus. There were no patients with distant metastasis prior to surgery. By March 2013, 99 patients had died while 16 patients remained alive. The median survival time was 27.2 months.

Western blotting. As previously described (12), fresh tissue samples of SCLC were ground into powder in liquid nitrogen and lysed in SDS-PAGE sample buffer. Equal amounts of protein samples (20 μ g) were separated on 12% SDS-polyacrylamide gels and transferred to PVDF membranes. Membranes were blocked with 5% fat-free milk in Tris-buffered saline containing 0.1% Tween-20 for 1 h at room temperature (RT). Membranes were incubated with the NQO1 antibody (1:5,000; Santa Cruz Biotechnology, Santa Cruz, CA, USA) overnight at 4°C and then with horseradish peroxidase-conjugated rabbit anti-mouse IgG. NQO1 expression was detected using the ECL Prime Western Blotting detection reagent (Amersham) according to the manufacturer's instructions. β -actin (Sigma, St. Louis, MO, USA) was used as a loading control. Protein bands were quantified using the LANE 1D system (Sage, China).

RNA extraction and qRT-PCR. Total RNA from fresh tissues was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). First-strand cDNA was synthesized using PrimeScript reverse transcriptase (Takara Biotechnology, Dalian, China) and oligo(dT), following the manufacturer's instructions. To examine expression, qPCR was performed with a Bio-Rad sequence detection system according to the manufacturer's instructions using a double-stranded DNA-specific SYBR Premix Ex Taq™ II kit (Takara Biotechnology). Double-

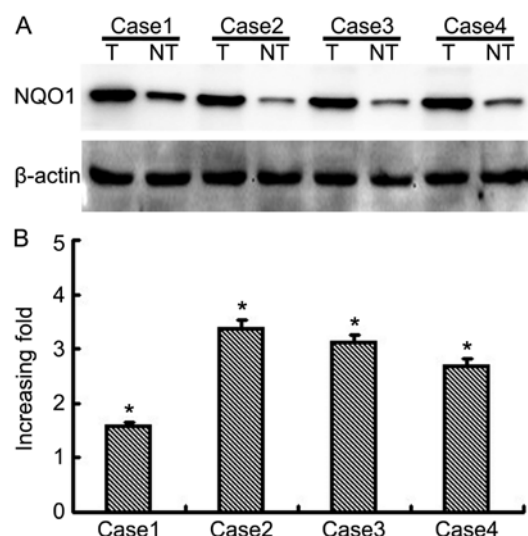


Figure 1. Western blot analysis of the NQO1 protein. (A) Images of western blot analyses of NQO1 protein expression in four matched pairs of SCLC (T) and adjacent non-tumor tissues (NT). (B) Relative T/NT ratios of NQO1 protein expression level in paired SCLC and adjacent non-tumor tissues (increased fold-change, * $P < 0.05$). NQO1, NAD(P)H:quinone oxidoreductase-1; SCLC, small cell lung cancer.

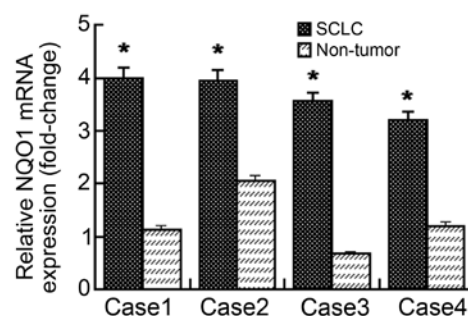


Figure 2. qRT-PCR analysis of NQO1 mRNA. SCLC and adjacent non-tumor tissues (non-tumor) were collected and subjected to qRT-PCR analysis of NQO1 mRNA levels. Data represent the mean of individual samples tested in triplicate relative to that of the normal control \pm SD (* $P < 0.05$). NQO1, NAD(P)H:quinone oxidoreductase-1; SCLC, small cell lung cancer.

stranded DNA-specific expression was tested using the comparative Ct method using $2^{-\Delta\Delta C_t}$ (13). NQO1 primers were: 5'-GGCAGAAGAGCACTGATCGTA-3' and 5'-TGATGGGATTGAAGTTCATGGC-3'. Primers for GAPDH, which was used as an internal control, were: 5'-GGTCTCCTCTGACTTCAACA-3' and 5'-ATACCAGGAAATGAGCTTGA-3'. All assays were performed in triplicate and repeated at least three times.

IHC analysis. IHC analysis was performed using the Dako LSAB kit (Dako A/S, Glostrup, Denmark). Briefly, to eliminate endogenous peroxidase activity, 4- μ m tissue sections were deparaffinized, rehydrated and incubated with 3% H_2O_2 in methanol for 15 min at RT. Antigen retrieval was performed at 95°C for 20 min by placing the slides in 0.01 M sodium citrate buffer (pH 6.0). The slides were then incubated with the NQO1 antibody (1:500; Santa Cruz) at 4°C overnight. After incubation with the biotinylated secondary antibody at RT for 30 min, the slides were incubated with streptavidin-peroxidase

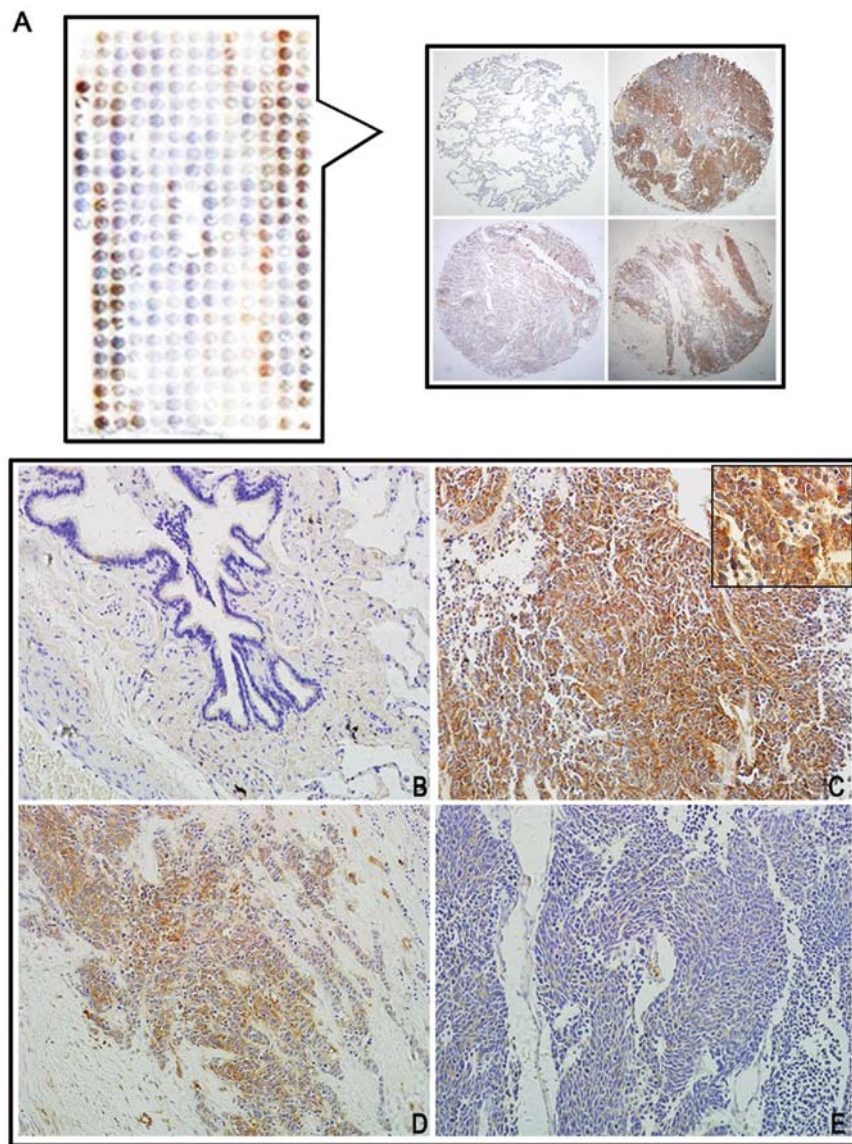


Figure 3. NQO1 protein expression in SCLC using IHC. (A) NQO1 protein expression in an SCLC tissue microarray. (B) NQO1 protein staining was negative in adjacent normal lung tissues. (C) NQO1 protein showed diffusely and strongly cytoplasmic (insert) positive staining in late-stage SCLC. (D) NQO1 protein staining was weakly positive in SCLC without metastasis. (E) NQO1 protein staining was negative in SCLC without metastasis. NQO1, NAD(P)H:quinone oxidoreductase-1; SCLC, small cell lung cancer; IHC, immunohistochemistry.

complex at RT for 30 min. Immunostaining was developed using 3,3'-diaminobenzidine, and Mayer's hematoxylin was used for counterstaining (14). We used mouse IgG as an isotope controls. In addition, positive tissue sections were processed while omitting the primary antibody (mouse anti-NQO1) as negative controls.

Two pathologists (T. Jin and Z. Li), blinded to all clinical data, independently scored all slides. Interpretation criteria were as previously described (13). Both cytoplasmic and nuclear NQO1 expression patterns were considered positive staining, and ++ or +++ scored samples were considered strongly positive. For survival data analysis, ++ or +++ scored samples were considered high and - or + scored samples were considered low NQO1 expression.

Statistical analysis. Statistical analyses were performed using SPSS 17.0. Correlation between NQO1 expression and

clinicopathological characteristics was evaluated using the χ^2 and Fisher's exact tests. Disease-free and 5-year survival rates after tumor removal were calculated by the Kaplan-Meier method, and difference in survival curves was analyzed by the log-rank test. Multivariate analysis was performed using the Cox proportional hazards regression model on all significant characteristics determined using univariate analysis. A P-value of <0.05 was considered to indicate a statistically significant result.

Results

NQO1 protein and mRNA expression in SCLC. Expression levels of NQO1 protein and mRNA were determined in four SCLC samples with matched adjacent non-cancerous fresh tissues. Western blotting data showed that the NQO1 protein was highly expressed in SCLC tissues compared with the

Table I. NQO1 protein expression in the SCLC cases.

| Diagnosis | No. of cases | NQO1 protein expression | | | | Positive rate (%) | Strongly positive rate (%) |
|---------------------|--------------|-------------------------|----|----|-----|--------------------|----------------------------|
| | | - | + | ++ | +++ | | |
| SCLC | 115 | 34 | 25 | 23 | 33 | 70.43 ^a | 48.70 ^a |
| Adjacent non-tumor | 115 | 85 | 18 | 7 | 5 | 26.09 | 10.43 |
| Normal lung tissues | 14 | 11 | 2 | 1 | 0 | 21.43 | 7.14 |

Statistical analyses were performed using Pearson's Chi-Square test. ^aP<0.01 compared with either adjacent non-tumor or normal lung tissues. NQO1, NAD(P)H:quinone oxidoreductase-1; SCLC, small cell lung cancer.

level in the matched adjacent non-cancerous tissues (Fig. 1). qRT-PCR data also confirmed the increased level of NQO1 mRNA expression in the SCLC samples when compared with the level in the adjacent non-cancerous tissues (Fig. 2).

NQO1 protein expression showed a mainly cytoplasmic staining pattern in SCLC using IHC. The positive and strongly positive rates of NQO1 protein expression were 70.43% and 48.70%, respectively, which was significantly higher than the rates in either adjacent non-cancerous or normal lung tissues (P<0.001, respectively) (Fig. 3, Table I).

Correlation between NQO1 expression and clinicopathological features of SCLC. The correlation between NQO1 protein expression and the major clinicopathological features of SCLC was analyzed. The results showed that the NQO1 high-expression rate was significantly correlated with large tumor size (P=0.019), late pathologic stage (P=0.001) and lymph node metastasis (P=0.001). However, NQO1 expression levels did not correlate with the pathological parameters of SCLC cases including age, gender, smoking status, location subtype or NSE level (P>0.05, respectively) (Table II).

NQO1 high-level expression predicts poor survival rates in patients with SCLC using the Kaplan-Meier method. To further confirm the role of NQO1 expression in SCLC progression, we analyzed disease-free survival and 5-year survival rates for 115 SCLC cases using the Kaplan-Meier method, and found that SCLC patients with NQO1 high-level expression had lower disease-free (log-rank=11.938, P=0.001) and 5-year survival rates (log-rank=12.899, P<0.001) than those with NQO1 low-level expression (Fig. 4).

To further substantiate the importance of NQO1 expression in SCLC progression, we analyzed the correlation between NQO1 high-level expression rate and clinical stages of SCLC. In early-stage SCLC, patients with NQO1 high-level expression had lower disease-free and 5-year survival rates compared with those with NQO1 low-level expression (P=0.045 and P=0.033, respectively) (Fig. 5A and B). However, in late-stage SCLC patients, disease-free and 5-year survival rates were not correlated with the NQO1 expression status (P=0.085 and P=0.104, respectively) (Fig. 5C and D).

NQO1 is an independent prognostic factor in SCLC using the Cox proportional hazards regression model. Upon univariate analysis, patients whose SCLC tumors with NQO1

Table II. Correlation between NQO1 expression and clinicopathological features of the SCLC cases.

| Variables | NQO1-positive cases (%) | | χ^2 | P-value |
|-----------------------|-------------------------|------------|----------|--------------------|
| | +++ / ++ | + / - | | |
| Age | | | | |
| ≤50 | 27 (47.37) | 30 (52.63) | 0.080 | 0.778 |
| >50 | 29 (50.00) | 29 (50.00) | | |
| Gender | | | | |
| Male | 35 (50.72) | 34 (49.28) | 0.284 | 0.594 |
| Female | 21 (45.65) | 25 (54.35) | | |
| Smoking status | | | | |
| Yes | 33 (45.83) | 39 (54.17) | 0.631 | 0.427 |
| No | 23 (53.49) | 20 (46.51) | | |
| Tumor size | | | | |
| ≤3 cm | 30 (40.54) | 44 (59.46) | 5.525 | 0.019 ^a |
| >3 cm | 26 (63.41) | 15 (36.59) | | |
| Location subtype | | | | |
| Central | 23 (52.27) | 21 (47.73) | 0.365 | 0.546 |
| Peripheral | 33 (46.48) | 38 (53.52) | | |
| Clinical stage | | | | |
| I-II | 25 (35.71) | 45 (64.29) | 12.066 | 0.001 ^b |
| III | 31 (68.89) | 14 (31.11) | | |
| Lymph node metastasis | | | | |
| Positive | 37 (63.79) | 21 (36.21) | 10.676 | 0.001 ^b |
| Negative | 19 (33.33) | 38 (66.67) | | |
| NSE level | | | | |
| ≥16.3 ng/ml | 37 (53.62) | 32 (46.38) | 1.677 | 0.195 |
| <16.3 ng/ml | 19 (41.30) | 27 (58.70) | | |

Statistical analyses were performed using Pearson's Chi-square test. ^aP<0.05, ^bP<0.01. NQO1, NAD(P)H:quinone oxidoreductase-1; SCLC, small cell lung cancer; NSE, neuron-specific enolase.

high-level expression had a significantly lower 5-year survival rate (P=0.001) than those with NQO1 low-level expression

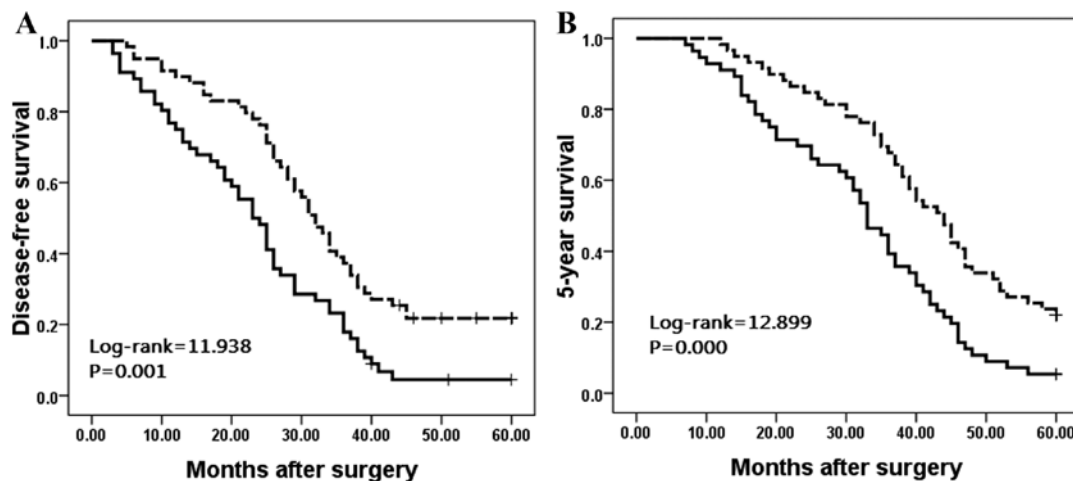


Figure 4. Kaplan-Meier analysis of SCLC patient survival rates in relation to NQO1 protein expression. Disease-free survival (A) and overall (5-year) survival rates (B) of patients with elevated (solid, n=56) and low (dashed, n=59) NQO1 expression. SCLC, small cell lung cancer; NQO1, NAD(P)H:quinone oxidoreductase-1.

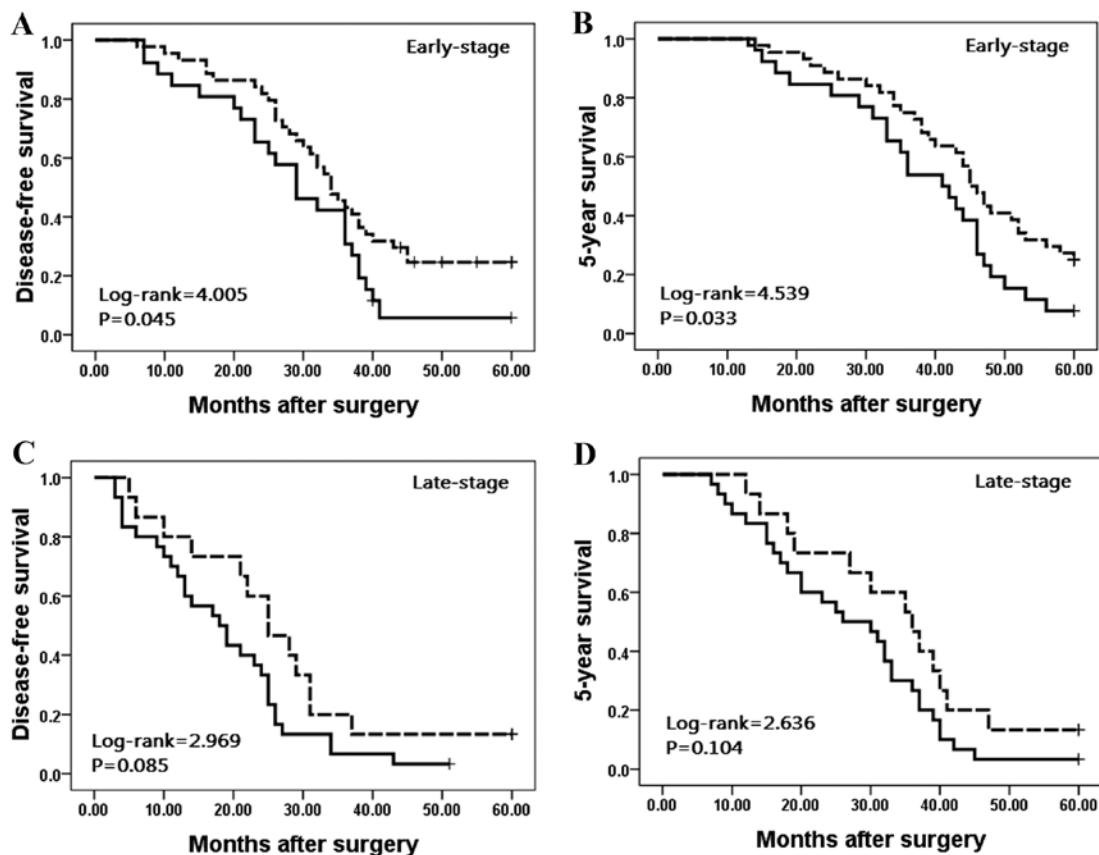


Figure 5. Kaplan-Meier analysis of the survival rates in patients with high- or low-level NQO1 expression with early- or late-stage SCLC. Disease-free survival (A) and overall (5-year) survival rates (B) were assessed in patients with early-stage SCLC concomitant with either high- (solid, n=25) or low-level (dashed, n=45) NQO1 expression. (C) Disease-free survival and (D) overall (5-year) survival rates were also assessed in patients with late-stage SCLC concomitant with high- (solid, n=31) or low-level (dashed, n=14) NQO1 expression. NQO1, NAD(P)H:quinone oxidoreductase-1; SCLC, small cell lung cancer.

tumors. Additionally, patient age ($P=0.025$), tumor location subtype ($P=0.014$), pathologic stage ($P<0.001$) and lymph node metastasis ($P=0.044$) were also associated with 5-year survival rates. Therefore, multivariate survival analysis was then performed using the Cox proportional hazards regression model for all significant variables in the univariate survival

analysis. We found that clinical stage (HR, 1.948; 95% CI, 1.292-2.938, $P=0.001$) proved to be an independent prognostic factor for 5-year survival rates in SCLC. More importantly, NQO1 expression also emerged as a significant independent prognostic factor in the prognosis of SCLC (HR, 1.577; 95% CI, 1.017-2.446, $P=0.042$) (Table III).

Table III. Univariate and multivariate analysis of clinicopathological factors for the 5-year survival rate of 115 patients with SCLC.

| Characteristics | B | SE | Wald | HR | 95% CI | | P-value |
|-----------------------|-------|-------|--------|-------|--------|-------|--------------------|
| | | | | | Lower | Upper | |
| Univariate analysis | | | | | | | |
| Age | 0.425 | 0.190 | 5.017 | 1.530 | 1.055 | 2.220 | 0.025 ^a |
| Gender | 0.078 | 0.192 | 0.167 | 1.081 | 0.743 | 1.574 | 0.683 |
| Smoking status | 0.083 | 0.188 | 0.196 | 1.087 | 0.752 | 1.571 | 0.658 |
| Tumor size | 0.354 | 0.194 | 3.349 | 1.425 | 0.975 | 2.082 | 0.067 |
| Location | 0.477 | 0.195 | 5.978 | 1.611 | 1.099 | 2.360 | 0.014 ^a |
| Stage | 0.810 | 0.197 | 16.917 | 2.248 | 1.528 | 3.308 | 0.000 ^b |
| LN metastasis | 0.383 | 0.190 | 4.061 | 1.467 | 1.011 | 2.130 | 0.044 ^a |
| NSE level | 0.215 | 0.192 | 1.250 | 1.240 | 0.851 | 1.806 | 0.264 |
| NQO1 | 0.643 | 0.192 | 11.229 | 1.902 | 1.306 | 2.771 | 0.001 ^b |
| Multivariate analysis | | | | | | | |
| Age | 0.327 | 0.199 | 2.703 | 1.387 | 0.939 | 2.048 | 0.100 |
| Location | 0.381 | 0.212 | 3.217 | 1.463 | 0.965 | 2.218 | 0.073 |
| Stage | 0.667 | 0.210 | 10.119 | 1.948 | 1.292 | 2.938 | 0.001 ^b |
| LN metastasis | 0.268 | 0.220 | 1.490 | 1.307 | 0.850 | 2.011 | 0.222 |
| NQO1 | 0.455 | 0.224 | 4.134 | 1.577 | 1.017 | 2.446 | 0.042 ^a |

Statistical analyses were performed using the Cox proportional hazards regression model. ^aP<0.05, ^bP<0.01. SCLC, small cell lung cancer; NSE, neuron-specific enolase; NQO1, NAD(P)H:quinone oxidoreductase-1.

Discussion

NQO1 is a mainly cytosolic enzyme that uses NADH or NADPH as substrates to catalyze the two-electron reduction of quinines to their hydroquinone forms (15), thus bypassing the toxic semiquinone intermediates, and these resultant hydroquinones are thus ready for further conjugation and excretion (16). In addition, the enzyme is present as a ubiquitous flavoprotein that is widely expressed in body tissues (17) and localized primarily in the cytosol, although it has been detected in the nucleus at lower levels (18). NQO1 is induced along with a battery of defense genes that provide protection against different stresses as well as carcinogen-induced organ tumorigenesis.

The activity of the NQO1 enzyme can be influenced by a major polymorphism involving a single C to T substitution at nucleotide 609 in exon 6 of the NQO1 gene that causes a Pro187Ser amino acid change in the protein (19). Some studies have shown that the polymorphism in the NQO1 gene affects the translation of the NQO1 protein. The NQO1 C609T polymorphism has been associated with an increased risk of various cancers such as renal (20), lung (21), esophageal (22), colorectal (23), and head and neck cancer (24). Therefore, since there is an increased incidence of disease and xenobiotic-induced toxicity in individuals carrying a polymorphism in NQO1, it has been suggested that it plays a role in chemoprotection.

Recently, however, it was reported that NQO1 protein and mRNA were abnormally elevated in many solid tumors. Mikami *et al* (25) demonstrated a close correlation between

protein expression and enzyme activity of NQO1 in colon cell lines and in colorectal tumor samples, and tumors with nodal metastases showed significantly higher NQO1 levels than did tumors without metastasis, raising the possibility that NQO1 expression is related to tumorigenesis and malignant progression of colorectal tumors.

Awadallah *et al* (10) and Lyn-Cook *et al* (8) reported that NQO1 is upregulated in pancreatic ductal adenocarcinoma (PDAC), and that combining NQO1 expression with cellular morphology assessment minimized the risk of a false-positive diagnosis. A study by Fagerholm *et al* (26) in breast cancer patients showed that NQO1 may predict the outcome of patients with overabundant expression of the Fanconi anemia group D2 (FANCD2) protein through its potential modification of prognostic DNA repair markers. A study by Li *et al* (27) highlighted that NQO1 was not only a 'biomarker', but also an exploitable and tumor-selective target for PDAC. However, little is known concerning the status of NQO1 in SCLC.

In the present study, qRT-PCR and western blotting data demonstrated that the levels of NQO1 mRNA and protein were significantly higher in SCLC samples compared with adjacent non-tumor tissues. Furthermore, we performed IHC staining and analysis of 115 cases of SCLC, and found that a strongly positive rate of NQO1 protein expression was significantly higher than that observed in either adjacent non-tumor or normal lung tissues, indicating that NQO1 plays a potentially important role in the progression of SCLC. Meanwhile, we analyzed the correlation between NQO1 expression and clinicopathological features of SCLC and found that NQO1 expression was significantly correlated with large tumor size,

late pathologic stage and lymph node metastasis ($P < 0.05$), but not with age, gender, smoking status, location subtype or NSE level ($P > 0.05$).

With regard to survival, we found that SCLC patients with high-level NQO1 expression had lower disease-free survival ($P < 0.01$) and 5-year survival rates ($P < 0.01$) than patients with low-level NQO1 expression. In early-stage SCLC, patients with high-level NQO1 expression had lower disease-free and overall survival rates compared with those with low NQO1 expression ($P < 0.05$). Multivariate survival analysis demonstrated that NQO1 expression emerged as a significant independent hazard factor for overall (5-year) survival in SCLC along with clinical stage. These results demonstrate that NQO1 is a potential effective predictor of poor prognosis, particularly in patients with early-stage SCLC.

Collectively, the present study implicates NQO1 as a putative new attractive biomarker for prognostic evaluation and as a potential molecular target in patients with SCLC. Additionally, Vasiliou *et al* (28) indicated that NQO1 was shown to be upregulated as part of the oxidative stress response and inexplicably overexpressed in particular types of tumors, whose function has not yet been elucidated. Garate *et al* (11) attempted to determine the expression and basic function of NQO1 in melanoma cell proliferation, and found that NQO1 significantly induced cell cycle progression by upregulating the expression of cyclin A2, B1 and D1, leading to the proliferation of melanoma cells *in vitro*. In spite of this, the molecular mechanism of NQO1 affecting tumor progression is still not clearly understood. NQO1 may constitute an undiscovered signaling molecule in cancer cells, and further studies are warranted to explore this possibility.

Acknowledgements

This study was supported by grants from the National Natural Science Funds of China (61371067), and the International Cooperative Project from the Jilin Provincial Science and Technology Department in China (20140414050GH).

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