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Association between ^{18}F-FDG uptake in PET/CT, Nrf2 and NQO1 expression and their prognostic significance in non-small cell lung cancer

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Two pentose phosphate pathway-related proteins, NF-E2-related factor 2 (Nrf2)/NAD(P)H dehydrogenase (Quinone) 1 (NQO1) regulate the expression of glucose metabolism and antioxidant genes. We evaluated the prognostic significance of NRF2, NQO1 and ^{18}F-fluorodeoxyglucose positron emission tomography (^{18}F-FDG PET) parameter and their relationship with non-small cell lung cancer (NSCLC) histology. A total of 241 patients, who underwent surgical resection for NSCLC, were reviewed retrospectively. Preoperative ^{18}F-FDG PET and immunohistochemical results of Nrf2 and NQO1 were evaluated. In squamous cell carcinoma (SQCC), the maximum standardized uptake value (SUVmax) was significantly higher in NQO1-high than in NQO1-low expression (p=0.023). In adenocarcinoma, SUVmax was not correlated with NQO1 expression. Patients with a high NQO1 expression showed poor recurrence-free survival (RFS) and overall survival (OS) than patients with a low NQO1 expression in SQCC (p=0.002 and p=0.014, respectively). NQO1 expression was not associated with clinical outcome in adenocarcinoma. Nrf2 expression was not correlated with prognosis in two types of NSCLC. High SUVmax was associated with poor RFS (p=0.03) but was not related to poor OS (p=0.569) in SQCC. In multivariate analyses, NQO1 expression and SUVmax were not independent prognostic factors in SQCC. However, in multivariate analysis combining NQO1 and SUVmax values, both low SUVmax and low NQO1 was independent prognostic factor for RFS and OS (HR=0.264, p=0.033 and HR=0.338, p=0.045, respectively). In conclusion, both low SUVmax and low NQO1 was an independent prognostic factor in SQCC alone. The sample size was small but there was a positive correlation between NQO1 expression and SUVmax in SQCC.

Key words: non-small cell lung cancer, NF-E2-related factor 2, NAD(P)H dehydrogenase (Quinone), positron emission tomography, prognosis

Non-small cell lung cancer (NSCLC) is a major cause of cancer-related death worldwide. Precision medicine depends on the exploration of useful biomarkers. Targeted therapies that bind to cancer specific targets, such as epidermal growth factor receptor (EGFR) and anaplastic lymphoma kinase (ALK) are widely used in clinical settings. Despite advances in targeted therapies and even immunotherapy, the 5-year survival rate of patients with NSCLC is still 15% [1]. Early detection and improved characterization of the disease using novel biomarkers can improve the outcomes by selecting optimal treatment for specific patients.

NF-E2-related factor 2 (Nrf2) is a basic leucine zipper transcription factor that regulates the expression of antioxidant proteins for protection against oxidative damage triggered by injury and inflammation [2]. Somatic mutations of Nrf2 are relatively frequent in malignant tumors [3] and Nrf2 plays an important role in tumor initiation and progression [4]. Several studies reported a prognostic significance of Nrf2 in malignant tumors of lung [5] and colon [6]. NAD(P)H dehydrogenase (Quinone) 1 (NQO1) is a conserved target of Nrf2 and NQO1 overexpression has been detected in solid tumors of the lung, thyroid, breast, ovary and colon [7]. NQO1 activation promoted cell cycle progression and led to cellular proliferation in melanoma cell lines [8].

The Nrf2/NQO1 pathway regulates the expression of lipid and glucose metabolism genes. Suppressing Nrf2 in lung cancer cells led to the downregulation of a series of genes involved in the pentose phosphate pathway (PPP) and

nicotinamide adenine dinucleotide phosphate (NADPH) synthesis [9]. The PPP is a branch of the glycolytic pathway and is required for the synthesis of nucleic acids and NADPH production in cells [10]. NADPH produced in oxidative PPP can be used for the detoxification of reactive oxygen species (ROS) [11]. Furthermore, a recent study using ^{18}F-fluorodeoxyglucose positron emission tomography (^{18}F-FDG-PET) revealed that carbohydrate/pentose related genes were elevated only in cells with high standardized uptake value (SUV) in lung adenocarcinoma [12]. Gene expression study revealed that the PPP was significantly correlated with FDG uptake in breast cancer cell lines [13].

Schuurbiers et al. suggested that the adenocarcinomas exhibit glycolysis under normal oxygen condition, whereas squamous cell carcinomas are exposed to hypoxia resulting in a very high anaerobic glycolytic rate [14]. The mechanism of development and progression varies according to histological type of NSCLC [15, 16]. However, no studies have evaluated the prognostic potential of Nrf2 or NQO1 based on NSCLC histology. There is also no study confirming the role of Nrf2 or NQO1 in glucose metabolism of NSCLC using FDG-PET. This retrospective study evaluated the relationship between Nrf2, NQO1 expression and SUV value, and examined their prognostic significance in NSCLC patients according to histological subtype.

Patients and methods

Patients. This study was approved by the Institutional Review Board of Ajou University School of Medicine. A total of 241 patients diagnosed as NSCLC after surgery between January 2009 and December 2013 were included. The patients' medical records, pathological and PET data were reviewed retrospectively. Bronchoscopy, pulmonary function testing, chest computed tomography (CT) and PET/CT were performed preoperatively. Pure ground-glass opacity lesions were excluded from the study. Postoperative chest CT scans were performed every 6 months and PET/CT scans were performed at 12-month intervals to detect recurrence.

FDG-PET/CT protocol and image analysis. All patients fasted for at least 6 h before PET/CT scan. Their blood glucose levels at the time of FDG injection were <150 mg/dl. FDG-PET/CT was performed using Discovery ST or Discovery STE (GE Healthcare; Milwaukee, WI, USA) PET/CT scanners. Before the PET scan, unenhanced CT scan was performed at 60 min after 5 MBq/kg FDG injection using an 8-slice or 16-slice helical CT (120 keV, 30–100 mA in AutomA mode; section width = 3.75 mm), and then an emission scan was acquired from thigh to head for 3 minutes per frame in 3-dimensional (3D) mode. Attenuation-corrected PET images using CT data were reconstructed by an ordered-subsets expectation maximization algorithm (20 subsets, 2 iterations). All PET/CT scans were reviewed by one nuclear medicine physician (SJL). Volumes of interest were placed on transaxial PET images using a dedicated workstation (GE Advantage Workstation 4.4, GE Healthcare) and the maximum SUV (SUVmax) of primary lung lesion was then calculated from the injected dose and body weight. Both scanners were concordant in SUVmax through software upgrades. We used the median as a cutoff point for SUVmax in survival analysis.

Histopathological analysis and immunohistochemistry. All histological and immunophenotypic data from the 241 patients with NSCLC were reviewed by two pathologists (JHH and YWK). In each case, a representative tumor paraffin block (donor block) was collected and two tumor cores 2 mm in diameter were obtained. An automatic immunohistochemistry staining instrument (Benchmark XT, Ventana Medical Systems; Tucson, AZ, USA) was used. Immunohistochemistry was performed using antibodies against cleaved Nrf2 (1:100 dilution, rabbit monoclonal, clone name EP1808Y, Abcam, Cambridge, UK) and NQO1 (1:1000 dilution, mouse monoclonal, clone name A180, Santa Cruz Biotechnology, CA, USA). Nrf2 and NQO1 intensities were evaluated as a four-value intensity score (0, 1, 2 and 3). The percentages of nuclear Nrf2 expression and cytoplasmic expression of NQO1 expression were evaluated. The overall score was obtained by multiplying the intensity and percentage of positive cells. Overall scores for Nrf2 and NQO1 were dichotomized based on the mean protein expression value. Pathological staging was recorded according to the seventh edition of the TNM classification.

Statistical analysis. All statistical analyses were performed using SPSS statistical software (version 18; SPSS, Chicago, IL, USA). A p value less than 0.05 was considered statistically significant. Overall survival (OS) and recurrence-free survival (RFS) were analyzed using the Kaplan-Meier curve and compared with the log-rank test. Multivariate prognostic analyses of OS and RFS were performed using the Cox proportional hazards regression model. Variables with a p value less than 0.05 in the univariate analysis were added in the final multivariate analysis. The enter method was used to determine the final Cox model for multivariate analysis. The chi-square test was used for categorical variables and the independent sample t-test was used for continuous variables.

Results

Patient demographics. The demographic data of the patients included in this study are listed in Table 1. The 241 patients included 167 (69.3%) males with the median age 64 years (range, 35–86). The majority of patients (87.5%) underwent anatomic resection (lobectomy or pneumonectomy). Diagnosis of the patients indicated that 114 (48.7%), 64 (27.4%) and 56 (23.9%) were at stages I, I, and III, respectively. 151 patients (62.7%) had adenocarcinoma and 90 patients (37.3%) had squamous cell carcinoma. The mean SUVmax values of primary lung lesions were 8.71±6.71 (range 0.50–51.46). The median follow-up period was 38.4 months.

Expression of NQO1 and Nrf2. A total of 64 patients (64/241, 26.6%) were assigned to the group with high Nrf2 and 165 patients (165/241, 68.5%) were assigned to the group with high NQO1. In squamous cell carcinoma, Nrf2 expression was significantly correlated with NQO1 expression (p=0.033). However, in adenocarcinoma, there was no correlation between Nrf2 and NQO1 expression (p=0.805).

Correlation between SUVmax, NQO1 and Nrf2 expression. We performed correlation analysis of SUVmax, Nrf2 and NQO1 expression because Nrf2 and NQO1 activation promoted glucose metabolism genes. In squamous cell carcinoma, SUVmax value was significantly higher in patients with elevated NQO1 compared with those manifesting a low NQO1 expression (mean and standard deviation, 12.85±5.04 vs. 10.04±6.08, p=0.023; Figure 1A). However, in adenocarcinoma, there was no difference in SUVmax in patients with high or low NQO1 expression (6.94±7.25 vs. 6.99±4.86, p=0.972; Figure 1B). In squamous cell carcinoma, there was no difference in SUVmax in patients with high or low Nrf2 expression (12.60±6.27 vs. 11.01±5.04, p=0.195). In adenocarcinoma, there was also no difference in SUVmax in patients with high or low Nrf2 expression (8.84±9.44 vs. 6.56±5.95, p=0.122).

Prognostic significance of NQO1, Nrf2 and SUVmax. In squamous cell carcinoma, patients with high NQO1 expression had an inferior 5-year RFS and 5-year OS compared with patients showing a low NQO1 expression (46.7% vs. 84.2%, p=0.002; Figure 2A and 45.2% vs. 75.6%, p=0.014; Figure 2B, respectively). However, NQO1-high and NQO1-low patients showed similar 5-year RFS and OS rates in adenocarcinoma (56.3% vs. 50.3%, p=0.403; Figure 2C and 67.9% vs. 64.1%, p=0.462; Figure 2D, respectively).

Nrf2 expression was not associated with RFS or OS rates in squamous cell carcinoma (p=0.34 and p=0.923, respectively). In adenocarcinoma patients, increased or decreased Nrf2 expression showed similar 5-year RFS and OS rates (p=0.242 and p=0.827, respectively).

Using the median as a cutoff point for SUVmax, the group with high SUVmax showed an inferior 5-year RFS compared with those with a low SUVmax in squamous cell carcinoma (46.2% vs. 73.5%, p=0.03; Figure 3A). However, both groups of patients showed similar 5-year OS rates in squamous cell

Table 1. Patient demographic and clinical characteristics.

Variable	Number (%)
Age, median (range) (years)	64 (35–86)
Male sex	167 (69.3%)
Smoking history	150 (65.8%)
Operation	
Pneumonectomy	14 (5.8%)
Lobectomy	197 (81.7%)
Sublobar resection	30 (12.4%)
Histologic subtype	
Adenocarcinoma	151 (62.7%)
Squamous cell carcinoma	90 (37.3%)
pT stage	
T1 / T2	41 (17%) / 181 (75.1%)
T3 / T4	16 (6.6%) / 3 (1.2%)
pN stage	
Nx / N0 / N1	7 (2.9%) / 138 (57.3%) / 43 (17.8%)
N2 / N3	51 (21.2%) / 2 (0.8%)
pTNM 7th edition	
Stage I	114 (48.7%)
Stage II	64 (27.4%)
Stage III	56 (23.9%)
Adjuvant chemotherapy	71 (29.5%)
Adjuvant EGFR TKI	16 (9.8%)
Adjuvant radiotherapy	86 (35.7%)

EGFR TKI, epidermal growth factor receptor tyrosine kinase inhibitor

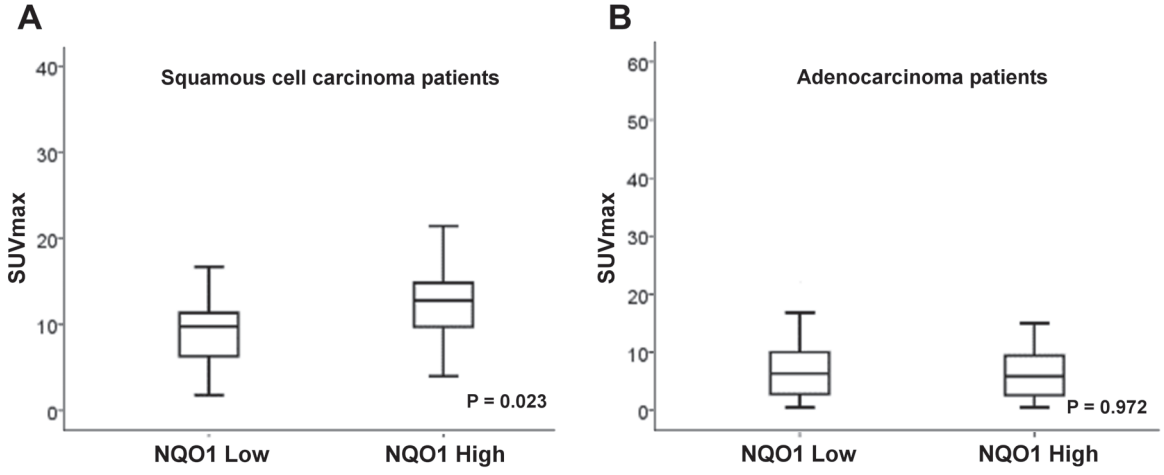

Figure 1. SUVmax according to NQO1 expression. A) The mean of SUVmax was significantly higher in patients with high NQO1 than with low NQO1 expression in squamous cell carcinoma. B) There was no statistically significant difference in SUVmax between high NQO1 and low NQO1 in adenocarcinoma.

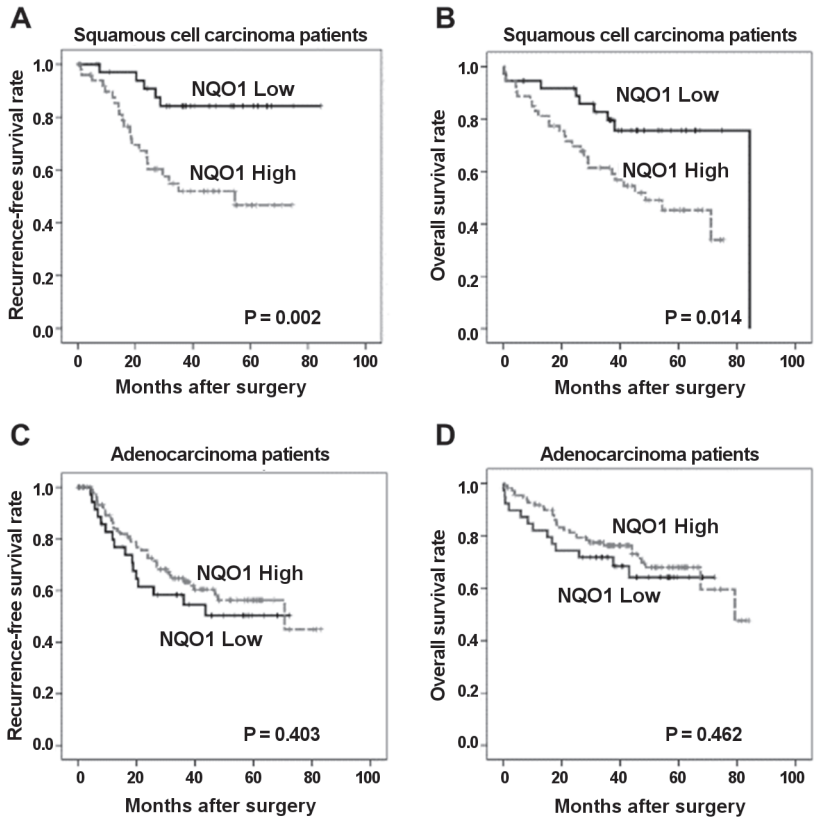

Figure 2. Comparison of survival rates according to NQO1 expression. Patients with high NQO1 expression had an inferior (A) recurrence-free survival and (B) overall survival compared with patients showing a low NQO1 expression in squamous cell carcinoma. NQO1-high and NQO1-low patients showed similar (C) recurrence-free survival and (D) overall survival in adenocarcinoma

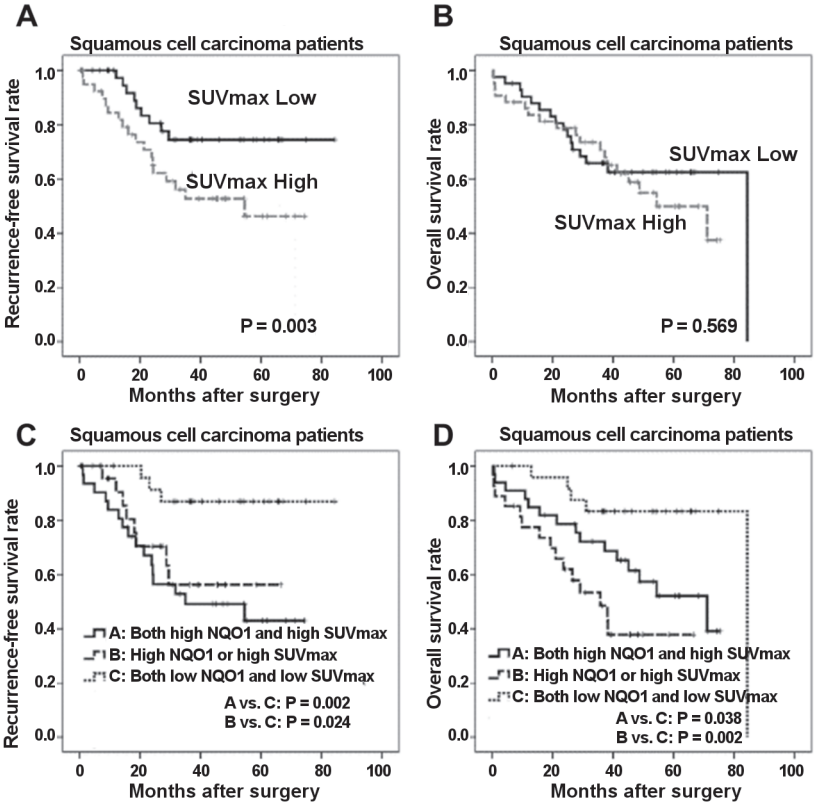

Figure 3. Comparison of survival rates according to SUVmax value. A) Recurrence-free survival rate was significantly lower in the SUVmax high cases in squamous cell carcinoma. B) Overall survival rate was not associated with SUVmax value in squamous cell carcinoma. Patients with both low NQO1 expression and low SUVmax had significantly longer (C) recurrence-free survival or (D) overall survival than patients with both high NQO1 expression and high SUVmax or patients with high NQO1 expression or high SUVmax in squamous cell carcinoma.

carcinoma (p=0.569; Figure 3B). The SUVmax was associated with RFS or OS rates in adenocarcinoma (p<0.001 and p<0.001, respectively).

We performed additional survival analyses using NQO1 and SUVmax due to the correlation between NQO1 expression and SUVmax in squamous cell carcinoma. We combined NQO1 expression and SUVmax value to perform subgroup analysis. We divided patients with squamous cell carcinoma into three groups (patients with both low NQO1 and low SUVmax, patients with both high NQO1 and high SUVmax and patients with high NQO1 or high SUVmax). Patients with both low NQO1 and low SUVmax had significantly longer RFS than patients with both high NQO1 and high SUVmax (p=0.002, Figure 3C) or patients with high NQO1 or high SUVmax (p=0.024, Figure 3C). Patients with both low NQO1 and low SUVmax had significantly longer OS than patients with both high NQO1 and high SUVmax (p=0.038, Figure 3D) or patients with high NQO1 or high SUVmax (p=0.002, Figure 3D).

We performed a multivariate analysis for patients with squamous cell carcinoma because of adverse prognostic effect of NQO1 and SUVmax in squamous cell carcinoma. In univariate analysis, TNM stage (p<0.001) and postoperative chemotherapy (p=0.001), postoperative radiotherapy (p<0.001), SUVmax (p=0.009) and NQO1 expression (p=0.005) were risk factors for RFS (Table 2). TNM stage (p=0.011) and NQO1 expression (p=0.018) were risk factors for OS (Table 2). In multivariate analysis, NQO1 expression

Table 2. Univariate analyses of recurrence-free survival and overall survival in squamous cell carcinoma.

Variables	Recur-free survival			Overall survival		
	HR	**95% CI**	**p-value**	**HR**	**95% CI**	**p-value**
Age (years) (<65 vs. ≥65)	0.96	0.456–1.88	0.832	1.61	0.859–3.039	0.136
Sex (female vs. male)	1.383	0.419–4.568	0.594	1.723	0.671–4.425	0.258
Smoking grade						
Non-smoker		reference			reference	
Light-smoker	0.353	0.111–1.122	0.078	0.362	0.136–0.961	0.041
Heavy-smoker	0.548	0.202–1.484	0.237	0.472	0.200–1.118	0.088
Pathologic stage						
I		reference			reference	
II	1.411	0.566–3.519	0.46	0.999	0.475–2.101	0.99
III	8.226	3.172–21.33	<0.001	3.296	1.445–7.519	0.005
Postoperative chemotherapy (– vs. +)	3.229	1.592–6.55	0.001	1.295	0.656–2.559	0.456
Postoperative radiotherapy (– vs. +)	5.333	2.374–11.98	<0.001	1.578	0.847–2.941	0.151
SUVmax (Low vs. High)	2.72	1.28–5.779	0.009	1.316	0.700–2.473	0.393
NQO1 (– vs. +)	4.084	1.543–10.81	0.005	2.600	1.180–5.728	0.018

NQO1: NAD(P)H dehydrogenase (Quinone) 1, SUVmax: maximum standardized uptake value

Table 3. Multivariate analyses of recurrence-free survival and overall survival in squamous cell carcinoma.

Variables	Recurrence-free survival			Overall survival		
	HR	95% CI	p-value	HR	95% CI	p-value
Postoperative chemotherapy (– vs. +)	1.164	0.425–3.194	0.768	–	–	–
Postoperative radiotherapy (– vs. +)	9.623	2.629–35.22	0.001	–	–	–
Pathologic stage						
II (vs. I)	0.415	0.132–1.305	0.133	0.883	0.380–2.049	0.771
III (vs. I)	1.518	0.443–5.204	0.507	2.599	1.080–6.251	0.033
SUVmax (Low vs. High)	3.544	0.961–7.240	0.06	2.021	0.818–4.991	0.127
NQO1 (– vs. +)	1.376	0.423–4.482	0.596	0.866	0.395–1.897	0.719
Postoperative chemotherapy (– vs. +)	1.161	0.449–3.003	0.758	–	–	–
Postoperative radiotherapy (– vs. +)	9.249	2.607–32.81	<0.001	–	–	–
Pathologic stage						
II (vs. I)	0.523	0.178–1.535	0.238	0.871	0.377–2.012	0.747
III (vs. I)	1.324	0.425–4.120	0.628	2.479	1.036–5.930	0.041
SUVmax/NQO1 (Both low vs. others)	0.264	0.077–0.901	0.033	0.338	0.117–0.977	0.045

NQO1: NAD(P)H dehydrogenase (Quinone) 1, SUVmax: maximum standardized uptake value

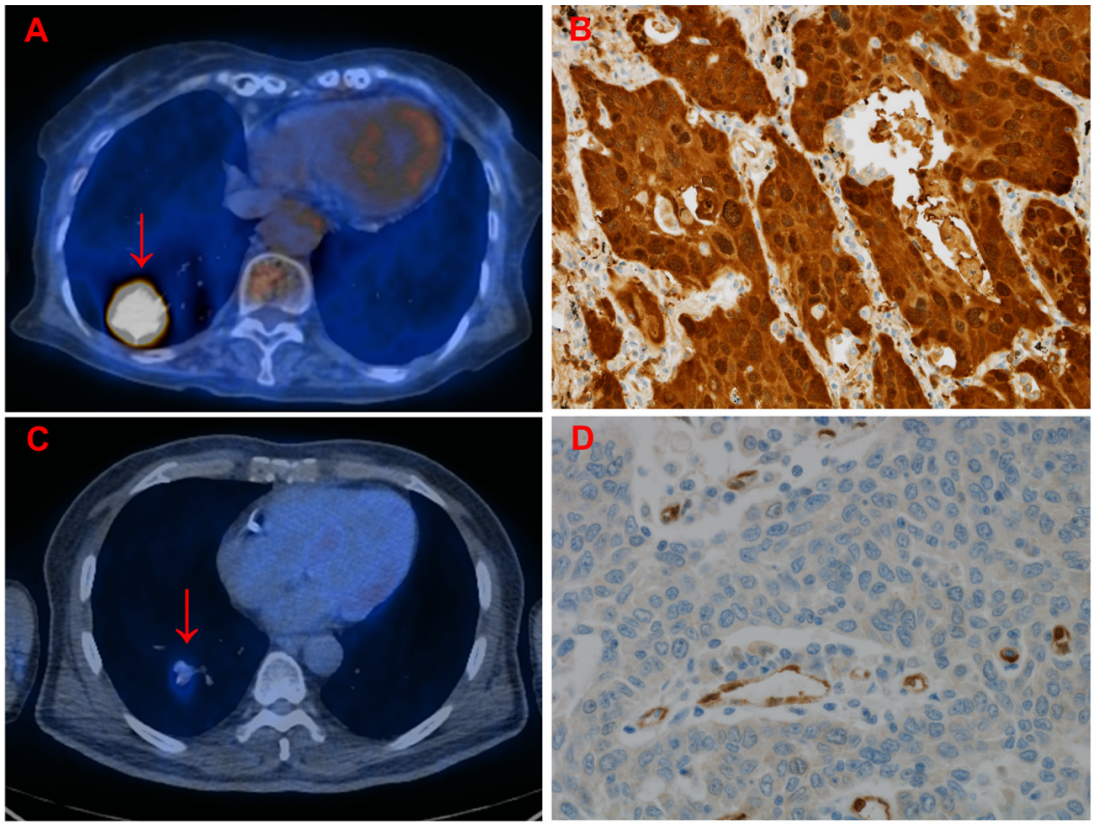

Figure 4. The representative cases of FDG uptake and NQO1 expression. Squamous cell carcinoma patients with A) high FDG uptake and B) NQO1 high expression, x400. Squamous cell carcinoma patients with C) low FDG uptake and D) NQO1 low expression, ×400.

was not an independent prognostic factor for RFS and OS (hazard ratio [HR]=1.376, p=0.596 and HR=0.886, p=0.719, respectively; Table 3). The SUVmax was not an independent prognostic factor for RFS and OS (HR = 3.544, p=0.06 and HR=2.021, p=0.127, respectively; Table 3). However, in multivariate analysis combining NQO1 and SUVmax values, both low SUVmax and low NQO1 was independent prognostic factor for RFS and OS (HR=0.264, p=0.033 and HR=0.338, p=0.045, respectively; Table 3).

78-year-old female patient with lung squamous cell carcinoma exhibited high metabolic activity on PET/CT (SUVmax = 16.77). This patient showed diffuse positivity for NQO1 (Figures 4A–B). Another 61-year-old male patient with lung squamous cell carcinoma exhibited low metabolic activity on PET/CT (SUVmax = 3.55). This patient showed negativity for NQO1 (Figures 4C–D).

Discussion

This study showed two novel findings. First, NQO1 expression was positively correlated with ^{18}F-FDG accumulation in patients with squamous cell carcinoma. Second, this study was the first of its kind to analyze the prognostic value of Nrf2 and NQO1 expression according to histological subtype of NSCLC. Both low SUVmax and low NQO1 was independent prognostic factor for RFS and OS. These results suggest that NQO1 plays an important role in glucose metabolism in patients with squamous cell carcinoma.

In our study, NQO1 expression was associated with poor prognosis in squamous cell carcinoma. Nrf2 is a potent transcription activator that is regulated by oxidative stress or xenobiotic stress. Under oxidative stress or high levels of ROS, Nrf2 is dissociated from Keap1 and translocates into the nucleus, binding the antioxidant response element and activating the transcription of the relevant gene including NQO1 [17]. The Nrf2/NQO1 pathway activates PPP to promote cancer cell survival. First, the oxidative PPP protects cancer cells from oxidative stress. In tumors, ROS are excessively generated by accelerated metabolism, DNA damage or hypoxia [18]. NADPH produced in oxidative PPP is the major antioxidant that maintains glutathione and thioredoxin in the reduced state and allows cancer cells to withstand oxidative stress [11]. This activity of PPP increases resistance to specific cancer therapies that increase oxidative stress or DNA damage. Sustained levels of elevated glucose-6-phosphate dehydrogenase and glutathione are characteristic of increased oxidative PPP following drug resistance [19, 20]. Second, the non-oxidative branch is

composed of a series of reversible reactions that recruit additional glycolytic intermediates such as fructose-6-phosphate and glyceraldehyde-3-phosphate into pentose phosphates [21, 22]. The PPP accelerates the oxidative branch and induces the non-oxidative branch to re-synthesize F6P from the pentose phosphate, for reconversion to glucose-6-phosphate to supplement the oxidation branch. Rapidly dividing cancer cells use non-oxidative PPP to generate ribonucleotides primarily for the synthesis of RNA and DNA [23]. K-Ras activation in a rat pancreatic cancer model showed that non-oxidative PPP was substantially activated without affecting the oxidative branch [24]. The levels of non-oxidative PPP, rather than oxidative PPP, were elevated in metastatic renal cell cancer [25]. The resistance to specific DNA damaging agents, such as 5-fluorouracyl, is associated with elevated non-oxidative PPP, and colon cancer cells resistant to 5-fluorouracyl showed high expression of non-oxidative PPP [26].

Schuurbiers et al. suggested that the adenocarcinomas exhibit glycolysis under normoxic condition, whereas squamous cell carcinomas are exposed to hypoxia resulting in a very high anaerobic glycolytic rate [14]. The expression of metabolic markers including glucose transporter 1 (GLUT1), carbonic anhydrase IX (CAIX) and monocarboxylate transporter 1 (MCT1) was also higher in squamous cell carcinomas than in adenocarcinomas [14]. Volume-dependent parameter such as total lesion glycolysis (TLG) was also higher in squamous cell carcinomas than in adenocarcinomas, however adenocarcinomas were better vascularized [14]. Furthermore, SUVmax value was also significantly increased in squamous cell carcinoma than in adenocarcinoma (11.7 vs. 6.95, $p<0.001$), in agreement with previous result of Schuurbiers et al. These results suggest that squamous cell carcinomas are more vulnerable to hypoxia compared to adenocarcinomas and have a higher glycolytic rate for survival. Hypoxic condition and accelerated metabolism in this status lead to excessive ROS levels [27], therefore squamous cell carcinomas will be more affected by ROS than adenocarcinomas. In our study, the NQO1 expression was associated with poor prognosis in squamous cell carcinoma but not adenocarcinoma. The NQO1 gene plays an important role in oxidative stress or glycolytic pathways. Because the microenvironment of squamous cell carcinoma is more vulnerable to hypoxia than adenocarcinoma, squamous cell carcinoma with NQO1 expression may increase survival rate by activating PPP over adenocarcinoma.

Dicumarol inhibits NQO1 and other reductases by competing with NADH for the binding site of the oxidized NQO1 form [28]. Dicumarol increases intracellular oxidative stress and increases cytotoxicity. Pancreatic cancer cells treated with dicumarol increased their intracellular superoxide production and inhibited cell growth and plating efficiency [29]. Thus, patients with high NQO1 expression in squamous cell carcinoma may benefit from targeted NQO1 therapy.

This study has several limitations. Our research may be associated with selective or information bias due to the retrospective design. Because of the small number of samples in our study, larger scale studies are needed for verification of our results. Tissue microarrays failed to reflect the entire tumor due to heterogeneous distribution.

In conclusion, this study analyzed the prognostic significance of Nrf2 and NQO1 and the correlation between Nrf2, NQO1 and SUVmax according to histological subtype of NSCLC. The sample size was small but there was a positive correlation between NQO1 expression and SUVmax in squamous cell carcinoma. NQO1 and SUVmax were not independent prognostic factors, but the combined variable was independent prognostic factor. These results suggest that NQO1 may help SUVmax to more accurately predict the prognosis of squamous cell carcinoma patients. Therefore, combining NQO1 and SUVmax value can be utilized to identify patients with squamous cell carcinoma who are at high risk of recurrence or progression and who may benefit from aggressive treatment modalities, including NQO1-targeted therapy.

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