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ORIGINAL ARTICLE

Cytoplasmic, but not nuclear Nrf2 expression, is associated with inferior survival and relapse rate and response to platinum-based chemotherapy in non-small cell lung cancer

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Abstract

Background: Several studies have previously indicated that nuclear factor erythroid 2-related factor 2 (Nrf2) expression may promote tumor progression when the Keap1/Nrf2 pathway is activated, but few reports have demonstrated the role of cytoplasmic Nrf2 on tumorigenesis.

Methods: Immunohistochemistry was conducted to evaluate Nrf2 expression in 167 tumors from surgically-resected patients with non-small cell lung cancer (NSCLC). Univariate and multivariate analyses were performed to examine the association of Nrf2 expression with patients' prognosis. This study was conducted to examine the association of Nrf2 expression with tumor response to cisplatin-based chemotherapy.

Results: Among these tumors, 56 and 32 of 167 tumors expressed Nrf2 in the cytoplasm (34% for C+/N-) and in the cytoplasm/nucleus (19% for C+/N+), but not in the nucleus of tumor cells. Nrf2 was negatively expressed in the remainder of the tumor samples (C-/N-, 79 of 167, 47%). Univariate analysis indicated that patients with Nrf2 positive tumors (C+/N- plus C+/N+) had worse overall survival (OS), but not relapse-free survival (RFS) than with Nrf2 negative tumors (C-/N-). However, patients with C+/N- tumors possessed worse OS and RFS than those with Nrf2 negative tumors (C-/N-). Multivariate analysis further confirmed the prognostic significance of patients with Nrf2 positive and C+/N- tumors on OS and RFS, but not on RFS for patients with Nrf2 positive tumors. Patients with Nrf2 positive and C+/N- tumors were determined to more frequently have an unfavorable response to cisplatin-based chemotherapy than those with Nrf2 negative tumors.

Conclusions: Cytoplasmic Nrf2 expression might potentially be used to predict poor prognosis and unfavorable response to cisplatin-based chemotherapy in patients with NSCLC.

Key points

- The expression of cytoplasmic Nrf2 showed a significant relationship with patients' response to cisplatin-based chemotherapy and influenced NSCLC prognosis.

- A proteasomal inhibitor such as carfilzomib might be used to improve the outcomes and therapeutic response to cisplatin-based chemotherapy in patients with tumors showing cytoplasmic Nrf2 expression.

Introduction

Nuclear factor (erythroid-derived 2)-like 2 (Nrf2) as a transcription factor stimulates the expression of genes which have an antioxidant response element (ARE) sequence in their promoters. These include genes such as heme oxygenase1 and NAD(P)H dehydrogenase (quinone) 1, which are known to prevent reactive oxygen species (ROS)-induced carcinogenesis and chemoresistance.[1–3] However, Nrf2 also promotes carcinogenesis by activating several oncogenes unrelated to antioxidant activity, such as matrix metallopeptidase 9, B-cell lymphoma 2 (Bcl-2), B-cell lymphoma-extra large (Bcl-xL), tumor necrosis factor α, and vascular endothelial growth factor A.[4]

One early report indicated that nuclear localization of Nrf2 (nNrf2) due to a Keap1 mutation could promote tumor progression and poor prognosis in patients with squamous cell carcinomas, but not in patients with adenocarcinomas.[5] Overall survival (OS) and progression free survival (PFS) were worse in patients with advanced non-small cell lung cancer (NSCLC) with high nNrf2 expression than with low nNrf2 expression.[6] Interestingly, nNrf2 immunostained tumors accounted for less than 10% of these tumor samples,[6] in agreement with our previous finding that cytoplasmic localization of Nrf2 (cNrf2), rather than nNrf2, appeared to be more responsible for tumor aggressiveness in colorectal cancer via activation of the NF-κB signaling pathway due to upregulation of PSMD4 expression.[7]

Nrf2 expression in cell models was suppressed at the transcription level by wild-type p53, but not by mutant p53. In addition, the p53 mutational status was associated with Nrf2 mRNA expression in tumor tissues from 109 NSCLC patients.[8] Nrf2 mRNA levels also had prognostic significance for OS and relapse free survival (RFS) in patients who received cisplatin-based chemotherapy.[8] However, the prognostic significance of cNrf2 immunostaining in lung tumors from surgically-resected NSCLC patients is still unidentified. Our preliminary data showed that nuclear p65 expression was higher in lung tumors with positive cNrf2 immunostaining than with negative cNrf2 immunostaining. This observation revealed the possibility that cNrf2 might promote the activation of NF-κB signaling pathway, and consequently to enhance tumor aggressiveness in NSCLC. We therefore hypothesized that cNrf2 expression could be associated with poor prognosis and unfavorable response to cisplatin-based chemotherapy in patients with NSCLC.

Methods

Study participants

There were 167 patients with NSCLC enrolled into the study. The inclusion criteria was a primary diagnosis with lung carcinoma; no metastatic disease at diagnosis; no previous diagnosis of carcinoma; no neoadjuvant treatment before primary surgery; and no evidence of disease within one month of the primary surgery. Tumor specimens were collected from patients who underwent resection at the Department of Thoracic Surgery, Taichung Veterans General Hospital (Taichung, Taiwan) between 1998 and 2004. The resected tissues were stored at −80°C until analysis. The study was approved by the Institutional Review Board of Chung Shan Medical University Hospital (CSMUH No: CS11177). The tumor stage of each specimen was histologically determined according to the World Health Organization (WHO) classification system (fourth edition, 2015). Cancer relapse data were obtained from chart review and confirmed by the surgeons. The clinical parameters of the patients and their overall survival (OS) data were collected from chart review and from the Taiwan Cancer Registry, Ministry of Health and Welfare, Executive Yuan, Republic of China. The survival time of each patient was taken as the period from the date of primary surgery to the date of death. The median follow-up time was 26.3 months (range 1–165.3 months) and the end of the follow-up period was December 2007. The relapse results were available for 133 patients. Over the course of the study, 105 patients died. Follow-up data indicated that 48 patients relapsed (25 had local recurrence, 15 had distant metastasis and eight had local and distant metastasis). Tumors frequently relapsed in the lung (24 patients), metastasized in the bone (seven patients), brain (five patients) and liver (five patients), and seven patients had tumors that metastasized to more than one organ.

Immunohistochemical analysis

The immunohistochemical procedures and quantification methods were as used in our previous report.[9] Nrf2 protein expression in lung tumors was detected using a specific antibody (GTX61763, GeneTex, Irvine, CA, USA). Specimens were formalin fixed and paraffin embedded. In brief, 3 μm sections were cut, mounted on glass, and dried overnight at 37°C. All sections were then deparaffinized in

xylene, rehydrated through alcohol, and washed in phosphate-buffered saline. This buffer was used for all subsequent washes. Sections were heated in a microwave oven twice for 5 minutes in citrate buffer (pH 6.0) and then incubated with antibody for 60 minutes at room temperature, followed by a conventional streptavidin peroxidase detection method (LSAB Kit K675, DAKO, Carpinteria, CA). Signals were developed with 3,3′-diaminobenzidine for 5 minutes and counterstained with hematoxylin. Negative controls were obtained by leaving out the primary antibody. The intensities of the signals were evaluated independently by three observers. There were 88%–90% cases with complete agreement of three observers for Nrf2 expression in cytoplasmic and/or nuclear localization of tumor cells. The internal consistency between three observers was calculated with Cronbach's alpha value.

Tumor response

Among the 167 enrolled patients, 48 had tumor recurrence and metastasis after surgical resection and were treated with cisplatin-based chemotherapy. Responses to chemotherapy were categorized as follows: Complete response (CR): a complete disappearance of all the tumors; partial response (PR): a decrease of 50% or more in the size or number of tumor lesions; progressive disease (PD): at least a 25% increase in the size or number of the tumor lesions; and stable disease (SD): neither sufficient shrinkage to qualify as a partial response nor a sufficient increase to qualify as progressive disease. Therefore, a favorable response (CR and PR) was a decrease in tumor size of least 50% or more, while an unfavorable response was PD or SD.

Statistical analysis

Statistical analysis was performed using the SPSS statistical software (Version 18.0; Chicago, IL.). The associations between clinical parameters and the nuclear and cytoplasmic expression of Nrf2 were analyzed with the chi-square test or Fisher's exact test. The degree of agreement between two pathologists was calculated using kappa coefficient. The internal consistency between three observers was calculated with Cronbach's alpha value. The prognostic value of Nrf2 expression on OS and RFS was assessed using the univariate Kaplan-Meier method, and differences between patient groups were determined by the log-rank test. Multivariate Cox regression analysis was performed to determine OS and RFS. The analysis was stratified for all known variables (age, gender, smoking status, and tumor stage) and nuclear and/or cytoplasmic Nrf2.

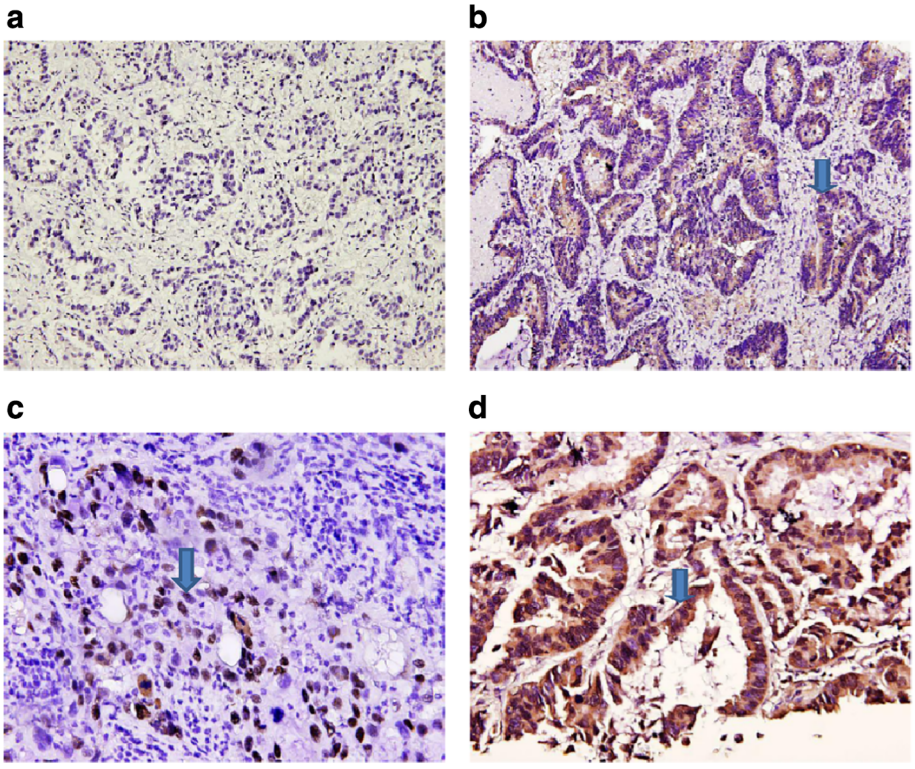

Figure 1 Representative immunostaining results of Nrf2 expression in lung tumors. (**a**) Negative immunostaining (C-/N-, x200); (**b**) Cytoplasmic Nrf2 immunostaining (C+/N-, x400) indicated by arrow; (**c**) Nuclear Nrf2 immunostaining (C-/N+, x400) indicated by arrow; and (**d**) Cytoplasmic and nuclear Nrf2 immunostaining (C+/N+, x400) indicated by arrow.

Results

Nrf2 positivity in tumors prominent in females, nonsmoker, older age, and advanced stage in NSCLC tumors

Representative cytoplasmic (C+/N-), nuclear (C-/N+), and nuclear plus cytoplasmic (C+/N+), and negative (C-/N-) Nrf2 immunostaining are presented in Fig 1. However, tumors with nuclear (C-/N+) Nrf2 immunostaining were too few for as a group and thus nuclear Nrf2 expressing tumors were excluded in this study population (n = 167). Among the 167 patients, 34% had C+/N- tumors, 19% had C+/N+ tumors, and 47% had C-/N- tumors (Table 1). The C+/N+ immunostained tumors were more commonly observed in female and nonsmoking patients than in male and smoking patients (P = 0.017 for genders, P = 0.028 for smoking status; Table 1). No correlation was observed between Nrf2 immunostaining and any other clinical parameters, including age, tumor types, stages, T, and N values (Table 1). Interestingly, C+/N- or C+/N+ tumors seemed to be more prevalent in older (> 65) or stage III patients than in younger or stage I + II patients (41% vs. 25% for age, 25% vs. 14% or 13%, respectively, for stages,), but these differences did not reach statistical significance (Table 1). These results suggested that tumors showing Nrf2 positive immunostaining (C+/N+ and C +/N- immunostaining tumors) were more prevalent in female, nonsmoker, older, and advanced stage patients.

Table 1 Relationships of Nrf2 immunostaining with clinical parameters in patients with NSCLC

		Nrf2 immunostaining			
Parameter	Patient No.	C+/N-	C+/N-	C-/N-	*P*-value
	167	32 (19)	56 (34)	79 (47)	
Age					
≦65	82	17 (21)	21 (25)	44 (54)	
>65	85	15 (18)	35 (41)	35 (41)	0.10
Gender					
Female	54	17 (31)	17 (31)	20 (69)	
Male	113	15 (13)	39 (35)	59 (52)	0.02
Tumor type					
AD	99	18 (18)	35 (35)	46 (47)	
SQ	68	14 (21)	21 (31)	33 (48)	0.82
Stage					
I	56	8 (14)	21 (38)	27 (48)	
II	31	4 (13)	11 (35)	16 (52)	
III	80	20 (25)	24 (30)	36 (45)	0.47
T value					
1	5	1 (20)	3 (60)	1 (20)	
2	117	19 (16)	41 (35)	57 (49)	
3	32	9 (28)	8 (25)	15 (47)	
4	13	3 (23)	4 (31)	6 (46)	0.59
N value					
0	72	10 (14)	27 (37)	35 (49)	
1	34	8 (24)	10 (29)	16 (49)	
2	57	14 (25)	17 (30)	26 (45)	
3	4	0 (0)	2 (50)	2 (50)	0.66
Smoking					
No	91	23 (25)	32 (35)	36 (40)	
Yes	76	9 (12)	24 (32)	45 (56)	0.03

C+/N+: Nrf2 cytoplasmic/ nuclear both positive immunostaining.
C+/N-: Nrf2 cytoplasmic positive/ nuclear negative immunostaining.
C-/N-: Nrf2 cytoplasmic/ nuclear both negative immunostainig.

Association of Nrf2 positive or C+/N- immunostaining with OS and RFS in NSCLC patients

We used univariate and multivariate analyses to examine the possibility that positive Nrf2 immunostaining could be associated with poor prognosis in NSCLC patients. Univariate analysis for OS and RFS indicated a shorter median survival and lower five-year survival in stage III patients than in stage I + II patients (OS: 18.6 months vs. 30.6 months for median survival, 23.7% vs. 40.3% for five-year survival; RFS: 9.7 months vs. 26.4 months for median survival, and 15.3% vs. 30.6% for five-year survival, P = 0.001; Table 2). The hazard ratio (HR) value for OS and RFS was significantly lower for stage III patients than for stage I + II patients (OS: HR, 1.531, 95% CI: 1.17–2.422, P = 0.016; RFS: HR, 2.141, 95% CI: 1.395–3.286, P = 0.001). Median survival and five-year OS were shorter in patients with Nrf2 positive tumors than with Nrf2 negative tumors (HR, 1.427, 95% CI: 1.003–2.231, P = 0.041), but RFS differences were not statistically significant (HR, 1.351, P = 0.132; Table 2). Interestingly, OS and RFS were worse in patients with C+/N- tumors than with Nrf2 negative tumors (OS: 21.5 months vs. 30.6 months for median survival, 21.5% vs. 37.5% for five-year survival, HR = 1.679, 95% CI: 1.077–2.615, P = 0.023; RFS: 13.8 months vs. 22.9 months for median survival, 18.8% vs. 33.7% for five-year survival, HR = 1.574, 95% CI: 1.024–2.419, P = 0.049; Table 2).

We further confirmed the prognostic value of stage parameter, Nrf2 positive immunostaining, and C+/N- immunostaining in this study population by multivariate analysis (Table 3). This analysis revealed a prognostic significance of stage, suggesting that the survival information of this study population can be confirmed by pathological examination. Worse OS and RFS were also confirmed in patients with C+/N- tumors than with Nrf2 negative tumors (OS: 21.5 months vs. 30.6 months for median survival, 21.8% vs. 37.6% for five-year survival, HR = 1.638, 95% CI: 1.059–2.535, P = 0.027; RFS: 13.8 months vs. 22.9 months for median survival, 18.8% vs. 33.7% for five-year survival, HR = 1.676, 95% CI: 1.074–2.614, P = 0.023), but a prognostic value for Nrf2 positive

Table 2 Univariate analysis for the influence of Nrf2 immunostaining on overall survival (OS) and RFS in patients with NSCLC

Parameter	Patient No.	Median survival month	Five-year survival%	HR	95% CI	*P*-value
OS						
Stage						
I, II	87	30.6	40.3	1.000	Referent	
III	80	18.6	23.7	1.531	1.017–2.422	0.016
Nrf2						
Negative	79	30.6	39.1	1.000	Referent	
Positive	88	20.4	24.6	1.427	1.003–2.231	0.041
Nrf2						
C-/N-	79	30.6	37.6	1.000	Referent	
C+/N-	56	21.5	21.8	1.679	1.077–2.615	0.023
RFS						
Stage						
I, II	69	26.4	30.6	1.000	Referent	
III	64	9.7	15.3	2.141	1.395–3.286	0.001
Nrf2						
Negative	65	22.9	29.7	1.000	Referent	
Positive	68	16.3	19.5	1.351	0.912–2.002	0.132
Nrf2						
C-/N-	63	22.9	29.7	1.000	Referent	
C+/N-	44	13.8	18.8	1.574	1.024–2.419	0.049

Negative: C-/N-.

Positive: C+/N- plus C+/N+.

Table 3 Multivariate Cox regression analysis for the influence of Nrf2 immunostaining on OS and RFS in patients with NSCLC

Parameter	Patient No.	Median survival month	5-year survival %	HR	95% CI	*P*-value
OS						
Stage						
I, II	87	30.6	40.3	1.000	Referent	
III	80	18.6	23.7	1.691	1.132–2.526	0.010
Nrf2						
Negative	79	30.6	39.1	1.000	Referent	
Positive	88	20.4	24.6	1.568	1.046–2.349	0.029
Nrf2						
C-/N-	79	30.6	37.6	1.000	Referent	
C+/N-	56	21.5	21.8	1.638	1.059–2.535	0.027
RFS						
Stage						
I, II	69	26.4	30.6	1.000	Referent	
III	64	9.7	15.3	2.141	1.395–3.286	0.001
Nrf2						
Negative	65	22.9	29.7	1.000	Referent	
Positive	68	16.3	19.5	1.609	0.874–1.533	0.087
Nrf2						
C-/N-	63	22.9	33.7	1.000	Referent	
C+/N-	44	13.8	18.8	1.676	1.074–2.614	0.023

Negative: C-/N-.

Positive: C+/N- plus C+/N+.

immunostaining was observed only for OS (20.4 months vs. 30.6 months for median survival, 21.8% *vs.* 37.6% for five-year survival, HR = 1.568, 95% CI: 1.046–2.349, $P = 0.029$) and not for RFS (Table 3). These results strongly supported previous studies indicating that Nrf2-positive immunostaining was associated with poor prognosis in NSCLC patients (5, 6, 9). Interestingly, this is the first report to reveal the independent prognostic significance of C+/N- immunostaining on OS and RFS in patients with NSCLC.

Table 4 Association of Nrf2 immunostaining with tumor response to cisplatin-based chemotherapy in patients with NSCLC

		Tumor response		
Nrf2	Patient No.	Unfavorable	Favorable	*P*-value
	48	23 (48)	25 (52)	
Nrf2				
Negative	19	5 (26)	14 (74)	
Positive	29	18 (62)	11 (38)	0.015
Nrf2				
C-/N-	19	5 (26)	14 (74)	
C+/N-	19	13 (68)	6 (32)	0.009

Negative: C-/N-.

Positive: C+/N- plus C+/N+.

Association of Nrf2 positive or C+/N- immunostaining with chemotherapeutic response in patients receiving cisplatin-based chemotherapy

Among the 167 surgically-resected patients, 48 patients had received cisplatin-based chemotherapy. The tumor response was examined to explore whether patients with Nrf2 positive or C+/N- immunostaining tumors differed in their response to cisplatin-based chemotherapy. Table 4 showed that the prevalence of unfavorable responses to cisplatin-based chemotherapy was higher in patients with Nrf2 positive or C+/N- tumors than with Nrf2 negative tumors (62% vs. 38% for Nrf2 positive immunostaining, $P = 0.015$, 68% vs. 32% for C+/N- immunostaining, $P = 0.009$). This is the first report of an association between cNrf2 expression (C+/N-) and an unfavorable response to cisplatin-based chemotherapy in NSCLC.

Discussion

Nrf2 activation caused by Nrf2/Keap1 mutations promotes Nrf2 nuclear translocation and subsequent transcriptional activation of antioxidant responses and expression of detoxifying genes for protection against ROS-induced carcinogenesis and chemoresistance in NSCLC.[5, 10–12] Therefore, Nrf2 expression in the nucleus is predominately caused by genetic aberration of the Keap1/Nrf2 pathway. Direct sequencing data have indicated that two of 67 (3.0%) and four of 145 (2.7%) patients had Keap1 and Nrf2 mutations in a subset of NSCLC patients (Table SS1). These results seemed to provide partial support for the observation that Nrf2 nuclear expression was nearly undetectable in the NSCLC tumors of this study population.

Localization of Nrf2 in the nucleus and cytoplasm is controlled by the Nrf2/Keap1 pathway, but it is also regulated by the chromosome maintenance 1, nitric oxide, and p53 mutational status.[13–15] The dual roles of Nrf2 in tumorigenesis might therefore be caused by Nrf2 shuttling between the nucleus and cytoplasm in cancer cells. The possibility of Nrf2 shuttling between the nucleus and cytoplasm has been confirmed by transfection of an Nrf2 expression vector in colon cancer cells.[7] Cytoplasmic Nrf2 expression may also promote a more aggressive colorectal cancer via activation of a NF-κB/AKT/β-catenin cascade due to increased PSMD4 expression. In an animal model, tumor formation induced by cytoplasmic Nrf2 expression may be completely suppressed by the proteasomal inhibitor carfilzomib.[7] In the present study, 88 of 167 (53%) tumors expressed Nrf2 in the cytoplasm and/or cytoplasm plus nucleus (C+/N- plus C+/N+), but did not express Nrf2 in the nucleus alone (Table 1). This observation was similar to that of previous studies indicating that less than 10%–15% of NSCLC tumors expressed Nfr2 in the nucleus.[6, 16] Moreover, univariate analysis has previously indicated that cytoplasmic Nrf2 expression is associated with poor OS.[16] This earlier finding has been partially supported by the results obtained in the present study indicating that cytoplasmic Nrf2 (C+/N-) expression may independently predict poor OS and RFS in patients with NSCLC (Table 3). We therefore suggest that a proteasome inhibitor, such as carfilzomib, might be used to improve the outcomes and overcome chemoresistance in NSCLC patients with tumors showing cytoplasmic Nrf2 expression.

Our previous report indicated that Nrf2 activation at the transcription level may confer chemoresistance in cell models and an unfavorable response to cisplatin-based chemotherapy in patients with NSCLC due to increased Bcl-2 and Bcl-xL expression.[8] In the present study, we provided evidence that OS and RFS are worse in patients with Nrf2 positive or C+/N- tumors than with Nrf2 negative tumors. The results of this study strongly support the findings of our previous report.

In summary, cytoplasmic Nrf2 expression may be a useful predictor of poor prognosis and an unfavorable response to cisplatin-based chemotherapy in patients with NSCLC.

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Disclosure

The authors declare that there are no conflict of interests.

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Supporting Information

Additional Supporting Informationmay be found in the online version of this article at the publisher's website:

Table S1 Direct sequencing of Nrf2 and Keap1 mutations in a subset of patients with NSCLC.

