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Original Article

Expression patterns and prognostic values of Nrf2 and Keap1 in nasopharyngeal carcinoma

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Abstract: Nuclear factor erythroid-2-related factor 2(Nrf2)-Kelch-like ECH-associated protein 1 (Keap1) signaling pathway is abnormally activated in response to cellular stress in various types of human cancers. In this study, we examined the immunohistochemical expression of nuclear Nrf2 and cytoplasmic Keap1 in 108 nasopharyngeal carcinoma (NPC) tissue specimens and investigated their correlations with clinicopathologic factors, including prognosis after radiotherapy (RT) or concomitant chemoradiotherapy (CRT) treatment. We found that the high rate of nuclear Nrf2 and cytoplasmic Keap1 expression were 36.1% (39/108), 58.3% (63/108), respectively, in NPC tissues. The results also showed that nuclear Nrf2 and cytoplasmic Keap1 expression were not associated with clinicopathologic parameters, but cytoplasmic Keap1 expression was likely an independent prognostic factor: high Keap1 level is associated with a longer DFS and OS outcome. These findings suggest that cytoplasmic expression of Keap1 in NPC may play a negative role in RT or CRT treatment.

Keywords: Nrf2, Keap1, nasopharyngeal carcinoma, biomarker, prognosis

Introduction

Nasopharyngeal carcinoma (NPC), a malignant neoplasm arising from the mucosal epithelium of the nasopharynx, has a unique set of etiological, epidemiological and biological characteristics in the head and neck area [1]. It is rare globally but common in southern China, Southeast Asia, northern Africa and Alaska [2, 3]. Currently, the main curative treatment strategy for NPC is radiotherapy (RT) for patients with early stage disease, and concomitant chemoradiotherapy (CRT) for those with locally advanced disease [4]. Despite the fact that NPC may be cured by RT or CRT, recurrence rate remains high, and the 5-year overall survival (OS) rate is around 80% [5]. Radioresistance remains a major obstacle to treat NPC successfully [6]. Hence, revealing the mechanisms underlying therapy resistance and identifying subgroup of radioresistance NPC patients are urgently needed for personalized therapy.

RT or CRT is mostly aimed at damaging DNA by generating electrophilics and reactive oxygen species (ROS) [7]. Most interestingly, the Nrf2 (erythroid-2 related factor 2)-Keap1 (Kelch-like ECH-associated protein 1) regulatory system plays a central role in cellular protection from electrophilics and ROS via its upregulation on various antioxidant response elements (ARE) genes [8]. The association of Nrf2 with Keap1 promotes Nrf2 degradation and prevents it from translocating to the nucleus and activating its target genes [9, 10]. Recently, Luisa M. Solis et al. [11] indicated that increased expression of nuclear Nrf2 and decreased expression of cytoplasmic Keap1 are common abnormalities in non-small cell lung carcinoma (NSCLC) and are associated with a poor outcome. However, to the best of our knowledge, no comprehensive analyses have been done for Nrf2 and Keap1 expressions in NPC, and no studies have determined the relationship between Nrf2 and Keap1 expressions and clinical outcomes after RT or CRT treatment.

Based on these informations, in this retrospective study, we characterized the expression levels of these two proteins in 108 NPC biopsy

specimens and investigated their effects on clinicopathologic characteristics, disease-free survival (DFS), and overall survival (OS) in patients treated with RT or CRT.

Materials and methods

Patients and data

We retrospectively searched the patient database of the Department of Otolaryngology, Zhangjiagang First People's Hospital, Affiliated Hospital of Soochow University, Suzhou, China, and identified patients with NPC who were treated from January 1999 to December 2010. A total of 108 patients with biopsy-proven non-keratinizing squamous cell carcinoma-undifferentiated type (NKSCC-UD) (Word Health Organization types of II or III) with available biopsy specimens for tissue microarray (TMA) construction were eligible for the study; no other malignant diseases; no distant metastases; Karnofsky performance score ≥70; without RT or CRT before biopsy. All patients signed written informed consent, and our study was approved and supervised by the ethical committee of the Zhangjiagang First People's Hospital, and the study followed the Declaration of Helsinki.

Treatment summary

All patients were treated as follows. The routine staging workup was composed of a detailed physical examination, including electronic optic nasopharyngoscopy, contrast-enhanced computed tomography (CT) or magnetic resonance imaging (MRI) of the entire head and neck, chest radiography, abdominal ultrasonography, hematology and biochemistry profiles. The clinical stage was defined on the basis of the International Union Against Cancer (UICC) and the American Joint Committee on Cancer (AJCC) [12]. Histological subtypes were evaluated by two different pathologists and classified according to the 6th edition of the TNM Classification of the UICC.

All 108 patients received RT or CRT as described previously [13, 14]. Briefly, all target volumes were outlined slice by slice in the treatment planning system based on enhanced CT scans. The radiation dose was 60-72 Gy at the nasopharyngeal region and 50-66 Gy at the regional lymph nodes. Concurrent chemotherapy consisted of cisplatin 80 mg/m^2 given on Day 1 and docetaxel 70 mg/m^2 given on Day 2 or 5-fluorouracil 750 mg/m^2 over 24 h on Days 2 to 6, repeated once every 3 weeks for three cycles.

Tissue microarrays construction (TMA)

TMA was constructed in accordance with a previously described method [15]. In brief, formalin-fixed, paraffin-embedded tissue blocks were prepared and TMAs were produced by Xinchao Bio-tech Co., Ltd (Shanghai, China). The representative cancer area was labeled in specific paraffin blocks in accord with hematoxylin and eosin staining results. Core tissue biopsies (2 mm in diameter) were taken from individual paraffin-embedded NPC and re-embedded into a recipient paraffin block at a defined position, using a tissue arraying instrument (Beecher Instruments, Silver Spring, MD, USA). The TMA was cut into 4-μm sections and placed on tissue microarray-specific adhesive-coated glass slides.

Immunohistochemical staining (IHC)

The tissue microarray slides for IHC were deparaffinized and rehydrated. For antigen retrieval, the sections were boiled under pressure in citrate buffer (pH 6.0) for 3 minutes at 100°C. Endogenous peroxidase activity was blocked with 3% hydrogen peroxide for 15 minutes, and non-specific binding of immunoglobulin was blocked with 10% goat serum for 20 minutes at room temperature. Then tissue sections were incubated with anti-Nrf2 (1:100 dilution; ab-62352, Abcam, Cambridge, UK) or anti-Keap1 (1:200 dilution; ab66620, Abcam, Cambridge, UK) antibody overnight at 4°C. After washing, the sections were incubated with biotinlabeled secondary antibody for 30 minutes at room temperature and subsequently incubated with streptavidin-conjugated horseradish peroxidase (HRP). Sections were colorized with 3,3-diaminobenzidine (DAB) chromogen solution and counterstained with hematoxylin. Sections were photographed under light microscope (Leica DM2500, Germany), and images were captured with a digital camera (Leica DFC295, Germany). Results were examined by two investigators in blinded manner.

Results were analyzed according to the method described as before [16, 17]. Briefly, nuclear Nrf2 and cytoplasmic Keap1 expressions were quantified using a four-value intensity score (0, no staining; 1, weak intensity; 2, moderate intensity; 3, strong intensity) and the ratio

Table 1. Patient clinicopathological information and demographic data (n=108)

Variable	No. (%)
Age (years)	
≤54.0	54 (50.0)
>54.0	54 (50.0)
Gender	
Female	37 (34.3)
Male	71 (65.7)
T category	
T1-T2	63 (58.3)
T3-T4	45 (41.7)
LN metastasis	
N0-N1	46 (42.6)
N2-N3	62 (57.4)
M category	
M0	108 (100.0)
M1	0 (0.0)
Clinical stage	
I-II	14 (13.0)
III-IV	94 (87.0)
Histologic differentitation	
DNKC	21 (19.4)
UNKC	87 (80.6)
Treatment	
RT	12 (11.1)
CRT	96 (88.9)
Death	
Yes	50 (46.3)
No	57 (52.8)
Loss of follow up	1 (0.9)

LN, lymph node; DNKC, differentiated non-keratinizing carcinoma; UNKC, undifferentiated non-keratinizing carcinoma; RT, radiotherapy; CRT, chemoradiotherapy.

(0-100%) of positively stained tumor cells. An immunohistochemical expression score was obtained by multiplying the intensity and reactivity extension values (range, 0-300%), and these expression scores were used to determine expression levels. Because the mean value of nuclear Nrf2 and cytoplasmic Keap1 expressions in NPC TMA cases were 50% and 150% respectively, so high nuclear Nrf2 expression was defined as a score >50%, and high cytoplasmic Keap1 expression was defined as a score >150%.

Follow-up

Follow-up data were obtained by phone, letter and outpatient clinical database. All the patients were followed from the date of diagnosis to death or the lasted census date. The patients were followed up every 3 months in the first 2 years, and every 6 months thereafter or until death. Local recurrence was established by electronic optic endoscopy, CT/MRI and biopsy. Distant metastases were diagnosed based on clinical symptoms, physical examination, and imaging methods including chest X-ray, CT/MRI, bone scan, and abdominal sonography or PET-CT. The following end points were assessed: DFS and OS.

Statistical analysis

For all statistical analyses, the SPSS 17.0 (SPSS Inc., Chicago, IL, USA) was used for the analysis. The association between categorical variables was analyzed by the Pearson Chi-square tests. DFS and OS curves were drawn using Kaplan-Meier method, and the differences between the survival curves were examined using the log-rank test. The Cox univariate and multivariate analyses were performed to explore the influences of different prognostic factors on DFS and OS times. In all analyses, a two-sided P value <0.05 was considered statistically significant.

Results

Demographic and clinicopathological characteristics

Detailed clinicopathologic information, including demographics, pathologic tumor-node-metastasis stage, DFS, and OS duration, were available for all of the patients. The patients' characteristics were summarized in **Table 1**. As shown, the median age was 54 years (range: 21-79 years), and the study cohort mainly consisted of male patients (65.7%). A total of 45 patients (41.7%) had advanced T-stage disease (T3 and T4), 94 patients (87.0%) were diagnosed at late clinical stages (III and IV), and there were no cases of distant metastasis. Pathological studies confirmed that 21 cases (19.4%) were differentiated non-keratinizing carcinoma (DNKC), namely WHO type II; 87 cases (80.6%) were undifferentiated non-keratinizing carcinoma (UNKC), namely WHO type III. Twelve (11.1%) stage I to IIa patients only received RT, and 96 (88.9%) stage Ib to IVa-b patients received platinum-based CRT.

The mean follow-up duration was 77.16 months (from 17 to 100 months). By the last follow-up,

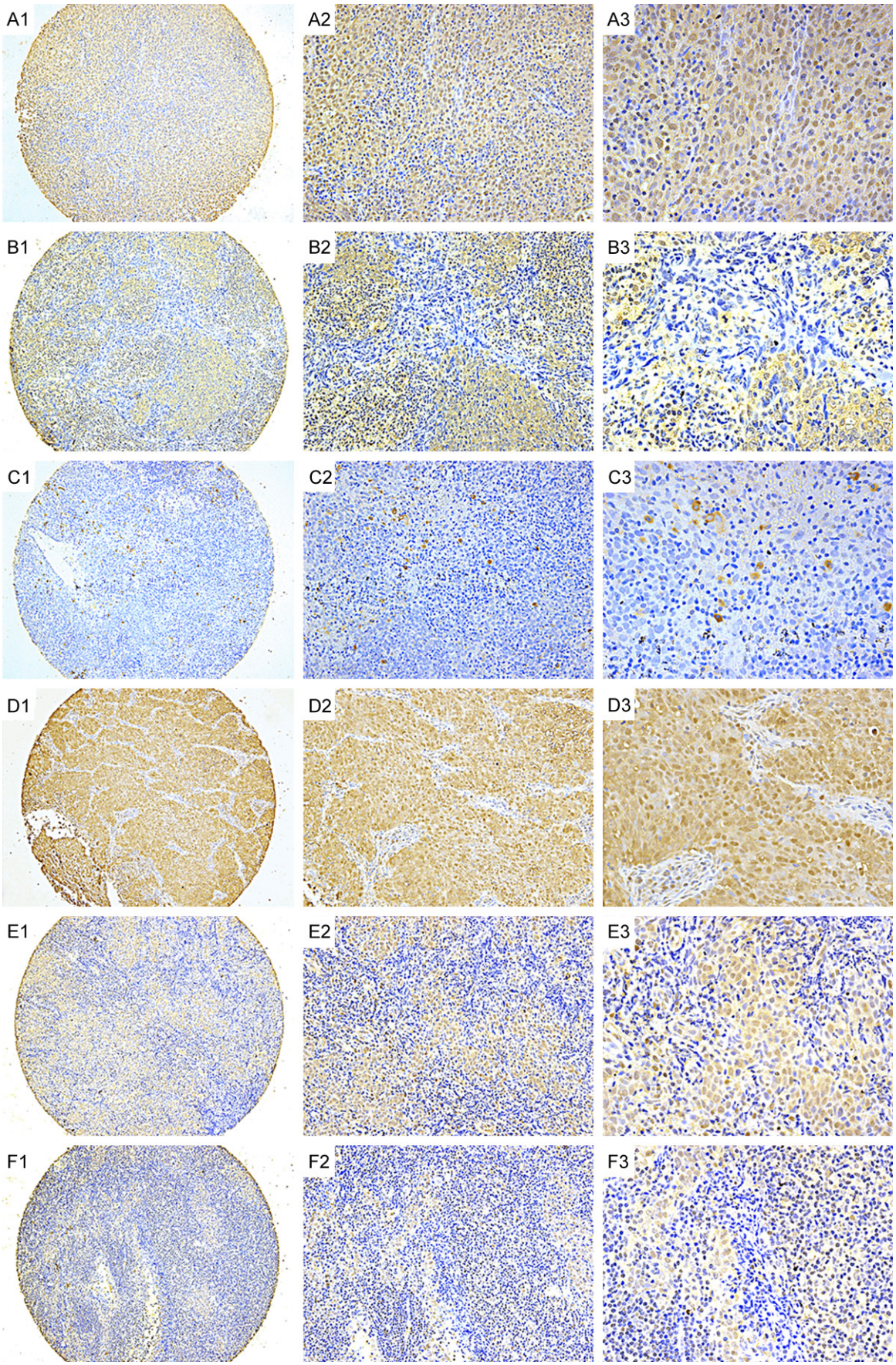
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Figure 1. Immunohistochemical expressions of Nrf2 and Keap1 in NPC. A1 to A3, typical immunohistologic features with high level of Nrf2 expression; B1 to B3, typical immunohistologic features with mild level of Nrf2 expression; C1 to C3, typical immunohistologic features with low level of Nrf2 expression; The Nrf2 staining localized predominantly to the nucleus. D1 to D3, typical immunohistologic features with high level of Keap1 expression; E1 to E3, typical immunohistologic features with mild level of Keap1 expression; F1 to F3, typical immunohistologic features with low level of Keap1 expression. Magnifications, ×100 (A1-F1), ×200 (A2-F2), ×400 (A3-F3).

Table 2. Clinicopathologic variables associated with different expression patterns of nuclear Nrf2 and cytoplasmic Keap1

Characteristics	No.	Nrf2 expression n (%)			Keap1 expression n (%)		
		Low	High	*P* value	Low	High	*P* value
Age (years)				0.161			0.172
≤54.0	54	38 (70.4)	16 (29.6)		26 (48.1)	28 (51.9)	
>54.0	54	31 (57.4)	23 (42.6)		19 (35.2)	35 (64.8)	
Gender				0.319			0.320
Female	37	26 (70.3)	11 (29.7)		13 (35.1)	24 (64.9)	
Male	71	43 (60.6)	28 (39.4)		32 (45.1)	39 (54.9)	
T stage				0.477			0.621
T1-T2	63	42 (66.7)	21 (33.3)		25 (39.7)	38 (60.3)	
T3-T4	45	27 (60.0)	18 (40.0)		20 (44.4)	25 (55.6)	
LN metastasis				0.804			0.263
N0-N1	46	30 (65.2)	16 (34.8)		22 (47.8)	24 (52.2)	
N2-N3	62	39 (62.9)	23 (37.1)		23 (37.1)	39 (62.9)	
Clinical stage				0.529			0.100
I-II	14	10 (71.4)	4 (28.6)		3 (21.4)	11 (78.6)	
III-IV	94	59 (62.8)	35 (37.2)		42 (44.7)	52 (55.3)	
Differentiation				0.768			0.711
DNKC	21	14 (66.7)	7 (33.3)		8 (38.1)	13 (61.9)	
UNKC	87	55 (63.2)	32 (36.8)		37 (42.5)	50 (57.5)	

P values were determined by Pearson Chi-square tests. LN, lymph node; DNKC, differentiated non-keratinizing carcinoma; UNKC, undifferentiated non-keratinizing carcinoma.

23 patients (21.3%) developed loco-regional recurrences, 33 patients (30.6%) developed distant metastases, and 5 patients (4.6%) developed both distant metastases and loco-regional recurrences. Among the 50 deaths, 48 (96.0%) patients died of NPC with recurrences or metastases, 2 (4.0%) died of cardiovascular disease.

IHC staining for the expression of Nrf2 and Keap1 in NPC TMA tissues

By using IHC to examine the protein expressions of nuclear Nrf2 and cytoplasmic Keap1 in NPC tissues, we found that the high rates of nuclear Nrf2 and cytoplasmic Keap1 expressions were 36.1% (39/108), 58.3% (63/108), respectively. Representative staining patterns of Nrf2 and Keap1 are shown in **Figure 1**. Nrf2 and Keap1 expressions were localized in both nuclei and cytoplasm in NPC cells, but Nrf2 mainly in nuclei.

Association of nuclear Nrf2 and cytoplasmic Keap1 expression with clinicopathological factors

Statistical analyses revealed that nuclear Nrf2 or cytoplasmic Keap1 expression had no significant association with clinicopathological features including age, gender, tumor T stage, lymph node (LN) metastasis, clinical stages, and differentiation (**Table 2**).

A high level of cytoplasmic Keap1 expression, but not that of nuclear Nrf2, correlates with increased DFS and OS

The overall 5-year DFS and OS rates were 57.4% and 70.2%, respectively. The 5-year DFS

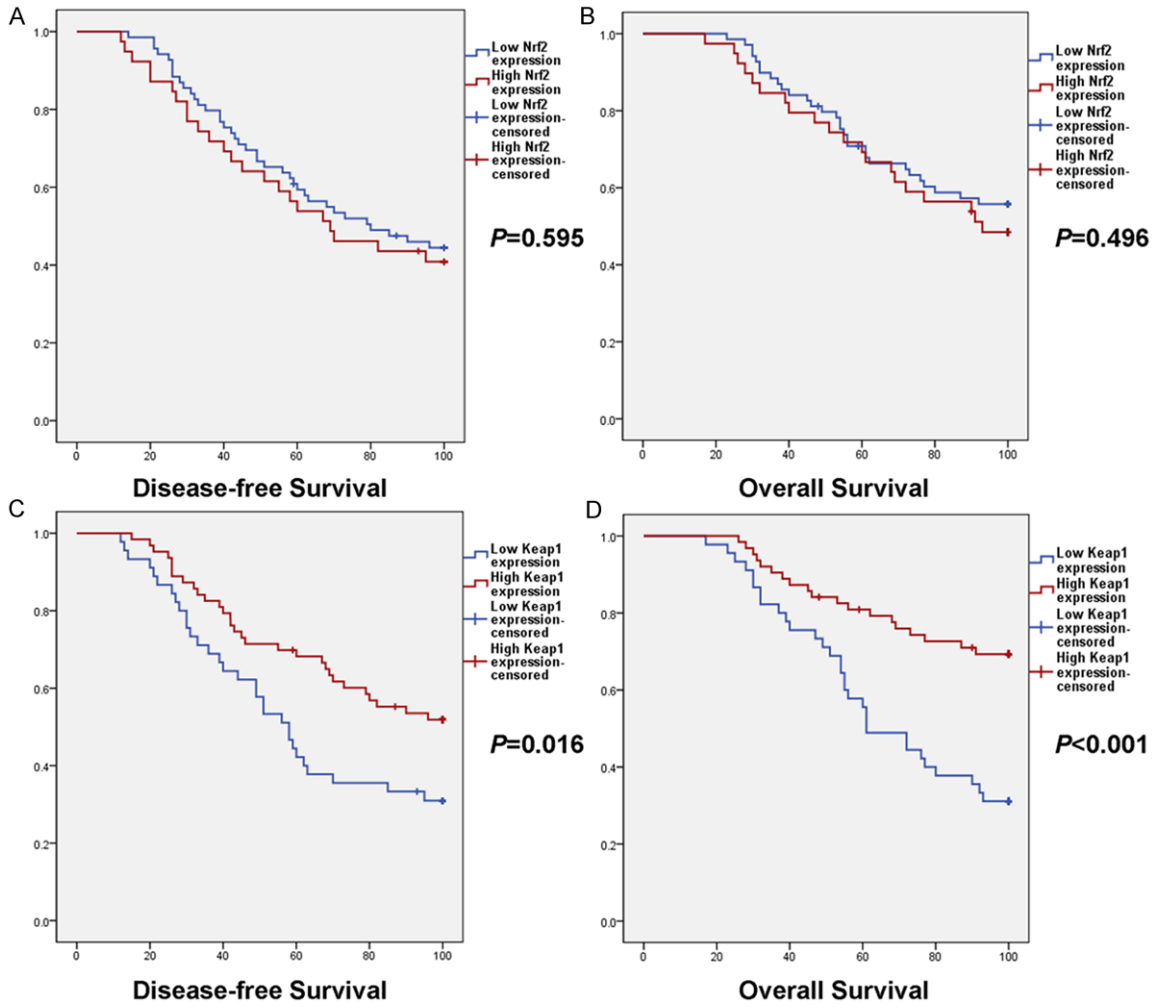

Figure 2. Kaplan-Meier curves for Nrf2 and Keap1 expressions in 108 patients with NPC. Patients with high level of Nrf2 expression did not show a better DFS rate (A) and OS rate (B). Patients with high level of Keap1 expression showed a significantly longer DFS rate (C) and OS rate (D).

rate was 59.4% in the low nuclear Nrf2 expression group, and 53.8% in the high nuclear Nrf2 expression group. The 5-year DFS rate was 42.2% in the low cytoplasmic Keap1 expression group, and 68.2% in the high cytoplasmic Keap1 expression group. The 5-year OS rate was 70.8% in the low nuclear Nrf2 expression group, and 69.2% in the high nuclear Nrf2 expression group. The 5-year OS rate was 55.6% in the low cytoplasmic Keap1 expression group, and 80.9% in the high cytoplasmic Keap1 expression group. The log-rank test showed that nuclear Nrf2 expression level didn't correlate with DFS (P=0.595, **Figure 2A**) and OS (P=0.496, **Figure 2B**). However, NPC patients with high level of cytoplasmic Keap1 expression had longer DFS (P=0.016, **Figure 2C**) and OS (P<0.001, **Figure 2D**).

Univariate and multivariate Cox proportional hazards regression analyses were performed to evaluate whether nuclear Nrf2 and cytoplasmic Keap1 expressions serve as prognostic markers in NPC patients treated with RT or CRT. As shown in **Table 3**, For univariate analysis, we found that Keap1 expression (Hazard ratio [HR]=0.544, 95% confidence interval [CI]: 0.329-0.900; P=0.018) was significantly correlated with DFS; while T stage (HR=1.943, 95% CI: 1.114-3.390; P=0.019), clinical stage (HR=4.673, 95% CI: 1.135-19.242; P=0.033), and Keap1 expression (HR=0.338, 95% CI: 0.190-0.599; P<0.001) were significantly correlated with OS. Multivariate analysis indicated that Keap1 expressionwas an independent prognostic factor for DFS (HR=0.544, 95% CI: 0.329-0.900; P=0.018) and OS (HR=0.362, 95% CI: 0.204-0.644; P=0.001).

Table 3. Results of univariate and multivariate Cox regression analyses in 108 patients

Parameter	Disease-free Survival		Overall Survival	
	HR (95% CI)	*P* value	HR (95% CI)	*P* value
Univariate analyses				
Age (≤54 vs. >54)	1.173 (0.709-1.942)	0.534	1.145 (0.657-1.995)	0.633
Gender (Female vs. male)	0.935 (0.548-1.597)	0.807	0.796 (0.435-1.458)	0.460
T stage (T1-T2 vs. T3-T4)	1.282 (0.773-2.126)	0.335	1.943 (1.114-3.390)	0.019*
LN metastasis (N0-N1 vs. N2-N3)	1.284 (0.768-2.147)	0.341	1.187 (0.674-2.090)	0.554
Clinical stage (I-II vs. III-IV)	1.840 (0.791-4.278)	0.157	4.673 (1.135-19.242)	0.033*
Differentiation (DNKC vs. UNKC)	0.588 (0.332-1.041)	0.069	0.604 (0.321-1.138)	0.119
Nrf2 expression (Low vs. high)	1.150 (0.685-1.930)	0.596	1.216 (0.690-2.141)	0.499
Keap1 expression (Low vs. high)	0.544 (0.329-0.900)	0.018*	0.338 (0.190-0.599)	<0.001*
Multivariate analyses				
T stage (T1-T2 vs. T3-T4)			1.443 (0.814-2.561)	0.210
Clinical stage (I-II vs. III-IV)			3.986 (0.964-16.477	0.056
Keap1 expression (Low vs. high)	0.544 (0.329-0.900)	0.018*	0.362 (0.204-0.644)	0.001*

HR, hazard ratio; CI, confidence interval; LN, lymph node. *$P<0.05$.

Discussion

Radiotherapy with or without chemotherapy remains the standard care for NPC patients [13]. Although technical improvements, such as intensity modulated radiotherapy (IMRT), image-guided radiotherapy (IGRT), and concomitant chemoradiotherapy (CRT), have been made in therapy, many patients still either fail to respond due to intrinsic resistance or develop therapy acquired resistance [6]. Recently, relationships between radioresistance and expressions of several genes, such as gp96 and GDF15 [18]; CCL5, STAT1-α, STAT2 and GSTP1 [6]; Maspin, GRP78, and Mn-SOD [19], have been reported. However, the molecular mechanisms are still unclear, and there are no effective biomarkers for predicting NPC radioresistance.

The Nrf2-Keap1 pathway has been reported to be impaired in several cancers, such as gastric cancer [16, 20], neuroblastoma [21], pancreatic cancer [22], lung cancer [23], and so on. In the present study, we investigated for the first time the clinical values of nuclear Nrf2 and cytoplasmic Keap1 expressions in NPC. We found that Nrf2 and Keap1 expressions were localized in both nuclei and cytoplasm in NPC cells, but Nrf2 mainly in nuclei. The high rate of nuclear Nrf2 expression was 36.1%, and the high rate of cytoplasmic Keap1 was 58.3%. Moreover, nuclear Nrf2 or cytoplasmic Keap1 expression had no significant association with clinicopathological features. These findings are consistent with previous findings of Nrf2 expression in stage I non-small cell lung cancer (NSCLC) [17]. However, Tinghua Hu et al. [24] reported that the expression of Nrf2 protein was positively correlated with larger tumor size, more advanced TNM stage, lymph node metastasis, and distant metastasis in colorectal cancer. We speculate that the status and role of Nrf2 expression maybe vary across tumor types, but further researches are required.

We further revealed that NPC patients with high level of cytoplasmic Keap1 expression had significantly higher DFS and OS rates, and Keap1 was an independent prognostic factor for DFS and OS. However, nuclear Nrf2 expression had not correlated with DFS and OS on univariate and multivariate analyses. Therefore, these results might help us to determine the prognosis of patients according to the expression level of Keap1 but not Nrf2. Although some reported that increased expression of Nrf2 and decreased expression of Keap1 are common abnormalities and are associated with a poor outcome [11]. However, our findings showed that Nrf2 level is not associated with DFS and OS.

Nrf2-Keap1 pathway can not only protect normal cells, but also can protect cancer cells fromcell stress [16]. Different views were held about the function of Nrf2-Keap1 pathway in tumor therapy. Previous studies have shown

that Nrf2 could be activated by ionizing radiation, knockdown of Nrf2 or elevated Keap1 could promote radiation induced apoptosis and Nrf2-mediated Notch signaling is an important determinant in radioresistance [25]. Upregulation of Nrf2 by the small chemical tertbutylhydroquinone (tBHQ) also enhanced the resistance of cancer cells [26]. Moreover, inhibition of Nrf2 may be a promising strategy to combat chemoresistance in many cancer types [16, 26, 27]. However, whether Nrf2-Keap1 pathway is involved in the germination and development of radioresistance in NPC has not been characterized. The current study only found that elevated Keap1, but not Nrf2, is an independent prognostic factor for DFS and OS. But this study has several shortcomings. Primarily, it was retrospective in design and included only a small number of patients. Secondly, the NPC TMA specimens were obtained from biopsy before RT or CRT, thus, these specimens can't illustrate Nrf2 and Keap1 expression patterns during therapy, because Nrf2-Keap1 system could be activated by ionizing radiation, and Nrf2 is highly unstable in the cytosol with a half-life of <20 minutes [28]. Finally, because the biopsy specimens have no matched noncancerous mucosa, we were unable to assess the Nrf2-Keap1 expression levels in adjacent noncancerous mucosa of the nasopharynx.

In summary, the present study retrospectively investigated the clinical values of Nrf2 and keap1 expressions in NPC tissues for the first time. We found that nuclear Nrf2 or cytoplasmic Keap1 expression had no significant association with clinicopathological features, and elevated Keap1, but not Nrf2, is an independent prognostic factor for DFS and OS in NPC. In order to verify the usefulness of this biomarker, further studies of larger patient scale are required to validate these findings. Considering the possible role of Nrf2 and Keap1 in radioresistance/chemoresistance, in our next project, experiments on NPC cell lines' survival in response to RT/CRT by elevating Keap1and/or decreasing Nrf2 will be explored.

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Disclosure of conflict of interest

None.

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