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Original Article

Prognostic significance of DNA-dependent protein kinase catalytic subunits in patients with nasopharyngeal carcinoma

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Abstract: *Objective:* This study was aimed to investigate the expression of DNA-dependent protein kinase catalytic subunits (DNA-PKcs) in nasopharyngeal carcinoma (NPC), and evaluate the prognostic value of DNA-PKcs in patients with NPC. *Methods:* From March 2009 to February 2011, all 64 untreated patients with NPC were recruited, and biopsy specimens from the 64 untreated patients with NPC were subjected to immunohistochemical detection, among which 8fresh NPC specimens and another 8 nasopharyngeal mucosa specimens were detected by using qRT-PCR and Western blot analysis. Kaplan-Meier survival curves analyzed the relationship between DNA-PKcs expression and prognostic factors. *Results:* The expression of DNA-PKcs was higher in NPC than in nasopharyngeal mucosa ($P< 0.05$). Statistically significant correlation was found between DNA-PKcs expression and histological differentiation ($P = 0.012$), TNM stages ($P = 0.018$), and lymph node metastasis ($P = 0.062$). There was a significant correlation between the expression level of DNA-PKcs and survival of patients ($P < 0.05$). Moreover, patients with positive expression of DNA-PKcs had a lower 5-year disease-free survival rate (23.4% *vs*. 45.2%, $P < 0.01$) and overall survival rate (33.0% *vs*. 53.1%, $P < 0.01$) than those with negative expression of DNA-PKcs. Multivariate Cox regression analysis revealed that DNA-PKcs expression was an independent prognostic factor for patients with NPC ($P < 0.01$). *Conclusions:* Our data suggest that the expression of DNA-PKcs is expected to become an independent prognostic factor of disease-free survival and overall survival in patients with NPC.

Keywords: Nasopharyngeal carcinoma, DNA-dependent protein kinase catalytic subunits, disease-free survival, overall survival, prognosis

Introduction

Nasopharyngeal carcinoma (NPC) is malignant tumorderived from nasopharyngeal mucosa, and multiple stages and various signal pathways are involved in the cancer development. There are approximately 80,000 incident cases and 50,000 deaths annually worldwide [1]. In contrast, NPC is a much more common cancer in Southern China and Southeast Asia [2]. There are 3different histologic subtypes according to the World Health Organization (WHO): keratinizing squamous cell carcinoma (WHO type I), nonkeratinizing differentiated carcinoma (WHO type II), and nonkeratinizing undifferentiated carcinoma (WHO type III). Nonkeratinizing undifferentiated carcinoma comprises 90% to 95% of cases in endemicregions, such as southern China. In the regions where Epstein-Barr virus (EBV) infectionis perhaps the most extensively studied on the aetiological factor of NPC.

In addition to viruses, various molecular biomarkers have been studied in attempt to predict the association with NPC. It is well known that DNA-PKcs is an indispensable component in the non-homologous end-joining (NHEJ) pathway of double-stranded DNA break (DSB) repair [3]. The causal link between DNA-PKcs and the development of NPC remains to be elucidated, despite the advance in molecular biology confirming the association. With the improvement in diagnostic techniques and multidisciplinary therapy, the 5-yearoverall survival rate of NPC is about 40% to 50% after radiotherapy [4], and

Table 1. Association of DNA-PKcs and clinical-pathological characteristics in patients with nasopharyngeal carcinoma

Characteristics	Patients *n* (%)	DNA-PKcs		
		Positive group (%)	Negative group (%)	*P* value
Gender				
Male	34 (53.13)	19 (55.88)	15 (44.12)	0.695
Female	30 (46.87)	17 (56.67)	13 (43.33)	
Age (years)				
< 45	31 (48.44)	17 (54.84)	14 (45.16)	0.849
≥ 45	33 (51.56)	19 (57.58)	14 (42.42)	
WHO pathology classification				
I (keratinizing)	7 (10.94)	4 (57.14)	3 (42.86)	0.012
II (nonkeratinizig)	27 (42.19)	15 (55.56)	12 (44.44)	
III (undifferentiated)	30 (46.87)	17 (56.67)	13 (43.33)	
TMN stage				
Stage I-II	35 (54.69)	20 (57.14)	15 (42.86)	0.018
Stage III-IVB	29 (45.31)	16 (55.17)	13 (44.83)	
Lymph node status				
Negative	21 (32.81)	13 (61.90)	8 (38.10)	0.062
Positive	43 (67.19)	23 (53.49)	20 (46.51)	
Therapy				
IMRT	35 (54.69)	20 (57.14)	15 (42.86)	0.018
CTR	29 (45.31)	16 (55.17)	13 (44.83)	

WHO, World Health Organization; IMTR, intensity modulated radiation therapy; CRT, concurrent chemoradiotherapy.

the main causes for the treatment failure are local recurrence and distant metastasis [5].

Up to now, there is always no extensive study about the prognostic significance of DNA-PKcs in the patients with NPC. We performed the study to detect the expression level of DNA-PKcs in NPC and further evaluate the association between DNA-PKcs expression and prognosis of patients with NPC.

Materials and methods

Patients and tissue specimens

All subjects were Chinese.64 untreated patients with nasopharyngeal cancer undergoing biopsy from April 2009 to March 2011 at the People's Hospital of Guizhou Province (Guiyang, China) were enrolled in this study. For pathologic diagnosis, biopsyspecimens were obtained from nasopharyngeal and lymph node tissues in 59 and 5 patients, respectively. All these tissue samples were detected by using immunohistochemical method, among which 8 fresh tumor tissues and another 8 fresh nasopharyngeal mucosa tissues were analyzed by using qRT-PCR and Western blot analysis. Patients with a history of other cancers or with distant metastasis were excluded. The tumors were staged according to the TNM classification as presented in the AJCC Cancer Staging manual (6th edition). Patients who had stage I-II disease were treated with intensity modulated radiation therapy (IMRT) alone and those with stage III-IVB disease were treated by concurrent chemoradiotherapy (CRT). Classification system of the nasopharyngeal cancer was based on the World Health Organization (WHO). Follow-up duration was defined as the time (in months) from the diagnosis of NPC to the final visit (from 60 months to 84 months). Written informed consent was obtained from all patients before biopsy. A summary of the clinicopathologic characteristics of the patients was summarized in **Table 1**.

Quantitative RT-PCR

Eight pairs of fresh tumor tissues and normal mucosa tissues which had various expressions of DNA-PKcs were selected for PCR assay. Total RNA of these tumor tissues and normal mucosa tissues was extracted by using Bio Flux kit (Japan) according to the manufacturer's protocol. cDNA was synthesized using Access RT-PCR System (promega, America). Real-time PCR was performed using SYBR Green ER quantitative PCR (qPCR) uperMix (Thomero) according to manufacturer's instructions. The primer sequences used for qPCR were as follows: DNA-PKcs (forward) 5'-GGCTTAGGCATGAGAATTGC-3' and (reverse) 5'-TCACACTCAGAGTACACTGC-3', with the length of PCR product of 385bp; β-actin (forward) 5'-CCTCGCCTTTGCCGATCC-3' and (reverse) 5'-GGATCTTCATGAGGTAGTCAGTC-3', with the length of PCR product of 626bp.Relative mRNA levels were calculated based on the cycle threshold(Ct) values according to the equation: $2^{-ΔCt}$[ΔCt=Ct(DNA-PKcs)-Ct(β-actin)]. Quantitative RT-PCR analysis was done intriplicate. Only the results using

β-actin as the reference gene were shown. A multiimage analyzer (Bio-Rad, Hercules, CA) was employed for analysis.

Western blot analyses

The protein levels of DNA-PKcs expression in the same tissues with PCR were detected with Western blotting. Nuclear proteins were extracted according to the manufacturer's protocol (keygen, China). Proteins (20 μg per lane) were electrophoresed on 6% SDS-polyacrylamide gels. The separated proteins were transferred to polyvinylidenefluoride membranes (Bio-Rad), which were blocked for 2 h in 5% milk dissolved Tris-buffered saline with 0.1% Tween and incubated overnight with the first antibody at 4°C. The filters were incubated with the first antibody (DNA-PKcs, 1:200 dilution, from Abcam, UK). β-actin (1:200 dilution; Abcam, UK) was used as the internal control. Antigen and the first antibody complexes were detected by the second antibody, horserdish peroxidase-conjugated antimouseIgG (cell signaling) (1:1000), and enhanced chemiluminescence (Amersham Pharmacia Biotech). The signal was measured using Image Lab™ software (BIO-RAD, America). The Western blot analysis was performed in triplicate.

Immunohistochemistry analyses

Paraffin-embedded slides of the tumor tissues and normal mucosa tissues were dried, deparaffinized, and dehydrated in a graded series of ethanol. The slides were treated with 3% H_2O_2 for 10 minutes, followed by treatment with goat serum for 30 min at room temperature. The slides were incubated with monoclonal mouse anti-human DNA-PKcs/FITC (1:100 dilution; Abcam, UK) and β-actin (1:200 dilution; Abcam, UK) antibodies diluted in blocking buffer at 4°C for 8-12 hour or overnight. After washed with PBS (3×2 min), the above samples were incubated with the secondary antibody (InVision™ two anti Goat anti pika universal reagent Poly-HRP) for 15-30 min at room temperature. After washed with PBS (3×2 min), they were stained with 0.01% DAB hydrogen peroxide for about 3-10 min, followed by washing thoroughly with tap water. The samples were counterstained with hematoxylin for immunohistochemistry analysis under the light microscope.

The staining slides were assessed blindly by two senior pathologists. DNA-PKcs was expressed in the nuclei, and brown or tan particles indicated positive. The following scoring system was used to evaluate DNA-PKcs expression according to the intensity and percentage of positive cells [6]: dark brownstaining (scored as 3), strong stainingobscuring the nuclei of tumor cells; yellow staining (scored as 2); primrose yellow staining (scored as 1); absent(scored as 0), no staining in tumor cells. At the same time, it was also scored according to the percentage of positive cells: negative, 0; positive cell rate ≤ 10%, 1; 11% - 50%, 2; 51% - 75%, 3; ≥ 75%, 4. The staining intensity score and percentage score were multiplied, and the final score was obtained. For the final score: 0-1, negative (-); 2-12, positive (+). The positive results were statistically analyzed.

Statistical analysis

Statistical analyses were performed with SPSS17.0 software and carried out using Graphpad Prism 6 software. Integral optical density (IOD) was measured by using the bands and the results were described as mean ± standard errors. The two groups of mean values were compared using t-test. The relationship of DNA-PKcs expression with clinicopathologic features was analyzed using Chi-square or the Fisher exact test.

Survival analysis was performed using the Kaplan-Meier method, and the curves were compared using the log-rank test. Disease-free survival (DFS) and overall survival (OS) were investigated by using univariate models, leading to crude estimates. Cox regression for multivariate analysis was performed to identify the prognostic factors that influenced actuarial survival. Disease-free survival was defined as the interval of disease until the first relapse or death from any cause or the last visit without a previous relapse. Overall survival was defined as the interval from the time of biopsy to the time ofdeath from any cause or tothe last follow-up. A two-sided *P*-value less than 0.05 was considered statistically significant.

Results

The expression of DNA-PKcs mRNA/Protein in NPC tissues

DNA-PKcs expression was higher in NPC tissues than innasopharyngeal mucosa tissues at

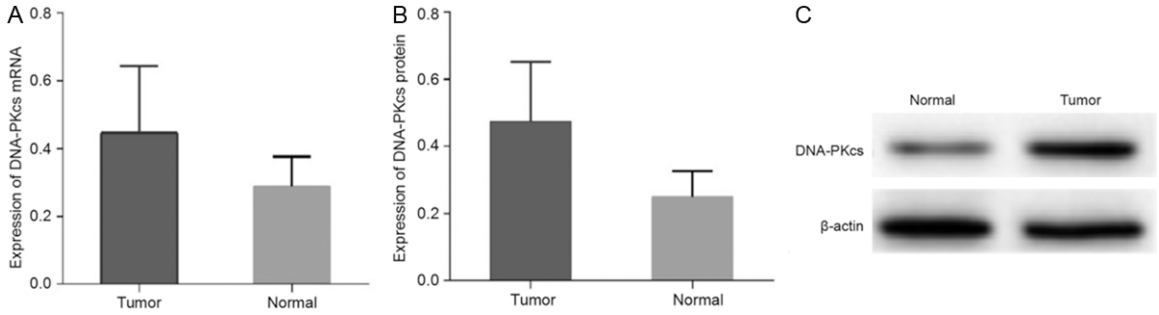

Figure 1. The expression level of DNA-PKcs in tumor tissues (Tumor) and normal mucosa tissue (Normal). A: The expression level of DNA-PKcs mRNA (qRT-PCR), B and C: The expression level of DNA-PKcs protein (Western blot analysis). β-actin as an internal reference.

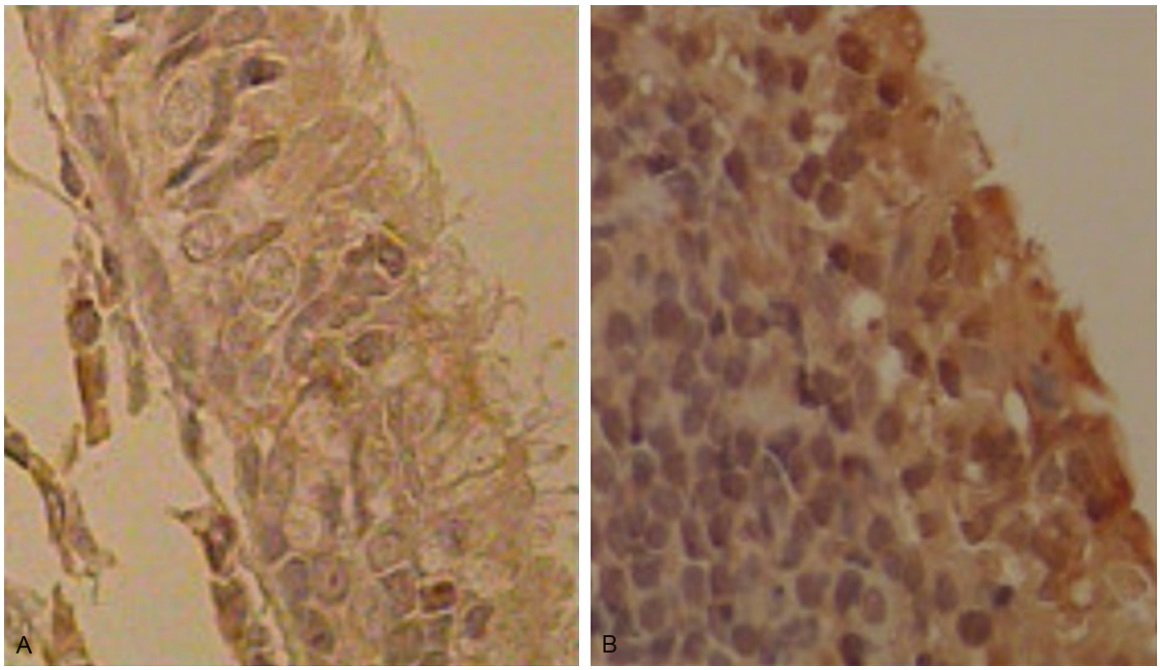

Figure 2. DNA-PKcs expression in normal mucosa tissue and tumor tissue using immunohistochemical analyses (InVision). A: Negative expression of DNA-PKcs in noamal mucosa tissue, B: Positive expression of DNA-PKcs in tumor tissue. Magnification, ×200.

the gene and protein level using quantitative RT-PCR (**Figure 1A**) and western blot (**Figure 1B** and **1C**). Average values of DNA-PKcs mRNA levels of the 8 tumor tissues (0.673 ± 0.331) were significantly higher than those of 8 normal mucosa tissues (0.391 ± 0.235) ($P < 0.05$). Average values of DNA-PKcs protein levels of the 8 tumor tissues (0.679 ± 0.371) were significantly higher than those of 8 nasopharyngeal mucosa tissues (0.338 ± 0.205) ($P < 0.05$). We also observed similar results at the expression level of DNA-PKcs using immunohistochemisty (**Figure 2** and **Table 1**).

Association between DNA-PKcs and clinicopathological parameters

The clinicopathological characteristics of these patients are summarized in **Table 1**. According to the expression level of DNA-PKcs, a total of 64 patients were assigned to the low expression group (DNA-PKcs negative) and the high expression group (DNA-PKcs positive). The expression level of DNA-PKcs was significantly associated with TNM stage and lymph node involvement. Positive expression of DNA-PKcs was mainly observed in advanced TNM stage

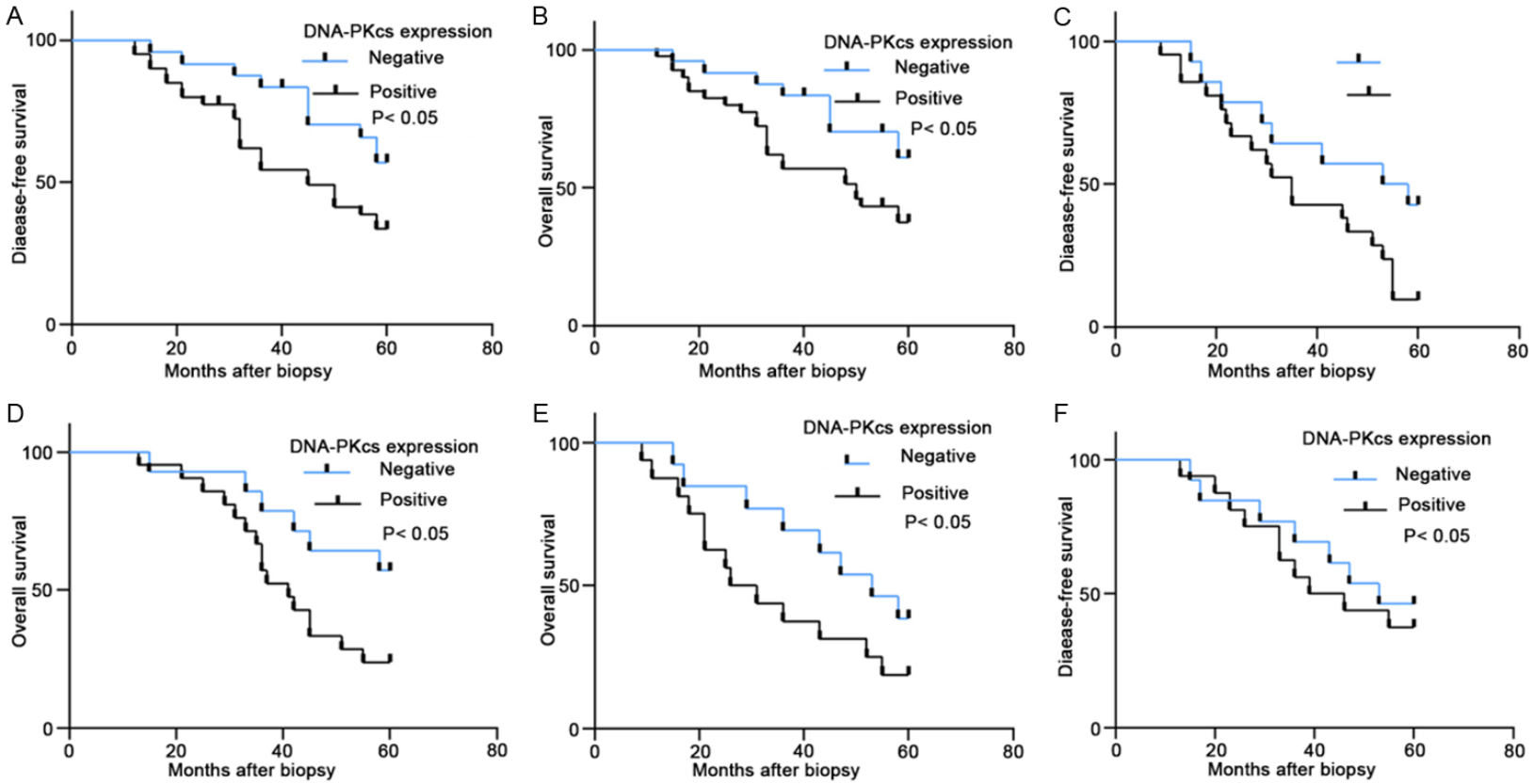

Figure 3. Kaplan-Meier curves for disease-free survival (DFS) and overall survival (OS) according to the expression level of DNA-PKcs. Patients with positive expression DNA-PKcs had poorer prognosis for DSF (A) and OS (B) than those with negative expression DNA-PKcs (log-rank test, $P < 0.05$). Stratified survival analysis according to the TNM stage revealed that the association with DNA-PKcs and DFS and OS in all stages. C: DFS in stage I-II, $P = 0.010$; D: OS in stage I-II, $P = 0.036$; E: DFS in stage III-IVB, $P = 0.001$; F: OS in stage III-IVB, $P = 0.001$.

Table 2. Univariate survival analysis of prognostic variables for nosapharyngeal carcinoma (disease-free survival and overall survival)

Characteristics	Patients *n* (%)	5year DFS	*P* value	5 year OS	*P* value
Gender					
Male	34 (53.13)	33.2 (2.4)	0.141	43.3 (2.5)	0.223
Female	30 (46.87)	42.6 (3.8)		46.7 (4.0)	
Age (years)					
< 45	31 (48.44)	37.9 (2.8)	0.190	50.1 (3.0)	0.054
≥ 45	33 (51.56)	33.6 (2.9)		37.8 (3.1)	
WHO pathology classification					
I (keratinizing)	7 (10.94)	42.3 (4.3)	0.067	52.6 (4.5)	0.071
II (nonkeratinizig)	27 (42.19)	38.9 (3.1)		46.0 (3.0)	
III (undifferentiated)	30 (46.87)	27.1 (3.6)		37.5 (3.6)	
TMN stage					
Stage I-II	35 (54.69)	67.9 (5.7)	0.045	68.5 (5.8)	0.041
Stage III-IVB	29 (45.31)	13.3 (2.5)		29.2 (3.2)	
Lymph node status					
Negative	21 (32.81)	65.1 (6.1)	0.826	63.9 (6.5)	0.530
Positive	43 (67.19)	30.3 (2.5)		40.2 (2.7)	
Therapy					
IMRT	35 (54.69)	37.1 (2.3)	0.579	42.9 (2.3)	0.259
CRT	29 (45.31)	27.9 (5.2)		49.8 (5.3)	
DNA-PKcs expression					
Negative group	28 (43.75)	53.1 (2.7)	0.031	45.3 (2.9)	0.047
Positive group	36 (56.25)	33.1 (3.3)		23.5 (2.7)	

Kaplan-Meier analysis (log-rank test); DFS, disease-free survival; OS, overall survival; s.e., standard error.

and lymph node metastasis patients. But we could not observe the association between the expression level of DNA-PKcs and age, gender, and histological differentiation (P > 0.05).

Association between DNA-PKcs expression and survival

Kaplan-Meier curves were plotted for disease-free survival and overall survival according to the expression level of DNA-PKcs (**Figure 3**). The positive expression DNA-PKcs group had a lower 5-year disease-free survival rate (23.4% vs. 45.2%, P < 0.01) and overall survival rate (33.0% vs. 53.1%, P < 0.01) than the negative expression group (**Table 2**). Patients with positive expression of DNA-PKcs had poorer prognosis of disease-free survival (log-rank test, P < 0.05) and overall survival (log-rank test, P < 0.05) than those with low-expression of DNA-PKcs (**Figure 3A** and **3B**). The expression level of DNA-PKcs still had remarkable influence ondisease-free survival and overall survivalin TNM stage I-II (**Figure 3C** and **3D**). We did not find the significant influence of gender (P = 0.141 and 0. 223, respectively), age (P = 0.190 and 0.054, respectively), histological differentiation (P = 0.067 and 0. 071, respectively), lymph nodeinvolvement (P = 0. 826 and 0.530, respectively) and radiotherapy (P= 0.579 and 0.259, respectively) ondisease-free survival and overall survival (**Table 2**).

Moreover, the multivariate analysis covered histological differentiation, TNMstage, lymph node status, and the expression level of DNA-PKcsto evaluate their prognostic significance for disease-free survival and overall survival. Cox's proportional hazards regression showed that the expression level of DNA-PKcs was an independent prognostic factor of NPC. The positive expression of DNA-PKcs really increased mortality compared with negative expression of DNA-PKcs. The positive expression of DNA-PKcs was independently associated with disease-free survival and overall survival in multivariate analysis. The hazard ratio was 1.358 (95% confidence interval [CI], 1.055-1.931) and 1.455 (95% confidence interval [CI], 1.257-1.901) (**Table 3**).

Discussion

DNA-dependent protein kinase (DNA-PK), which consists of the Ku and the DNA-PK catalytic subunit (DNA-PKcs), plays a major role in the repair of double-stranded DNA breaks (DSBs) by non-homologous end-joining (NHEJ) and site-specific V(D) Jrecombination [7-9]. Ku is a heterodimeric protein composedof 70- and 86-kD subunits (Ku70/Ku80), which was first identified as an autoantigen in patients with polymyositis syndrome [10]. The gene of DNA-PKcs, a 460 kDa serine/threonine protein kinase, is mainly located at 8q11, which is an

Table 3. Multivariable survival-analysis of prognostic variables for nasopharyngeal carcinoma (disease-free survival and overall survival)

Multivariable analysis*	Disease-free survival		Overall survival	
	HR (95% CI)	*P* value	HR (95% CI)	*P* value
Differentiation (III *vs.* II vs. I)	0.967 (0.840-1.109)	0.658	0.991 (0.829-1.153)	0.823
TNM stage (III-IVB *vs.* I-II)	1.197 (1.006-1.800)	0.048	1.416 (1.024-1.700)	0.044
Lymph node status (negative *vs.* Positive)	0.456 (0.254-0.948)	0.673	0.486 (0.251-0.994)	0.547
DNA-PKcs (negative *vs.* positive)	1.358 (1.055-1.931)	0.035	1.455 (1.257-1.901)	0.031

*Cox regression model. HR (95% CI), hazard ratio (95% confidence interval).

necessary component of the NHEJ DNA repair pathway [11-15]. In DNA DSBs repair, at least two major repair mechanisms, homologous recombination (HR) and NHEJ, have been reported [11]. In NHEJ pathway, DNA DSBs are directly, or after processing of the DNA ends, rejoined at an appropriate chromosomalend. At the same time, DNA-PKcs can maintain normal immune function, regulate DNA repair, and prevent further malignant transformation of cells [16].

It is well known that DNA-PKcs is necessary for the NHEJ pathway of DSBs. DNA-PKcs is strongly expressed in human cells [17]. Recently, the DNA-PKcs of overexpression was reported in various malignancies [18-21], and its expression level was also reported to correlate with the differentiation and proliferation status of some cell types or the development of productive tissues [22-24]. In contrast, DNA-PKcs deficiency results in severe combined immunodeficiency (SCID) in mammals [25]. However, in gastric and ovarian cancer, low expression of DNA-PKcs is linked to adverse outcome in patients [26]. The role of DNA-PKcs in DNA repair, however, remains controversial.

The expression of DNA-PKcs expression was detected in 8 pairs of fresh nasopharyngeal tumor tissues and non-tumor tissues. The results confirmed that the expression level of DNA-PKcs can be checked by RT-PCR, western blot analysis and immunohistochemical method, and the expression levels of DNA-PKcs are significantly higher in tumor tissues than in non-tumor tissues ($P < 0.05$) (**Figures 1** and **2**). In this study, only the immunohistochemical method was selected to further investigate the association of the DNA-PKcs expression with the clinicopathological features of NPC. This method was practical and efficient for paraffin-embedded tumor tissues. Because most cases of NPC are diagnosed only based on the examination of small punch-biopsy specimens, the previously used primary cell culture method was clinically impractical. Our study indicated that higher DNA-PKcs expression was mainly present in patients with advanced stage III-IVB and lymph node metastasis. However, there was no significant difference between the expression level of DNA-PKcs and gender, age, histologic differentiation, and T stages ($P > 0.05$).

Our data indicated that positive expression of DNA-PKcs was significantly associated with poor survival. Univariate analyses demonstrated a statistically significant difference between the positive expression and negative expression groups ($P < 0.01$, **Figure2**). Patients with positive expression of DNA-PKcs had markedly lower 5-year disease-free survival (23.4% *vs.* 45.2%, $P < 0.01$) and overall survival rates (33.0% *vs.* 53.1%,$P < 0.01$) than those with negative expression of DNA-PKcs. Multivariate survival analysis indicated that the expression level of DNA-PKcs was an independent prognostic factor in patients with NPC [27]. But some studies did not agree with these results. For example, Friesland *et al.* reported that the survival of patients with tumors expressing high levels of DNA-PKcs was significantly longer than that of patients with tumors expressing low levels of DNA-PKcs [28]. In addition, the previous studies conducted by Lee SW *et al*. did not find significant associations between the level of DNA-PKcs expression and the clinical outcomes [29]. Thus further studies are required to confirm our results.

The TNM stage system is thought topredict the survival most accurately. However, patients who have the same TNM stagediseasemay have dramatically different prognosis. Apart from the TNM staging system, more and more

molecular biomarkers have been extensively studied in attempt to predict the survival outcomes more accurately in NPC [30-33]. In this study, it was found that positive expression of DNA-PKcs was mainly present in advanced TNM stage. However, patients with positive expression of DNA-PKcs had significant lower 5-year disease-free survival ($P < 0.05$) and overall survival rates ($P < 0.05$) than those with negative expression of DNA-PKcs in TNM stage I-II. Multivariate analyses revealed that expression of DNA-PKcs was not associated with all TNM stage.

As previously described, lymph node status was aprognostic factor for patients with NPC. Our research also showed that the influence of DNA-PKcs on prognosis may derive from the association between DNA-PKcs and lymph node status. Univariate analyses demonstrated that patients with positive expression of DNA-PKcs tended to have a higher rate of neck lymph node metastasis than those with negative expression of DNA-PKcs in NPC (**Figure 3A**). Patients with positive expression of DNA-PKcs had markedly lower 5-year disease-free survival ($P < 0.05$) and overall survival rates ($P < 0.05$) than those with negative expression of DNA-PKcs. Moreover, in multivariate survival analysis, the basic expression of DNA-PKcs had no obvious correlation with disease-free survival and overall survival as well as lymph node metastasis. Koyula *et al*. reported that there was a direct association between DNA damage repair and cancer metastasis [34]. However, their result was inconsistent with this study. Yang *et al*. indicated that NPC patients with negative expression of DNA-PKcs tended to have a higher rate of lymph node metastasis than those with positive expression of DNA-PKcs [27] (**Figure 3A**).

Finally, we found that the DNA-PKcs expression level was not a significant prognostic factor in NPC patients treated with RT and combined chemotherapy. NPC can be frequently cured by RT. Thus, prognostic tests for the RT outcome based on biologic markers are of particular interest to radiation oncologists, especially for nondisseminated NPC. Therefore, we examined the DNA-PKcs molecule involved in the repair of damaged DNA, which was the component of DNA-PKcs, a key enzyme of DNA damage repair. The results indicated that the positive expression of DNA-PKcs provided a strong molecular marker of improved disease-free survival and overall survival in patients with NPC who were treated with IMRT or CRT. However, it is still controversial whether DNA-PKcs expression can be aprognostic factor for NPC patients with response to RT. Kasten *et al*. reported that there was nocorrelation between DNA-PKcs expression and radiosensitivity [35]. However, multivariate survival analysis indicated that the expression level of DNA-PKcs was not significantly associated with the survival of NPC patients after RT. These inconsistent results suggest that further studies are required to clarify these associations.

In conclusion, positive expression of DNA-PKcsmay be correlated with advanced TNM stages in patients with NPC. Therefore, we concluded that NPC patients with a higher expression level of DNA-PKcs have poorer disease-free survival and overall survival. DNA-PKcs may be a classical prognostic biomarker for disease-free survival and overall survival in NPC patients.

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Disclosure of conflict of interest

None.

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