Overexpression of Ku80 correlates with aggressive clinicopathological features and adverse prognosis in esophageal squamous cell carcinoma

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Abstract. Ku80, a subunit of the heterodymeric Ku protein, is clearly implicated in nonhomologous end joining DNA repair, chemoresistance and radioresistance in malignant tumors. In the present study, the clinicopathological significance of Ku80 in esophageal squamous cell carcinoma (ESCC) was investigated. The expression levels of Ku80 were determined by reverse transcription-quantitative polymerase chain reaction and immunohistochemistry in ESCC specimens and normal esophageal mucosa. The mRNA and protein levels of Ku80 were significantly higher in ESCC tissues than in normal esophageal mucosa, and were significantly associated with tumor differentiation, local invasion, lymph node metastasis and tumor-node-metastasis (TNM) stage. However, overexpression of Ku80 mRNA and protein levels were not significantly correlated with age, gender, tumor site or tumor size. Cox proportional hazards regression model demonstrated that tumor local invasion, lymph node metastasis, TNM stage and Ku80 mRNA and protein levels were independent risk factors indicating the overall survival of patients with ESCC. The present study demonstrated that aberrant Ku80 overexpression is observed in ESCC. In addition, high expression levels of Ku80 are associated with adverse clinicopathological features and unfavorable prognosis in ESCC patients.

Introduction

Esophageal squamous cell carcinoma (ESCC) is a malignant disease with uneven geographical distribution worldwide. Notably, the age of patients at the time of diagnosis is reducing (1). In East Asia (China, Japan and South Korea), ESCC has high incidence and mortality rates, and accounts for 90% of all cases of esophageal carcinoma (2). Despite advances in combined modality therapies for ESCC, including surgery, chemotherapy and radiotherapy, the prognosis for patients remains poor, and the 5-year overall survival rate is 20-30% (3). Although a number of genetic and epigenetic alterations associated with ESCC have been described (for example, p53, Bcl-2, epidermal growth factor receptor, RUNX3 and esophageal cancer related gene 1) (4-6), its oncogenesis and pathogenesis remain largely unknown. Optimal and commonly-used molecular markers to facilitate the comprehensive management of patients, including early diagnosis and prognostic evaluation, have not been proposed thus far. The prognosis of ESCC patients is tumor-node-metastasis (TNM) stage-specific, but the TNM stage is not sufficiently sensitive to evaluate the prognosis of patients (7). Therefore, the discovery of biomarkers for therapeutic development and prognostic prediction is required.

ESCC is characterized by genome instability through severe DNA damage caused by several factors, including the consumption of food or beverages at high temperatures, tobacco smoking, poor nutrition, infection and heredity (8). Ku80, one of the subunits of the Ku80/Ku70 heterodimer, is a central element in the nonhomologous end joining (NHEJ) DNA repair pathway. Double-strand DNA breaks (DSB) activate the catalytic subunit of the DNA-dependent protein kinase and trigger NHEJ repair activities (9). It has been demonstrated that the silencing of Ku80 proteins prevents DSB repair and telomere maintenance, and results in cell cycle arrest, apoptosis, chemosensitivity and radiosensitivity (10,11). Previous studies have demonstrated that Ku80 is associated with pathological processes in certain malignant tumors, and have provided valuable information regarding the expression levels of Ku80 and its pathological role in ESCC (11-14). Yang *et al* (11) reported that Ku80 participates in tumorigenesis, radioresistance and chemoresistance in esophageal cancer cells. Tonotsuka *et al* (14) observed that Ku80 protein was expressed in the nuclei of basal cell layers and luminal cell layers in esophageal cancer tissues. However, the protein expression pattern of Ku80 and its clinicopathological significance in ESCC is not well

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established. In the present study, the expression levels of Ku80 in ESCC tissues were analyzed, and the association of Ku80 expression with the clinicopathological features and prognosis of patients affected with ESCC were further investigated.

Materials and methods

Ethics statement. The current study protocol was reviewed and approved by the Research Ethics Committee of the Provincial Hospital Affiliated to Shandong University (Jinan, China) (2003-063). All participants provided written informed consent for the detection of Ku80 in the tissue-derived samples, subsequent data analysis and publication of the results.

Patients and samples. From January until May 2003, 119 patients with ESCC (41 females and 78 males; mean age, 57.8±12.3 years) were screened in the Provincial Hospital Affiliated to Shandong University. These patients were diagnosed with ESCC by histopathological detection at the Pathology Department of the hospital, precluding esophageal leiomyoma or other benign disease and malignant tumors originated from organs other than the esophagus. Eligibility was granted if primary diagnosis occurred ≤6 months prior to study enrollment and patients had not received chemotherapy, radiotherapy or biotherapy prior to sample collection. Full medical examinations were recorded, and the clinicopathological characteristics of the patients were analyzed. All the ESCC patients included in the present study were restaged according to the 2009 International Union Against Cancer TNM staging guidelines for esophageal cancer (15). The histopathological evaluation of the samples was performed according to the criteria proposed by the World Health Organization (16).

In the control group, 109 volunteers (35 females and 74 males; mean age, 56.6±13.2 years) from the Provincial Hospital Affiliated to Shandong University were screened as normal subjects without malignant disease. Their medical records indicated the absence of drug, tobacco and alcohol abuse. All individuals were Han Chinese without consanguineous relationships. Detailed questionnaires were completed by the subjects participating in the present study. The questionnaires collected information about the individuals, including medical and family history, use of over-the-counter medications and exposure to dietary carcinogens. The detailed questionnaires were used to measure average dietary intake 1 year prior to the date of selection for the current study. No significant differences were observed between the 119 ESCC patients and the 109 healthy volunteers (regarding age, gender, medical and family history, smoking status, exposure to dietary carcinogens and dietary habit). All patients fasted ≥12 h and had not smoked for 6 months prior to the collection of tissue samples.

In the ESCC group, 119 pairs of samples were collected from the 119 patients by gastroscopy. Each pair of samples consisted of ESCC tissue and corresponding healthy mucosa (CHEM).

The corresponding healthy esophageal mucosa was harvested from a position >5 cm in distance from the margin of the ESCC. A total of 109 normal esophageal mucosa (NEM) samples from the control group were harvested via gastroscopy. The macroscopic examination of the healthy mucosa revealed no signs of deterioration and necrosis. Light microscope examination demonstrated that the healthy mucosal tissues were free of tumor and any detectable concurrent disease, including esophagitis and dysplasia. Following encapsulation in tin foil wrapper, the tissue samples were rinsed in cold NaCl (0.9%), and immediately stored at -80˚C until further use.

RNA extraction and reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was isolated from esophageal mucosal tissues using TRIzol (Invitrogen Life Technologies, Carlsbad, CA, USA), according to the manufacturer's instructions. The RT system included 2 μg RNA, oligo-dT15 primer and M-MLV reverse transcriptase (Takara Bio, Inc., Shiga, Japan) in a volume of 20 μl diethylpyrocarbonate-treated water (Takara Shuzo Co., Ltd., Kyoto, Japan). Gene-specific primer sequences (Dingguo Changsheng Biotechnology Co., Ltd., Beijing, China) were as follows: Ku80, F 5'-ACGATTTGGTACAGATGGCACT-3' and R 5'-GCTCCTTGAAGACGCACAGTTT-3' (product, 497 bp); GAPDH, F 5'-GAAGGTGAAGGTCGGAGTC-3' and R 5'-GAAGATGGTGATGGGATTTC-3' (product, 300 bp). RT-qPCR was performed in a LightCycler 480 (Roche Diagnostics, Nutley, NJ, USA) under the following conditions: 1 cycle for 3 min at 94˚C followed by 30 cycles of 30 sec at 94˚C, 30 sec at 57˚C and 60 sec at 72˚C. The reactions were terminated at 4˚C, following 5-min elongation at 72˚C. The expression levels of GAPDH were used as an internal control for RNA quantity and quality. The relative quantity of mRNA (RQ) was calculated as the calibrator-normalized ratio using the LightCycler 480 software, version 1.5 (Roche Diagnostics), applying the following formula: $RQ = 2^{-\Delta\Delta Ct}$, where $\Delta\Delta Ct = (Ct_{targeted\ gene} - Ct_{GAPDH})$targeted sample - $(Ct_{targeted\ gene} - Ct_{GAPDH})$calibration sample. All the experiments were repeated ≥3 times, and included controls without cDNA, primers or thermophilic polymerase. The specificity of the assay was determined by PCR, using all the primer pairs on each cloned template cDNA to exclude cross-reactivity.

Immunohistochemistry (IHC). IHC staining for Ku80 was performed on 4-μm tissue sample sections using an UltraVision Quanto detection system (Thermo Fisher Scientific, Inc., Fremont, CA, USA) following the manufacturer's instructions. Briefly, the sections were incubated with antigen retrieval solution (pH 7.0; Beijing Solarbio Science & Technology Co., Ltd., Beijing, China) for 10 min at 95˚C followed by incubation with UltraVision hydrogen peroxide block (Thermo Fisher Scientific, Inc.) for 10 min to block any endogenous peroxidase activity. Next, the sections were incubated with UltraVision protein block (Thermo Fisher Scientific, Inc.) for 5 min to reduce nonspecific background staining. Following overnight incubation at 4˚C with a rabbit monoclonal antihuman Ku80 primary antibody (1:500; cat. no. ab80592; Abcam, Cambridge, UK), the specimens were washed with phosphate-buffered saline (PBS), and incubated with the primary antibody amplifier Quanto (Thermo Fisher Scientific, Inc.) for 10 min, followed by

10-min incubation with horseradish peroxidase polymer Quanto (Thermo Fisher Scientific, Inc.). Next, the sections were washed with PBS and deionized water, incubated with 3,3-diaminobenzidine (Dingguo Changsheng Biotechnology Co., Ltd.) and counterstained with hematoxylin (Beyotime Institute of Biotechnology, Nantong, China). The specificities of the primary antibodies had been previously confirmed, and their use as positive controls in HeLa cells has also been validated, since previous studies have demonstrated that Ku80 is overexpressed in these cells (17). The control sections were incubated with PBS instead of the primary antibodies and used as negative controls.

The immunohistochemical scoring of Ku80 was performed using a semiquantitative system as previously reported (18). The specimens were examined under a light microscope. In 5 randomly selected fields per section, the number of immunoreactive cells/100 cells was assessed and quantified as a percentage. Next, the average percentage of the 5 fields was used to assess the proportion score in a 6-category grading system (0, negative; 1, 1-10%; 2, 11-25%; 3, 26-50%; 4, 51-75%; and 5, >75%). The intensity score for staining was estimated using a 4-category grading system (0, negative; 1, weak; 2, moderate; and 3, strong). The IHC score was defined as the proportion score x the intensity score. The scientists were blinded to the patients data, and scored the samples independently, then reached agreement by repeated analysis and discussion.

Receiver operating characteristics (ROC) curve. The cut-off scores for Ku80 mRNA and protein overexpression levels were screened based on the ROC curve. The raw data corresponding to Ku80 mRNA and protein expression levels in the ESCC and the control groups were analyzed by the MedCalc statistical software package, version 13.0.2.0 (MedCalc Software bvba, Ostend, Belgium). The score closest to the point with maximum sensitivity and specificity was selected as the cut-off score leading to the largest number of patients correctly classified with or without overexpression of Ku80 mRNA and protein levels.

Statistical analysis. All statistical analyses were performed using SPSS software, version 17.0 (SPSS Inc., Chicago, IL, USA). Statistical comparisons between the Ku80 mRNA expression levels in the different groups were performed by analysis of variance (ANOVA). The Mann-Whitney U test was used to determine the differences in Ku80 protein expression levels across the groups. Associations between categorical variables were analyzed using the χ^2 test. The survival time was calculated from the date of the diagnosis until mortality or the end of the study. The survival curves were calculated by the Kaplan-Meier method. Univariate log-rank test and Cox regression model analysis were performed to identify prognostic factors. A 2-tailed P<0.05 was considered to indicate a statistically significant difference.

Results

High expression levels of Ku80 mRNA in ESCC tissues. The mRNA expression levels of Ku80 were examined in 119 pairs of samples from the ESCC group and in 109 samples from the control group. The relative mRNA expression levels of Ku80 in ESCC tissue, CHEM and NEM were 5.348±1.480, 3.327±1.106 and 3.149±1.092, respectively (Fig. 1A). The mRNA levels of Ku80 in ESCC were significantly higher than in CHEM and NEM (ANOVA; P<0.001). However, no clear difference in the mRNA levels of Ku80 between CHEM and NEM was observed (P=0.866). According to the ROC curve (Fig. 1B), the threshold value of 4.35 was the closest to the point with maximum sensitivity and specificity of 74.8 and 87.2%, respectively; thus, it was selected as the cut-off value. The area under the ROC curve (AUC) was 0.878 [95% CI, 0.829-0.918]. Therefore, samples with a calibrator-normalized ratio >4.35 were identified as high expression levels of Ku80 mRNA, whereas the remaining samples were considered to have low levels. Consequently, patients were divided into 2 groups; high (n=84, 70.6%) and low (n=35, 29.4%) mRNA expression groups. However, in the control group there were 20 (18.3%) cases of high expression of Ku80 mRNA and 89 (81.7%) cases of low expression. Overall, the frequency of high Ku80 mRNA expression levels was significantly increased in ESCC compared with NEM (χ^2 test; P<0.001) (data not shown).

High expression levels of Ku80 protein in ESCC tissues. The IHC scores in ESCC tissue, CHEM and NEM were 9.656±4.633, 5.608±3.759 and 5.532±3.741, respectively (Fig. 1C). The IHC scores of Ku80 in ESCC was significantly higher than those in CHEM and NEM (Mann-Whitney U test; P<0.001). However, there was no clear difference in IHC scores between CHEM and NEM (P=0.268). According to the ROC curve analysis (Fig. 1D), the cut-off score was 9, with maximum sensitivity of 63.0% and specificity of 87.2%. The AUC was 0.756 (95% CI; 0.695-0.811). Consequently, the patients were divided into 2 groups; high (n=75, 63.0%) and low (n=44, 37.0%) protein expression groups. However, there were 18 (15.1%) cases of high expression of Ku80 mRNA and 101 (84.9%) cases of low expression in the control group. The difference in frequency of high protein expression levels between the ESCC tissues and NEM was observed to be statistically significant (χ^2 test; P<0.001). Spearman bivariate correlation indicated a positive correlation between the mRNA and the protein expression levels of Ku80 (r=0.923; P<0.001) (data not shown). Visual analysis of the IHC staining was performed, and positive expression of Ku80 protein was revealed as yellow or brownish yellow stain in the nuclei of tumor cells. Strong positive Ku80 staining was observed in the cancer cell nuclei, whereas negative or weak staining was observed in CHEM and NEM (Fig. 2).

Clinicopathological characteristics of ESCC patients and Ku80 expression levels. The clinicopathological features (age, gender, tumor site, tumor size, differentiation degree, local invasion, lymph node metastasis and TNM stage) of the 119 patients with ESCC in the current study are summarized in Table I. χ^2 analysis indicated that the Ku80 mRNA expression levels positively correlated with differentiation degree (P=0.016), local invasion (P=0.016), lymph node metastasis (P=0.002) and TNM stage (P=0.001), but not with age (P=0.840), gender (P=0.980), tumor location (P=0.351) or tumor size (P=0.407) (Table I). In agreement with the mRNA

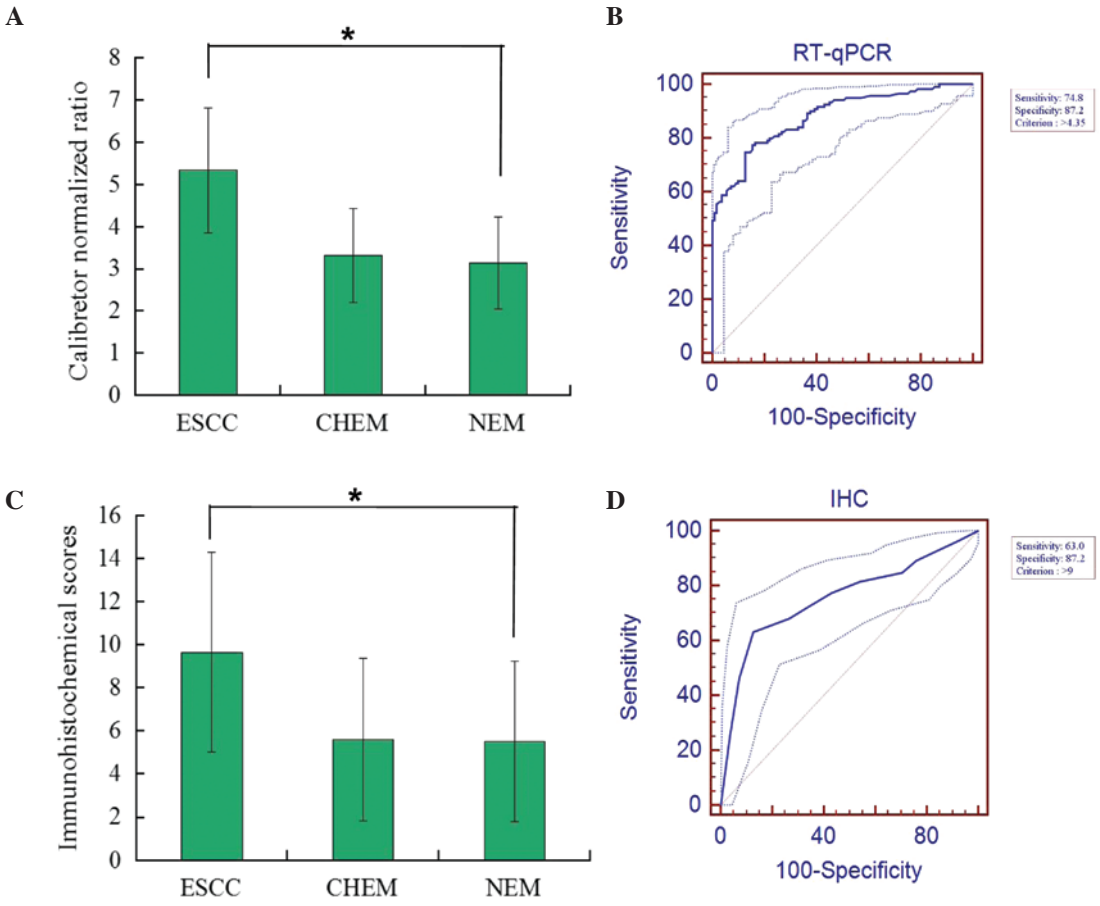

Figure 1. Ku80 is overexpressed in ESCC. (A) RT-qPCR assay of Ku80 mRNA expression levels in 119 cases of ESCC vs. CHEM tissues and 109 cases of NEM. *P<0.001, ESCC vs. NEM. (B) ROC curve analysis for Ku80 mRNA expression levels and selection of the cut-off score. (C) IHC assay of Ku80 protein expression levels in ESCC tissues vs. CHEM and NEM. *P<0.001, ESCC vs. NEM. (D) ROC curve analysis for Ku80 protein expression levels and selection of the cut-off score. The data are presented as the mean ± standard deviation. ESCC, esophageal squamous cell carcinoma; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; CHEM, corresponding healthy mucosa; NEM, normal esophageal mucosa; ROC, receiver operating characteristics; IHC, immunohistochemistry.

expression pattern, high expression levels of Ku80 protein presented significant correlations with differentiation degree (P=0.001), local invasion (P=0.004), lymph node metastasis (P=0.007) and TNM stage (P=0.002), but no correlation was observed between the protein expression levels and age (P=0.849), gender (P=0.842), tumor location (P=0.906) or tumor size (P=0.113).

Ku80 expression levels are associated with prognosis in patients with ESCC. Patients with ESCC were followed during for a median period of 65.8 months, ranging from 5 to 132 months. The overall 1-, 3-, 5- and 10-year survival rates of the 119 patients were 75.6, 54.6, 42.0 and 5.0%, respectively. The median survival time was 46.0 months (95% CI, 32.8~59.2 months). As indicated in Table II and Fig. 3, univariate analysis revealed that local invasion (P=0.014), lymph node metastasis (P<0.001), TNM stage (P<0.001) and Ku80 mRNA and protein levels (P<0.001) were all significant prognostic factors. However, the age of the patient (P=0.406), gender (P=0.719), tumor location (P=0.248), tumor size (P=0.339) and differentiation degree (P=0.241) were not observed to be significant (Table II). Low aggressive local invasion, negative lymph node metastasis, early TNM stage and low Ku80 mRNA and protein expression levels implicated significantly improved prognosis. To exclude confounding factors, the Cox proportional hazards model was used to identify factors associated with overall survival of patients with ESCC. Multivariate analysis revealed that local invasion (P=0.011), lymph node metastasis (P=0.009), TNM stage (P<0.001), Ku80 mRNA and protein levels (P=0.024 and 0.007, respectively) were independent significant prognostic factors.

Discussion

Numerous literature reports have demonstrated genomic and proteomic changes in ESCC (4-7). However, the processes underlying the carcinogenesis and progression of ESCC remain unclear. A previous study indicated that genome instability through severe DNA damage is associated with the carcinogenesis and development of ESCC (8). Ku80 is involved in telomere maintenance, maintenance of chromosomal integrity, recombination of the V, D and J segments during immunoglobulin production, regulation of glucose-regulated peptide 78 gene transcription and cell survival (19-22). Additionally, a number of notable findings regarding the involvement of the Ku80 gene in cancer have been reported previously: The overexpression of Ku80 is clearly associated with the carcinogenesis and the progression of several types

Table I. Associations between Ku80 expression levels and multiple clinicopathological parameters in ESCC.

		Ku80 mRNA levels			Ku80 protein levels		
Characteristics	Cases (119)	Low (35)	High (84)	P-value	Low (44)	High (75)	P-value
Age (years)				0.840			0.849
≥60	54	15	39		19	35	
<60	65	20	45		25	40	
Gender				0.980			0.842
Male	78	23	55		28	50	
Female	41	12	29		16	25	
Tumor location				0.351			0.906
Upper	12	3	9		4	8	
Middle	77	26	51		28	49	
Lower	30	6	24		12	18	
Tumor size				0.407			0.113
≥50 mm	41	10	31		11	30	
<50 mm	78	25	53		33	45	
Differentiation degree				0.016			0.001
G1	29	14	15		19	10	
G2	57	16	41		17	40	
G3	33	5	28		8	25	
Local invasion				0.016			0.004
T1+ T2	57	23	34		29	28	
T3+ T4	62	12	50		15	47	
Lymph node metastasis				0.002			0.007
Positive	71	13	58		19	52	
Negative	48	22	26		25	23	
TNM stage				0.001			0.002
I + II	53	24	29		28	25	
III + IV	66	11	55		16	50	

ESCC, esophageal squamous cell carcinoma; TNM, tumor-node-metastasis.

of invasive cancer, including bladder carcinoma (12), gastric carcinoma (13), colorectal carcinoma (23) and breast carcinoma (24). These results suggest that Ku80 may act as an oncogene in various types of cancer.

In the present study, the role of Ku80 in ESCC was explored. It was demonstrated that the mRNA expression levels of Ku80 were increased in ESCC tissues compared with those in CHEM and NEM (Fig. 1). The RT-qPCR results were confirmed by IHC, which revealed that the immunohistochemical scores of Ku80 protein in ESCC were also higher than in CHEM and NEM. Spearman bivariate correlation analysis indicated that the expression levels of Ku80 mRNA were positively correlated with Ku80 protein expression levels. This is consistent with the results reported by Yang *et al* (11) and Tonotsuka *et al* (14), who demonstrated that Ku80 was overexpressed in ESCC tissues and cell lines, and suggested that Ku80 may participate in the development of malignant clinicopathological processes in ESCC.

To further explore the clinicopathological significance and prognostic value of Ku80, the 119 ESCC patients were divided into 2 groups of high and low expression, based on the levels of Ku80 mRNA and protein expression. It is difficult to determine the extent to which Ku80 expression is pathologically and clinically relevant, as previous studies had defined the expression levels of Ku80 using different classification systems (10-12). There was no universal and commonly accepted classification system, and the cut-off score was often set arbitrarily. This may lead to numerous errors in studies aiming to evaluate the association of Ku80 expression levels with the clinicopathological features of ESCC. In the present study, an objective, rapid and reproducible scoring method to set the cut-off score based on a ROC curve analysis was used. The value closest to the point with maximum sensitivity and specificity was selected as the cut-off score. By ROC curve analysis, the largest number of ESCC patients was correctly classified into different groups using statistical significance. Thus, there were 84 and 35 patients with ESCC in the high and low Ku80 mRNA level groups, respectively, and 75 and 44 patients in the high and low Ku80 protein level groups, respectively. χ^2 test indicated

Table II. Univariate and multivariate survival analyses for patients with ESCC.

	Univariate analysis		Multivariate analysis		
Variable	χ^2	P-value	HR	95% CI	P-value
Age (years)					
≥60 vs. <60	0.689	0.406	-	-	-
Gender					
Male vs. female	0.129	0.719	-	-	-
Tumor location					
Upper vs. middle vs. lower	2.791	0.248	-	-	-
Tumor size (mm)					
≥50 vs. <50	0.916	0.339	-	-	-
Differentiation degree					
G1 vs. G2 vs. G3	2.848	0.241	-	-	-
Local invasion					
T1+T2 vs. T3+T4	6.075	0.014	1.706	1.133-2.568	0.011
Lymph node metastasis					
Positive vs. negative	29.168	<0.001	1.898	1.173-3.073	0.009
TNM stage					
I+II vs. III+IV	26.936	<0.001	2.612	1.638-4.163	<0.001
Ku80 mRNA levels					
Low vs. high	13.166	<0.001	1.701	1.074-2.694	0.024
Ku80 protein levels					
Low vs. high	17.169	<0.001	1.765	1.164-2.676	0.007

ESCC, esophageal squamous cell carcinoma; HR, hazard ratio.

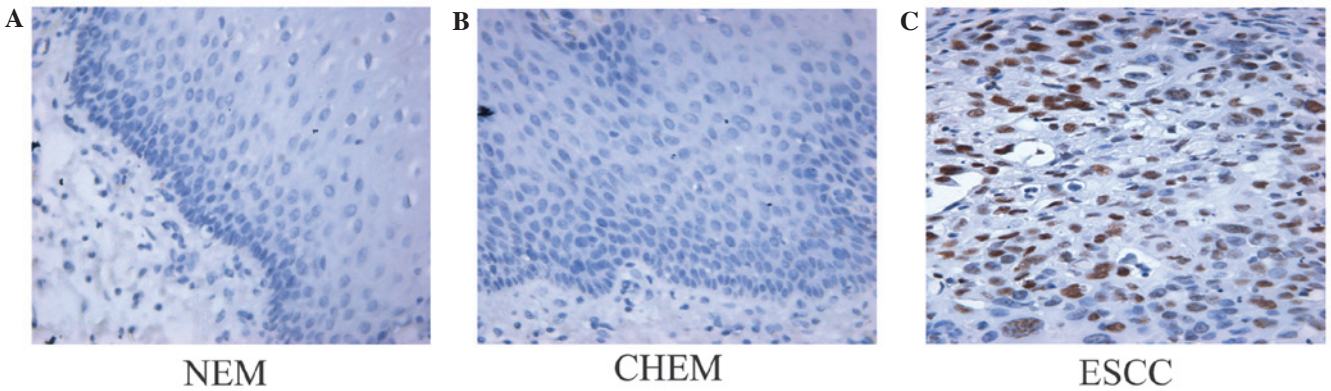

Figure 2. Immunohistochemical staining of Ku80 in ESCC specimens. (A) Representative image of negative Ku80 protein expression in NEM at a magnification of x400. (B) Representative image of negative Ku80 protein expression in CHEM at a magnification of x400. (C) Representative image of negative Ku80 protein expression in ESCC tissues at a magnification of x400 (right panel). NEM, normal esophageal mucosa; CHEM, corresponding healthy mucosa; ESCC, esophageal squamous cell carcinoma.

that the frequency of overexpression was significantly higher in ESCC tissues than in NEM. Additionally, high expression levels of Ku80 were significantly associated with adverse clinicopathological features, including low differentiation degree, aggressive local invasion, lymph node metastasis and advanced TNM stages. Consistent with these results, the data from the IHC analysis indicated that increased Ku80 protein expression levels were associated with low differentiation degree, aggressive local invasion, lymph node metastasis and advanced TNM stages. However, no correlation was observed between the Ku80 mRNA or protein expression levels and several other clinicopathological parameters, including age, gender, tumor location and tumor size, in the current patient cohort (Table I). These findings are consistent with previous reports, which demonstrated that Ku80 expression was associated with important clinicopathological characteristics

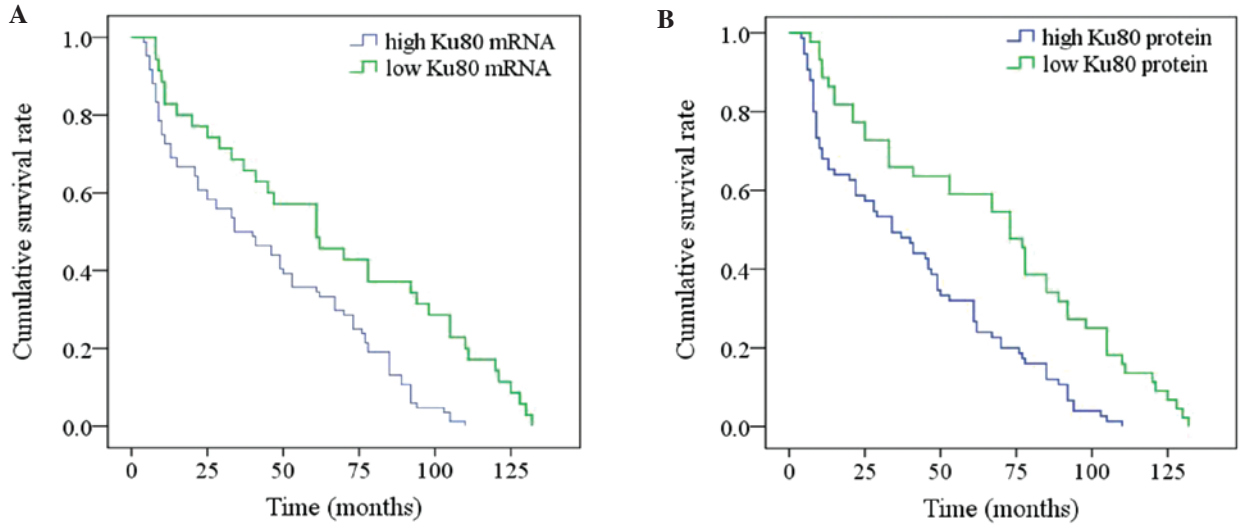

Figure 3. Kaplan-Meier graphs representing the probability of cumulative overall survival in patients with ESCC based on Ku80 expression. High Ku80 (A) mRNA and (B) protein levels were significantly associated with reduced overall survival. ESCC, esophageal squamous cell carcinoma.

in lung adenocarcinoma (25), colorectal cancer (26) and breast cancer (13). These findings suggested that Ku80 was a *bona fide* oncogene that may be used as a biomarker and a therapeutic target in ESCC.

Previous studies evaluating the prognostic significance of Ku80 expression levels in several types of human cancer reported different conclusions: In lung adenocarcinoma, Ma *et al* (25) demonstrated an association between Ku80 expression levels and patient outcome in terms of progression-free survival or overall survival. In colorectal cancer, Grabsch *et al* (26) did not observe any association between high expression levels of Ku80 and long-term survival. In studies of cases of breast cancer, although Alshareeda *et al* (27) have reported an association with disease-free survival, other authors did not observe such an association (28). These results may reflect the complexity of the DNA damage repair mechanisms in different types of cancer. Therefore, the role of Ku80 in different pathways and networks and its effect on clinical outcome should be analyzed in this complex context. In order to investigate whether Ku80 may be pursued as a novel biomarker for prognostic prediction in patients with ESCC, the prognosis of 119 patients were analyzed in the present study using the Kaplan-Meier method. The current data indicated that the long-term survival of the patients was associated with low aggressive local invasion, negative lymph node metastasis, early TNM stage and low Ku80 mRNA and protein expression levels. Notably, multivariate analysis demonstrated that local invasion, lymph node metastasis, TNM stage and Ku80 mRNA and protein expression levels had independent prognostic influence in patients with ESCC. Therefore, assessment of Ku80 expression in ESCC may provide valuable information regarding the outcome and follow-up management.

Nevertheless, there were various limitations to the present study as follows: Expression of Ku80 in the esophageal mucosa of the individuals investigated may be differentially induced by DNA-damage mutagens. Although numerous potential mutagens, including dietary habits, medicine, alcohol and tobacco were eliminated, the expression levels of Ku80 are affected by environmental mutagens to different extents. The disease-associated alterations in diet and medication were of small concern, since the patients were diagnosed 6 months prior to the study and did not receive any therapeutic treatment. Human Ku80 is strictly regulated by complex transcriptional and translational mechanisms (17,21). Furthermore, genetic differences among patient cohorts of different geographical origins may result in differences in Ku80 expression. One limitation of the present study is that all participants, who are Han Chinese, come from the same geographical area and have similar genetic background. Adjuvant chemotherapy or radiotherapy may also affect the prognosis of patients. Regarding the treatment modalities, their efficacy was not discussed, since there was no randomized clinical trial. Another limitation of the present study is the limited sample size of the controls used to evaluate the role of Ku80 expression in ESCC. Although, to the best of our knowledge, this is the first report demonstrating that the expression of Ku80 is an independent prognostic factor in patients with ESCC, questions remain unanswered regarding the mechanism of action of Ku80 during the initiation and progression of ESCC. Therefore, further studies with larger samples of cases at the various stages of ESCC are warranted to assess the clinicopathological significance of Ku80.

In conclusion, the present study provides unique perspectives regarding the involvement of Ku80 in esophageal carcinogenesis. The data indicate that Ku80 is overexpressed in a significant proportion of cases of ESCC, and it is associated with key clinicopathological features and patient prognosis. In conclusion, the present study provides evidence about the unrecognized roles that Ku80 may perform in tumorigenesis, and its potential use as a novel biomarker and therapeutic target for ESCC.

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