Ku80 Is Differentially Expressed in Human Lung Carcinomas and Upregulated in Response to Irradiation in Mice

Jian Ye,^{1,*} Zhenyi Ren,^{1,*} Qing Gu,² Limin Wang,¹ and Jiaoli Wang¹

Based on the role of Ku80 in mediating radiation-induced DNA repair, we investigated Ku80 expression in human lung cancers of different pathological types and evaluated the effect of radiotherapy on Ku80 expression levels in a mouse model. We used immunohistochemistry and real-time PCR to determine Ku80 protein and mRNA levels, respectively. We inoculated nude mice with A549 cells and subjected the tumor-bearing mice to varying doses of irradiation. Lung carcinoma tissue exhibited higher Ku80 mRNA and protein levels when compared with normal tissue. Among the tumor subtypes, lung adenocarcinoma and lung squamous carcinoma showed higher levels of Ku80 protein and mRNA, compared with small-cell lung carcinoma. There was a dose-dependent and time-dependent increase in *Ku80* mRNA levels in nude mice that were inoculated with A549 cells and exposed to varying doses of irradiation. Ku80 may play an important role in the DNA damage response pathway. Higher Ku80 levels in lung squamous carcinoma and adenocarcinoma may explain their lower radiosensitivity when compared with small-cell lung carcinoma. Ku80 expression levels could be useful in predicting radiosensitivity of lung tumors and inhibition of Ku80 may be an interesting target to improve radiosensitivity in lung cancer patients.

Introduction

LUNG CANCER, which has the highest mortality among all malignancies worldwide, has a current 5-year survival rate of <15% and constitutes a major threat to human health (Greenlee *et al.*, 2000; Xing *et al.*, 2008). Radiotherapy with gamma irradiation is one important strategy used in the treatment of lung cancers and has been shown to cause DNA damage, cell cycle arrest, and apoptosis. However, a number of tumors develop radioresistance, which is an important reason for poor outcome and therapeutic failure among lung cancer patients (Komuro *et al.*, 2002). Cancers of different pathological types are also known to differ in their sensitivity to radiation therapy and their ability to repair radiation injury (Nakagawa *et al.*, 2003; Bergqvist *et al.*, 2004).

DNA double-strand breaks (DSBs) resulting from ionizing radiation are among the most severe types of DNA damage (Xing *et al.*, 2008), with unrepaired DSBs leading to oncogenic transformation and genetic instability. The two major pathways for DSB repair are homologous recombination and nonhomologous end joining (NHEJ) (Kotnis *et al.*, 2009). The NHEJ machinery plays a more prominent role in most mammalian cells and consists of the catalytic subunit of DNA protein kinase (DNA-PK), Ku70/Ku80, Artemis, XRCC4, and DNA ligase IV (Collis *et al.*, 2005). Single-nucleotide polymorphisms within the NHEJ genes are

known to modulate the risk and prognosis of non-small-cell lung carcinoma (Tseng *et al.*, 2009).

The heterodimeric Ku antigen, which acts as a molecular detector of DSBs, consists of two subunits of 70 kDa (Ku70) and 80 kDa (Ku80 or Ku86) and has been reported to activate DNA-PK by binding directly to free DNA termini in a non-sequence-specific manner (Chai *et al.*, 2002; Postow *et al.*, 2008; Suzuki *et al.*, 2010). Removal of Ku from the DSB is thought to occur via a ubiquitin-mediated mechanism (Postow *et al.*, 2008). Cells deficient in DNA-PK or *Ku80* have been reported to have defective DSB repair systems (Gu *et al.*, 1997).

Expression of Ku protein was previously shown to be upregulated in human invasive breast cancer and bladder cancer cells when compared with normal tissues (Pucci *et al.*, 2001), suggesting that it played a role in tumor progression. A number of studies have explored the effect of *Ku* deficiency on radiosensitivity of different cell lines. Human cervical cancer cells (Hela), human colon cancer cells (HCT116), and human mammary epithelial cells (MCF10A) with siRNA-mediated knockdown of *Ku* all displayed increased radiosensitivity (Ayene *et al.*, 2005; Vandersickel *et al.*, 2010). It was also reported that Tob1-mediated downregulation of *Ku70* and *Ku80* resulted in extremely radiation-responsive phenotypes (Jiao *et al.*, 2007). Previous reports showed that the xrs-5 cell line, derived from Chinese hamster ovary cells

¹Department of Pneumology and ²Emergency Department, The First People's Hospital, Hangzhou, China.

*These authors equally contributed to this work.

(CHOK1) carried a mutation of the *Ku80* gene and was extremely radiosensitive, whereas overexpression of the *Ku80* gene in these cells decreased their sensitivity to radiation damage to levels comparable with the parental CHOK1 cells (Singleton *et al.*, 1997; Matheos *et al.*, 2003). Downregulation of *Ku70* and *Ku80* in A549 cells treated with roscovitine in combination with radiotherapy sensitized them to radiotherapy, possibly because of inhibition of DNA-PK complex formation and subsequent inhibition of DNA repair (Zhang *et al.*, 2008).

In vivo, decreased expression of *Ku80* was shown to result in reduced DNA repair capacity and increased sensitivity of tumor cells to ionizing radiation (Komuro *et al.*, 2002). Taken together, these data suggest that targeted knockdown of *Ku80* in lung adenocarcinoma cells could lead to increased radiosensitivity in these tumors and that analysis of *Ku80* gene structure and function will be valuable in understanding the molecular mechanisms underlying radiosensitivity of lung tumors.

As different cancers vary in their ability to respond to radiation and in their DNA damage response pathways, we elected to study the expression of *Ku80* in different histological types of lung cancer. We used immunohistochemistry and real-time PCR to evaluate *Ku80* protein and mRNA levels in normal human lung tissues, lung squamous cell carcinoma, adenocarcinoma, and small-cell lung cancer tissues. We also irradiated nude mice that were inoculated with A549 human lung adenocarcinoma cells and compared *Ku80* protein and mRNA expression levels before and after radiotherapy. Our findings lead us to speculate that *Ku80* may play a role in repair of radiation injury.

Materials and Methods

Collection of human lung cancer tissue

Malignant tumor tissues were collected from 24 patients with primary lung cancer who underwent surgery for lung cancer at the Hangzhou First People's Hospital between January 2008 and October 2009. None of the patients received anticancer therapy prior to admission. Based on the WHO (2004) (Travis, *et al.*, 2004) classification of lung tumor pathology, the samples consisted of 10 adenocarcinoma tissues, 8 squamous cell carcinoma tissues, and 6 small-cell carcinoma tissues. We also collected six normal lung tissues as negative controls. These tissues were taken from the adjacent normal tissues (at a distance of 5 cm from the tumor) in patients with adenocarcinoma or squamous carcinoma and confirmed by pathology as normal lung tissues. The harvested tissue was divided into two pieces and stored at -70°C for immunohistochemical and real-time PCR analysis of *Ku80* protein and mRNA expression, respectively. The human study was approved by the institutional review board of the First People's Hospital, Hangzhou, and written informed consent was obtained from all the patients.

Reagents and equipment

The *Ku80* rabbit polyclonal antibody was obtained from Bioworld Technology, Inc. The PV-6000 two-step immunohistochemistry kit and reagents were purchased from Beijing ZhongShan Golden Bridge Biotechnology Co. PBS was used as the negative control. The PrimeScript One Step reverse

transcription PCR kit was purchased from TAKARA. Quantitative PCR was performed with the One Step SYBR PrimeScript reverse transcription PCR kit (Perfect Real Time; TAKARA) on an ABI 7500 quantitative PCR Instrument (Life Technologies). PCR primers were purchased from Shanghai Biological Engineering Co. The Nikon eclipse 80i optical microscope was used to visualize immunohistochemical images and the Carl Zeiss Imaging System software (Carl Zeiss, Inc.) was used for image analysis. Radiotherapy was performed with the Siemens mevatron KD2 linear accelerator.

Immunohistochemistry

Immunohistochemical detection of *Ku80*. Tissues were fixed overnight in 4% paraformaldehyde and embedded in paraffin. Tissue sections (4 μm thickness) were evaluated for *Ku80* protein expression using the two-step method. Briefly, the sections were baked at 60°C for 2 h and then dewaxed and washed with distilled water for 2 min. The sections were fixed with 0.01 M citrate buffer (pH 6.0) using the pressure cooker method and then cooled. The sections were then incubated with 3% H_2O_2 peroxidase blocking solution for 10 min, washed, and incubated with primary anti-*Ku80* antibody (1:100; Bioworld Technology, Inc.) for 1 h at 37°C . Sections were washed extensively and incubated with secondary antibody (Dako Elivision™ Plus Two-step System PV-6000 kit; Zymed, Co.). The sections were washed extensively and color was developed with DAB (Sigma-Aldrich) for 2 min. The sections were then stained, fixed, and visualized. Primary antibody was replaced by PBS in the negative control samples.

Scoring of immunohistochemical staining. Nuclei with light yellow, brownish yellow, or brown granular staining of *Ku80* protein were scored positive. The negative control samples demonstrated only blue-stained nuclei. The Carl Zeiss Imaging Systems High-Definition Color pathology report management system was used for quantitative analysis of *Ku80* protein expression. The average optical density, positive staining area, and total area were measured in five nonoverlapping, randomly selected, high-magnification fields (400 $\times$) and the positive area ratio was calculated. The average optical density and the average positive area ratio of five fields per section were also calculated as previously described (Thapa *et al.*, 2004). Average optical density is equal to average optical density of positive stained area, which reflects the positive intensity of area expressing *Ku80* protein. The positive area ratio is calculated as (the total positive area per unit area/the total cell area per unit area) $\times 100\%$, which reflects the scope of area with positive *Ku80* expression.

Real-time fluorescence quantitative PCR. Samples were flash frozen in liquid nitrogen, ground, and incubated in 1 mL precooled TRIzol reagent (Life Technologies) at room temperature for 5 min. RNA was extracted according to the manufacturer's instructions and resuspended in 20 μL DEPC water. Total RNA (2 μg) was reverse transcribed according to the manufacturer's instructions in the presence of first-strand reaction buffer, oligo dT primer, and reverse transcriptase at 37°C for 20 min. The reaction was terminated by heating to

85°C for 15 s and the cDNA was stored at -20°C. The *Ku80* primer sequences used were (F) 5' TGGTGGCGTCGGG GAATA 3' and (R) 5' CAGAAAGGGGATTGTCAGTGC 3'. The β -actin primer sequences used were (F) 5' TGGCACC CAGCACAATGAA 3' and (R) 5' CTAAGTCATAGTCCGC CTAGAAGCA 3'. The expected sizes of amplified *Ku80* and β -actin products were 207 and 186 bp, respectively. Intron-spanning primers were used to rule out genomic contamination. PCR conditions for *Ku80* amplification were denaturation at 94°C for 5 min followed by 40 cycles of 15 s at 94°C and 45 s at 60°C. The sizes of the amplicons were confirmed by gel electrophoresis. The fluorescence value and melting curves were detected at 60°C. *Ku80* expression in the control group was set as 1.000 and the relative expression levels of *Ku80* in the experimental groups were calculated. Quantitative analysis was done using the $2^{-\Delta\Delta CT}$ value of each experimental group ($2^{-\Delta\Delta CT}$ was the relative change in *Ku80* expression in the experimental groups compared with the control group) (Livak and Schmittgen, 2001). The specificity of the PCR products was confirmed each time by the melting curve assay.

Animals and cells. Forty-two pathogen-free female BALB/c nude mice (age: 4 weeks; weight: 20 ± 5 g) were purchased from the Animal Center of Zhejiang University of Traditional Chinese Medicine. The human lung adenocarcinoma A549 cell line was provided by the Experimental Center of Zhejiang University of Traditional Chinese Medicine. The animal study was approved by the Institutional Animal Care and Use Committee of the First People's Hospital, Hangzhou.

Experimental design

Inoculation of nude mice with A549 tumor cells. A549 lung adenocarcinoma cells were cultured in DMEM medium supplemented with 10% calf serum in the presence of 5% carbon dioxide (both from Life Technologies). Cells in the logarithmic phase of growth were trypsinized and harvested in DMEM containing 10% calf serum. The cell density was adjusted to 5×10^7 mL⁻¹ and 42 female nude mice were subcutaneously injected in the lateral left lower extremity with 0.2 mL of the cell suspension. Subcutaneous tumors grew to a size of 1 cm $\times$ 1 cm at approximately 3 weeks after inoculation.

Irradiation of tumor-bearing mice. The mice were randomized into seven groups: one control group ($n=6$) and six radiation groups ($n=6$ in each group). Group 1 was the control group. Groups 2 and 3 mice were sacrificed at 24 and 48 h after receiving 1 Gy irradiation, which were designated as 1 Gy/24 h and 1 Gy/48 h, respectively. Groups 4 and 5 mice were sacrificed at 24 and 48 h after receiving 4 Gy irradiation, which were designated as 4 Gy/24 h and 4 Gy/48 h, respectively. Groups 6 and 7 mice were sacrificed 24 and 48 h after receiving 8 Gy irradiation, which were designated as 8 Gy/24 h and 8 Gy/48 h, respectively. Tumor tissues were collected and divided into two pieces for quantification of Ku80 protein and mRNA by immunohistochemical staining and quantitative PCR, respectively.

Statistical analysis

Continuous data, including age and Ku80 expression, were summarized by mean $\pm$ standard deviation (SD). Because of the small sample size in each group of mice or patients, nonparametric Kruskal-Wallis tests and Mann-Whitney tests were performed for the comparisons in all groups and *post hoc* tests for pairwise groups. Categorical data, including patients' gender and tumor stages, were summarized by count (percentage); the associations between them and the groups were tested by Fisher's exact test. The comparisons between control normal lung tissues and the carcinoma groups within patients were performed by nonparametric Wilcoxon signed-rank test. All statistical analyses were set with a significance level of 0.05 and performed using SPSS 15.0 statistics software (SPSS, Inc.).

Results

Ku80 expression in lung cancer

Of the 24 lung cancer patients who participated in this study, there were 10 cases of adenocarcinoma, 8 cases of squamous carcinoma, and 6 cases of small-cell carcinoma. The mean ages of the three groups were 53.20 ± 9.65 , 65.63 ± 5.93 , and 53.00 ± 9.01 years, respectively (Table 1). The patients with squamous carcinomas were significantly older than the other two groups. There were no significant differences in gender and tumor stages between the three

TABLE 1. SUMMARY OF DEMOGRAPHIC DATA, CLINICAL DATA, AND Ku80 EXPRESSION IN HUMAN LUNG CANCER TISSUE

	Adenocarcinoma (n=10)	Squamous carcinoma (n=8)	Small-cell carcinoma (n=6)	p-Value
Age (years)	53.20 $\pm$ 9.65	65.63 $\pm$ 5.93 ^a	53.00 $\pm$ 9.01	0.016*
Gender				
Female	6 (60.0%)	1 (12.5%)	3 (50.0%)	0.134
Male	4 (40.0%)	7 (87.5%)	3 (50.0%)	
Tumor stage				
I	5 (50.0%)	5 (62.5%)	3 (50.0%)	0.625
II	2 (20.0%)	3 (37.5%)	2 (33.3%)	
III	3 (30.0%)	0 (0.0%)	1 (16.7%)	
Ku80 expression				
Positive area rate (%)	32.57 $\pm$ 7.66	29.70 $\pm$ 9.52	13.07 $\pm$ 3.28	0.001*
Optical density	0.38 $\pm$ 0.02	0.40 $\pm$ 0.01	0.39 $\pm$ 0.04	0.091
<i>Ku80</i> mRNA expression ($2^{-\Delta\Delta CT}$)	91.75 $\pm$ 85.19	84.21 $\pm$ 42.67	7.35 $\pm$ 2.10	0.001*

* $p < 0.05$ by Kruskal-Wallis test.

^aSignificant difference compared with the adenocarcinoma group.

FIG. 1. Immunohistochemical evaluation of Ku80 protein expression in human lung cancer tissues. All images are shown at 400 $\times$ magnification. (A) Normal human lung tissues. (B) Human adenocarcinoma. (C) Human squamous cell carcinoma. (D) Human small-cell lung cancer.

FIG. 2. Bar charts for Ku80-positive area rates in human lung cancer tissues. (A) Comparison of Ku80-positive area rate between normal tissue and human lung cancer tissue (average of adenocarcinoma, squamous cell carcinoma, and small-cell carcinoma). (B) Comparison of Ku80-positive area rate among the three different carcinomas. Data are expressed as mean $\pm$ standard deviation (SD).

groups. Table 1 presents the relative *Ku80* mRNA levels based on tumor type. Our data showed significantly lower levels of *Ku80* mRNA in small-cell carcinomas compared with adenocarcinomas ($p < 0.001$).

We used immunohistochemistry to show that Ku80 protein was expressed at low levels in normal human lung tissues (Fig. 1A). We showed nuclear brown-yellow granular staining of Ku80 protein in human adenocarcinoma (Fig. 1B) and brown-yellow granular staining distributed in clumps in human squamous cell carcinoma (Fig. 1C). Human small-cell cancer tissue was also positive for Ku80 (Fig. 1D).

We evaluated differences between normal tissue and carcinoma tissue in the expression of Ku80 protein (Figure 2A). We used gel electrophoresis to confirm the size of the Ku80 amplicons (Figure 3A), and the differences between normal tissue and carcinoma tissue in the Ku80 mRNA expression were subsequently explored (Figure 3B). Our data showed that carcinoma tissues had significantly higher Ku80-positive areas and $2^{-\Delta\Delta CT}$ values when compared with normal tissues (Figs. 2A and 3B, respectively). However, small-cell carcinoma tissues had significantly lower Ku80-positive ar-

eas and $2^{-\Delta\Delta CT}$ values when compared with adenocarcinoma and squamous carcinoma tissues (positive area rate: 13.07 ± 3.28 in small-cell carcinoma vs. 32.57 ± 7.66 in adenocarcinoma and 29.70 ± 9.52 in squamous carcinoma; $2^{-\Delta\Delta CT}$: 7.35 ± 2.10 in small-cell carcinoma vs. 91.75 ± 85.19 in adenocarcinoma and 84.21 ± 42.67 in squamous carcinoma) (Figs. 2B and 3C, respectively). We found no significant difference in Ku80 optical density when we compared normal tissues with carcinoma tissues. There was also no significant difference in Ku80 optical density between the three carcinoma groups (data not shown).

Ku80 expression in nude mice inoculated with A549 cells and irradiated

We evaluated *Ku80* mRNA levels in nude mice that were inoculated with A549 cells and exposed to irradiation. Ku80 expression levels in mice from the seven experimental groups are summarized in Table 2. All six radiotherapy groups had significantly higher levels of Ku80 expression reflected by higher positive area rates, optical density, and $2^{-\Delta\Delta CT}$ values when compared with the control group. Mice exposed to

FIG. 3. (A) Gel electrophoresis to confirm the size of the *Ku80* PCR amplicon. (B) Comparison of *Ku80* mRNA expression ($2^{-\Delta\Delta CT}$) between normal tissue and human lung cancer tissue (average of adenocarcinoma, squamous cell carcinoma, and small-cell carcinoma). (C) Comparison of *Ku80* mRNA expression ($2^{-\Delta\Delta CT}$) among the three different carcinomas. Data are expressed as mean $\pm$ standard deviation (SD).

TABLE 2. SUMMARY OF KU80 EXPRESSION IN NUDE MICE INOCULATED WITH A549 CELLS AND SUBJECTED TO RADIOTHERAPY

	Control (n=6)	1 Gy/24 h (n=6)	1 Gy/48 h (n=6)	4 Gy/24 h (n=6)	4 Gy/48 h (n=6)	8 Gy/24 h (n=6)	8 Gy/48 h (n=6)	p-Value
KU80 expression								
Positive area rate (%)	25.90±1.82	44.64±4.21 ^a	43.15±4.80 ^a	47.71±5.39 ^a	46.94±5.00 ^a	50.20±5.16 ^a	51.88±4.04 ^a	0.001*
Optical density	0.176±0.002	0.189±0.003 ^a	0.219±0.004 ^{a,b}	0.231±0.002 ^{a,b,c}	0.248±0.003 ^{a,b,c,d}	0.260±0.007 ^{a,b,c,d}	0.294±0.002 ^{a,b,c,d,e,f}	<0.001*
Ku80 mRNA expression (2 ^{-ΔΔCT})	1.05±0.12	2.78±0.44 ^a	5.98±0.98 ^{a,b}	13.67±2.26 ^{a,b,c}	25.20±5.64 ^{a,b,c}	37.20±4.15 ^{a,b,c,d,e}	69.49±15.32 ^{a,b,c,d,e,f}	<0.001*

**p* < 0.05 by Kruskal–Wallis test.^aSignificant difference compared with the control group.^bSignificant difference compared with the 1 Gy/24 h group.^cSignificant difference compared with the 1 Gy/48 h group.^dSignificant difference compared with the 4 Gy/24 h group.^eSignificant difference compared with the 4 Gy/48 h group.^fSignificant difference compared with the 8 Gy/24 h group.

irradiation for a longer period of time had significantly higher optical density and 2^{-ΔΔCT} values of *Ku80* expression. The 1 Gy/48 h group had significantly higher *Ku80* optical density (0.219±0.004 vs. 0.189±0.003) and 2^{-ΔΔCT} values (5.98±0.98 vs. 2.78±0.44) when compared with the 1 Gy/24 h group. The 4 Gy/48 h group had significantly higher *Ku80* optical density (0.248±0.003 vs. 0.231±0.002) and 2^{-ΔΔCT} values (25.2±5.64 vs. 13.67±2.26) when compared with the 4 Gy/24 h group. The 8 Gy/48 h had significantly higher *Ku80* optical density (0.294±0.002 vs. 0.260±0.007) and 2^{-ΔΔCT} values 69.49±15.32 versus 37.20±4.15 when compared with the 8 Gy/24 h group. Among the groups that received radiotherapy for 24 h, the mice exposed to higher intensity radiation showed higher *Ku80* expression levels. The 8 Gy/24 h group had significantly higher *Ku80* optical density (0.260±0.007) and 2^{-ΔΔCT} values (37.20±4.15) when compared with the 4 Gy/24 h group (optical density: 0.231±0.002 and 2^{-ΔΔCT} 13.67±2.26) and the 1 Gy/24 h group (0.189±0.003 and 2^{-ΔΔCT} 2.78±0.44).

We showed by immunohistochemistry (Fig. 4) that *Ku80* protein was mainly expressed in nuclei of human A549 lung adenocarcinoma xenografts, with occasional cytoplasmic expression (Fig. 4A). *Ku80* protein was visualized as nuclear-located brown granules distributed as clumps or a diffuse mass. Increased *Ku80* nuclear staining was observed in mice receiving 1 Gy irradiation and then being sacrificed 24 hours later when compared with control mice (Fig. 4B). We also demonstrated a dose-dependent increase in *Ku80* expression in xenografts from mice receiving 8 Gy irradiation and then being sacrificed 24 h (Fig. 4C) and 48 h later (Fig. 4D).

Discussion

In this study, we evaluated the expression of *Ku80* in patients with lung adenocarcinoma, lung squamous carcinoma, small-cell lung carcinoma, and normal lung tissue. We found higher levels of *Ku80* in lung cancer tissue when compared with normal tissue. We also showed higher levels of *Ku80* protein and mRNA in lung adenocarcinoma and lung squamous cell carcinoma compared with small-cell lung carcinoma. Using an *in vivo* model in which nude mice were inoculated with A549 cells and irradiated at varying intensities, we showed that *Ku80* expression levels in the xenografts were directly proportional to intensity of dose and time after irradiation exposure.

Our data demonstrated that *Ku80* was primarily localized in the nuclei of lung adenocarcinomas, squamous cell carcinomas, and small-cell lung cancer tissue. Normal lung tissue and small-cell lung cancer tissue had significantly lower levels of *Ku80* protein and mRNA when compared with lung adenocarcinoma and squamous cell carcinoma tissue. These data are consistent with previous reports showing that lung adenocarcinoma and squamous cell carcinomas have a significantly poorer outcome after radiotherapy when compared with small-cell lung cancer (Nakagawa *et al.*, 2003; Bergqvist *et al.*, 2004). Given the role of *Ku80* protein in mediating radiation-induced DNA repair, these data may also explain why adenocarcinomas and squamous cell carcinomas have a lower radiosensitivity when compared with small-cell lung cancer.

Nude mice inoculated with human A549 lung adenocarcinoma cells were subjected to increasing doses of irradiation and were sacrificed either 24 or 48 h after

FIG. 4. Immunohistochemical staining to detect Ku80 in human lung adenocarcinoma A549 xenografts before and after irradiation. **(A)** Ku80 protein was expressed in A549 human lung adenocarcinoma xenografts as brown granules distributed as clumps or a diffuse mass in the nuclei ($\times 400$). **(B)** Ku80 protein expression in human A549 lung adenocarcinoma xenografts in the Gy1/24 h group. **(C)** Ku80 protein expression in human A549 lung adenocarcinoma xenografts in the 8 Gy/24 h group. **(D)** Ku80 protein expression in human A549 lung adenocarcinoma xenografts in the 8 Gy/48 h group.

irradiation. We showed that Ku80 was primarily localized in the nuclei of tumor masses in these mice. Our data also showed a time-dependent and dose-dependent increase in Ku80 expression. These data agreed with earlier *in vitro* work (Shintani *et al.*, 2003) demonstrating that irradiation of oral squamous cell carcinoma cell lines resulted in a significant upregulation of Ku80 protein expression, leading to resistance to radiation.

DNA double-strand breaks constitute the most lethal form of DNA damage, and prompt repair of such breaks is essential to maintain genomic integrity (Harper and Elledge, 2007). DSBs are the major form of damage caused by exposure of cells to genotoxic agents such as ionizing radiation. Unrepaired DSBs are known to activate cell cycle arrest as well as cell death signaling pathways (Harper and Elledge, 2007). The Ku protein, which is a key member of the DNA recombination repair system, plays an important role in homologous as well as nonhomologous end integration of the DSB repair pathway (Willmore *et al.*, 2008).

Recent studies have focused on understanding the Ku gene expression and functions, as well as its role in tumor development. Increased expression of Ku80 protein was previously shown to result in increased resistance of chronic lymphocytic leukemia cells and myeloma mouse leukemia cells to radiation (Muller and Salles, 1997). Nuclear Ku70/Ku80 proteins were upregulated during gastric cancer cell hyperplasia in a COX-2-dependent manner, and inhibition of Ku80 using COX-2 inhibitors suppressed cell proliferation

(Lim *et al.*, 2002). Suppression of Ku80 was also shown to inhibit cell proliferation and induce apoptosis in human colon cancer cells (Li *et al.*, 2002), and downregulation of Ku80 along with other proteins involved in the DNA damage response enhanced the radiosensitivity of lung cancer cells exposed to selenium and ionizing radiation (Shin *et al.*, 2007). Introduction of antisense Ku70 into a human squamous cell lung carcinoma cell lines was also previously shown to increase the radiosensitivity as well as chemosensitivity of these cells (Omori *et al.*, 2002). These data suggest that the Ku proteins are a potential therapeutic target. Manipulation of Ku levels may be a useful strategy to overcome the resistance of tumor cells to radiation and enhance the antitumor effect of radiotherapy.

Our findings suggest that Ku80 expression levels can be used for prediction of radiosensitivity of lung tumors in the clinic. The time- and dose-dependent increase in Ku80 levels in irradiated mice indicate that Ku80 may play a key role in the DNA damage response pathway.

An important limitation of our present study is the small patient population analyzed. Our future studies will focus on understanding *Ku80* gene expression and function at the molecular level. It will be interesting to explore whether inhibition of Ku80 expression would result in an improved response to radiotherapy in a large population of lung cancer patients and whether Ku80 can be developed as a novel target in lung cancer treatment. It will also be very interesting to explore the effect of age on the expression of Ku80.

Conclusion

Taken together, our data suggest that Ku80 may be important in radioresistance in lung tumors and that expression levels can be used for prediction of radiosensitivity of lung tumors in the clinic. Our *in vivo* data demonstrating a time- and dose-dependent increase in Ku80 levels in response to radiation therapy indicate a potentially important role for Ku80 in the DNA damage response pathway.

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Disclosure Statement

No competing financial interests exist.

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Address correspondence to:

Jian Ye, M.D.

Department of Pneumology

The First People's Hospital

Hangzhou 310006

China

E-mail: jianye17@126.com

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