Expression of DNA-dependent protein kinase catalytic subunit in laryngeal squamous cell carcinoma and its importance

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Abstract. The aim of the present study was to explore the expression and distribution of DNA-dependent protein kinase catalytic subunit (DNA-PKcs) in tumor tissues and adjacent normal mucosa tissues of patients with laryngeal squamous cell carcinoma (LSCC), and further analyze the association between the expression and the clinicopathological parameters of patients with LSCC. Clinical data of tumor tissues and corresponding adjacent normal mucosa tissues of pathologically diagnosed LSCC in 96 cases were collected in the present study. Of these specimens, the mRNA and protein expression levels of DNA-PKcs in LSCC tissues and the adjacent normal mucosa tissues were analyzed via reverse transcription-quantitative polymerase chain reaction and western blot analysis. Immunohistochemistry was used to detect expression and distribution of DNA-PKcs protein in LSCC tissues and corresponding adjacent normal mucosa tissues. The association between DNA-PKcs expression and the specific clinicopathologic features was evaluated by the $\chi^2$ test. Kaplan-Meier and Cox proportional hazards regression models were used to analyze the data. It was revealed that the expression of DNA-PKcs mRNA and protein was significantly higher in LSCC tissues than the adjacent normal mucosa tissues (P<0.05). DNA-PKcs was expressed predominantly in the nucleus. DNA-PKcs expression showed significant correlation with the differentiation degree of LSCC (P<0.05), and changes of DNA-PKcs expression gradually increased with the decrease of the differentiation degree. However, DNA-PKcs expression was not significantly associated with sex, age, lymph node metastasis or TNM stage (P>0.05). Patients with LSCC exhibited higher DNA-PKcs expression had markedly shorter survival than those with lower DNA-PKcs expression.

In conclusion, the present results suggested that the expression levels of DNA-PKcs were significantly increased in LSCC tumor tissues than in adjacent normal mucosa. DNA-PKcs expression was correlated with differentiation of LSCC, and may become a novel prognostic marker for patients with LSCC.

Introduction

Laryngeal squamous cell carcinoma (LSCC), which is the second most prevalent cancer of the head and neck region worldwide (1), predominantly affects individuals aged 60-70 years old, which has a high morbidity and mortality, with ~650,000 new cases and ~350,000 instances of mortality every year (2). Patients with LSCC typically have poor survival, which has not been improved in the last 30 years (3). Invasion and metastasis remain the factors which typically lead to mortality. Management of LSCC depends primarily on early detection and appropriate surgical resection, and adjunctive treatment of radiotherapy and chemotherapy; however the benefit and optimal timing of the combined chemotheraphy and radiotherapy remain uncertain (4). Therefore, in order to improve its detection and treatment, an improved understanding of the pathogenesis and biological features of LSCC is required. Although several diagnostic and prognostic biomarkers for LSCC have been described previously (5,6), there is still a lack of optimal biomarkers that may be widely used in the clinical setting.

DNA-dependent protein kinase catalytic subunit (DNA-PKcs), a 460 kDa serine/threonine protein kinase, the gene of which is primarily located in 8q11, is an essential component of the non-homologous end-joining (NHEJ) DNA repair pathway (7-11). In DNA double-strand breaks (DSBs) repair, at least two major repair mechanisms, homologous recombination (HR) and NHEJ, have been reported (7). In NHEJ pathway, DNA DSBs are rejoined directly at an appropriate chromosomal end or following processing of the DNA ends, and DNA-PKcs serve an important role in DNA DSBs repair by NHEJ throughout the cell cycle (8). DNA-PKcs is the catalytic subunit of DNA-dependent protein kinase (DNA-PK) complex and a member of the phosphatidylinositol 3-kinase family (12). Previous studies have identified the products of the X-ray repair cross-complementing protein 5 (XRCC5) and XRCC7 DSB repair genes as subunits of the DNA-PK complex, and the XRCC7 gene appears to encode DNA-PKcs (9,10). A

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unique feature of DNA-PKcs is that its enzymatic function is only active when in the presence of DNA ends, and it is suggested that catalytic activity is enhanced when substrate and enzyme are colocalized to the same DNA molecule (13). Furthermore, DNA-PKcs is able to maintain normal immune function, regulate DNA repair, and prevent further malignant transformation of cells (14). It is well known that DNA-PKcs is required for the NHEJ pathway of DSBs (15). Recently, the overexpressed DNA-PKcs was detected in various human tumors (16-19), and its expression level was correlated with the differentiation and proliferation of some cell types or the development of productive tissues (20-22). In contrast, DNA-PKcs deficiency results in severe combined immunodeficiency in mammals (23). However, in gastric and ovarian cancer, low expression of DNA-PKcs is associated with adverse outcome in patients (24). The role of DNA-PKcs in carcinogenesis, however, remains to be characterized.

At present, the clinicopathological importance of the expression of DNA-PKcs in LSCC is unknown. In the present study, reverse transcription-quantitative polymerase chain reaction (RT-qPCR), western blot analysis and immunohistochemical analyses were performed to explore the expression of DNA-PKcs mRNA and protein in LSCC tissues and in paired adjacent normal mucosa tissues, and to detect the subcellular localization of DNA-PKcs in LSCC cells. The aim of the study was to elucidate whether DNA-PKcs served a role in development of LSCC and a novel diagnostic biomarker for LSCC.

Materials and methods

Patients and tissue specimens. All patients recruited in the present study were of Chinese ethnicity. Tumor tissue and corresponding adjacent normal mucosa specimens were obtained from 96 consecutive patients with LSCC who underwent total or partial laryngeal resection at the Department of Otolaryngology-Head and Neck Surgery, People's Hospital of Guizhou Province (Guiyang, China) between 2009 and 2011. These tumor tissue samples and corresponding adjacent normal mucosa tissues were randomly harvested from LSCC tissues stored at -80°C (for qPCR and western blot assays) or in 4% paraformaldehyde (for immunohistochemistry analyses) in 2012. Adjacent normal mucosa samples, which were used as a control group, were harvested from 1 cm within the border of the tumor and were pathologically excluded from cancer cell infiltration. Patients who received any anti-cancer treatment before operation were excluded from cancer cell infiltration. Patients who received total or partial laryngeal resection at the Department of Otolaryngology-Head and Neck Surgery, People's Hospital of Guizhou Province, and written informed consent was obtained from all patients prior to surgery. A summary of the clinicopathological characteristics of the patients is presented in Table I.

qPCR. The expression levels of the DNA-PKcs of the four groups were analyzed by qPCR assay. Total RNA of tumor and adjacent normal mucosa tissue specimens were extracted using total RNA extraction kit (BioFlux Corporation, Tokyo, Japan) according to the manufacturer's protocol. cDNA was synthesized using an Access RT-PCR system (Promega Corporation, Madison, WI, America). qPCR was performed using SYBR® Green ER qPCR SuperMix-UDG with ROX (Thermo Fisher Scientific, Inc., Waltham, MA, USA) according to the manufacturer's instructions. The following thermocycling conditions were used: 50°C for 2 min; 95°C for 2 min; 95°C for 15 sec; and 60°C for 30 sec for 40 cycles. The primer sequences used for qPCR were as follows: DNA-PKcs, forward 5′-AGCATC ATGGTACACCGCACT-3′ and reverse 5′-TCCATACGGCAGCTTCACTC-3′; and β-actin, forward 5′-CCTCGCTTTTGC CGATCC-3′ and reverse 5′-GGATCTTTCGAGGGTACG TCACTG-3′. The length of the PCR products of DNA-PKcs and β-actin were 383 and 626 bp, respectively. Relative mRNA levels were calculated based on 2−ΔΔCt method (26). All experiments were performed in triplicate.

Western blot analysis. The protein levels of DNA-PKcs expression in tissue samples were detected by western blotting. Nuclear proteins were extracted using the nuclear protein extraction kit (Nanjing KeyGen Biotech Co., Ltd., Nanjing, China) and concentrations were analyzed using the bicinchoninic acid protein assay kit (Beyotime Institute of Biotechnology, Shanghai, China) according to the manufacturer's protocol. Proteins (20 μg per lane) underwent electrophoresis on 6% SDS-PAGE. Following electrophoresis, the separated proteins were transferred to polyvinylidene fluoride membranes (Bio-Rad Laboratories, Inc.). The membranes were blocked for 2 h at room temperature in 5% milk dissolved in Tris-buffered saline with 0.1% Tween, and were subsequently incubated overnight at 4°C with primary antibodies against DNA-PKcs (ab32566, 1:2,000; Abcam, Cambridge, UK) and β-actin (ab8224, 1:1,000; Abcam) as the internal control. Antigen and the first antibody complexes were detected by the horseradish peroxidase (HRP)-conjugated anti-mouse immunoglobulin G (no. 7076, 1:1,000) and anti-rabbit secondary antibody (no. 7074, 1:1,000) Cell Signaling Technology, Inc., Danvers, MA, USA), and enhanced chemiluminescence reagent was used for band detection (GE Healthcare, Chicago, IL, USA). The signal was measured using Image Lab software 4.1 (Bio-Rad Laboratories, Inc.). Western blot analyses were performed in triplicate.

Immunohistochemistry analyses. The specimens of the four groups were immersed in 4% paraformaldehyde solution for >24 h at room temperature, embedded in paraffin and cut into 5-μm-thick sections. The paraffin embedded sections were dried at 65°C for 30 min, deparaffinized with xylene, and dehydrated in a graded series of ethanol. The sections were treated with 3% H2O2 for 10 min, followed by treatment with normal goat serum (Beyotime Biological Technology Co., Ltd., Shanghai, China) for 10 min at room temperature. The slides were incubated with monoclonal mouse anti-human DNA-PKcs (1:100), diluted in antibody diluent buffer (Boster Biological Technology Co., Ltd., Wuhan, China) at 4°C for 8-12 h or overnight. Following washing three
times with PBS (2 min each), the samples were incubated with the secondary antibody (859643, Invitrogen; Thermo Fisher Scientific, Inc.) for 15-30 min at room temperature. Following washing three times with PBS (2 min each), the samples were stained with 0.01% DAB hydrogen peroxide for 3-10 min at room temperature, washed with tap water, and then counterstained with hematoxylin (AR0005, Boster Biological Technological Co., Ltd., Wuhan, China) for 1 min at room temperature. For immunohistochemistry analysis under light microscopy.

Immunohistochemical staining was assessed by two senior pathologists who were blinded to the patients' characteristics. Any discrepancies were resolved by consensus. DNA-PKcs was expressed in the nucleus, and brown or tan particles were defined as positive. The following scoring system was used to evaluate DNA-PKcs expression according to the staining intensity of positive cells (27): Strong staining (scored as 3), dark brown staining which obscured the nucleus of tumor cells; yellow staining (scored as 2); primrose yellow staining (scored as 1); and absent (scored as 0), no marked staining in tumor cells. DNA-PKcs was also scored according to the percentage of positive cells as follows: Negative, 0% positive cells (scored as 0); ±10% positive cells (scored as 1); 11-50% positive cells (scored as 2); 51-75% positive cells (scored as 3); and ≥75% positive cells (scored as 4). The staining intensity score and percentage score were added together, and the final score was obtained. For the final score, 0-1 indicated negative staining (-); 2-3 indicated weak positive staining (+); 4-6 indicated moderate positive staining (++; >6 indicated strong positive staining (+++).

Statistical analysis. Statistical analyses and creation of graphs were performed using GraphPad Prism 7.0 software (GraphPad Software, Inc., La Jolla, CA, USA). The expression of DNA-PKcs between the four groups were presented as the mean ± standard deviation. Mean values were compared using the Student's t-test. The association between DNA-PKcs expression and clinicopathological features was analyzed using the χ² test or Fisher's exact test. Survival curves were plotted using the Kaplan-Meier method and compared using the log-rank test. Cox regression models were used for multiple factor analysis. All prognostic factors were evaluated individually using univariate analyses and then analyzed in combination with multivariate models. The primary end point in the present study was percent survival. The significant association between DNA-PKcs expression and percent survival in univariate analyses was used as the criterion for including DNA-PKcs expression in the multivariate backward stepwise elimination procedure. The final multivariate model retained some prognostic factors. P<0.05 was considered to indicate a statistically significant difference.

Results

qPCR analysis. The expression of DNA-PKcs between the four groups by qPCR assay were presented in Fig. 1. DNA-PKcs mRNA expression levels were significantly higher in tumor tissues compared with the corresponding adjacent normal mucosa tissues (P<0.05) and significantly correlated with tumor differentiation (P=0.025). The mRNA expression levels of DNA-PKcs between the well- and moderately-differentiated LSCC groups were similar, however, the mRNA expression levels in the poorly-differentiated LSCC were markedly higher.

Figure 1. Expression of DNA-PKcs mRNA in the well-, moderately- and poorly-differentiated laryngeal squamous cell carcinoma, and the corresponding adjacent normal mucosa tissues were demonstrated by reverse transcription-quantitative polymerase chain reaction. β-actin was used as the loading control. *P<0.05. ns, not significant.
compared with well- and moderately-differentiated groups (P<0.05).

**Western blotting analysis.** Similar to the observations of qPCR, the protein expression levels of DNA-PKcs were also higher in tumor tissues compared with the corresponding adjacent normal mucosa tissues and correlated with tumor differentiation (Fig. 2). The protein expression levels of DNA-PKcs between the well- and moderately-differentiated LSCC groups were similar, however, the protein expression levels in the poorly-differentiated LSCC group were significantly higher compared with well- and moderately-differentiated groups (P<0.05; Fig. 2).

**Immunohistochemistry analysis.** As shown in Fig. 3, immunohistochemistry analysis was performed to evaluate DNA-PKcs expression in tumor tissue samples and corresponding adjacent normal mucosa tissue samples. Regarding DNA-PKcs staining, brown or tan particles in tissues indicated the presence of DNA-PKcs, which was predominantly localized to nuclei. Strong positive DNA-PKcs staining was observed in tumor tissues, whereas weak positive or negative staining was observed in adjacent normal mucosa tissue samples. DNA-PKcs expression was significantly higher in LSCC tissue compared with adjacent normal mucosa (P=0.008; Table I).

**Association between DNA-PKcs expression and clinico-pathological parameters.** As shown in Table I, 78 of the 96 (81.25%) LSCC patients had positive scores for DNA-PKcs staining in tumor tissues. The expression of DNA-PKcs demonstrated significant correlation with differentiation of LSCC. There was no significant difference between well- and moderately-differentiated cancer (P=0.332); however, there was a significant difference between well- or moderately-differentiated cancer and poorly-differentiated cancer (P=0.028 and P=0.026, respectively). The expression of DNA-PKcs showed no significant correlation with TMN stage or lymph node metastasis (P=0.118 and P=0.262, respectively). There was no statistically significant correlation between DNA-PKcs expression and age or sex (P=0.116 and P=0.570, respectively).

**Association between DNA-PKcs positive expression and the survival rate of patients with LSCC.** Patients were followed up for 12 to 60 months. Of the 96 subjects involved with the present study, 35 succumbed to mortality, 23 of which were due to tumor recurrence or metastasis. The remaining 13 cases succumbed to other unrelated factors.

To evaluate whether DNA-PKcs expression in LSCC was associated with patient survival, survival curves were plotted via the Kaplan-Meier method and compared using the log-rank test. The results suggested that patients with high DNA-PKcs expression had lower survival rates than those with low expression. The 3- and 5-year survival rates in patients with low DNA-PKcs expression were 82.5 and 72.1%, respectively, compared with 61.1 and 49.0% in patients with high DNA-PKcs expression. Furthermore, the survival rate of patients with high DNA-PKcs expression was lower compared with those with lower expression, and a significant difference was observed in the percent survival curves between the high and low DNA-PKcs expression groups (Fig. 4A; log-rank test, $x^2=3.998; P=0.045$).

Fig. 4B demonstrates that the percentage survival of patients with poorly-differentiated LSCC was the most severe out of all groups as the survival rate was the lowest. Furthermore, the survival rate was markedly higher in the patients with well- and moderately-differentiated LSCC. Kaplan-Meier analysis of well-, moderately- and poorly-differentiated LSCC patients revealed the 5-year percent survival rates were 83.1, 73.2 and 30.8%, respectively, which varied with the degree of differentiation and decreased gradually. Univariate Cox regression analysis revealed that DNA-PKcs expression and tumor differentiation were significantly associated with survival (Table II; P=0.035). Furthermore, multivariate Cox regression analyses demonstrated that DNA-PKcs expression remained an independent prognostic factor for disease progression. Notably, subgroup analyses based on tumor differentiation suggested that a significant difference was observed between high and low DNA-PKcs expression groups in well- or...
Discussion

In mammalian cells, DNA DSBs are widely considered to be the most lethal form of DNA damage (28,29). NHEJ is a major repair mechanism for DSBs. The capture of both broken DNA ends and presenting them together to the DNA-protein complex is an initial and important step of NHEJ (30), and DNA-PKcs is the main component of NHEJ. The DNA repair mechanism serves a key role in the maintenance of genomic integrity, and the DNA-PKcs is predominantly associated with the repair of DNA DSBs in mammalian Cells (28,29).

In the present study, tumor tissues from 96 LSCC patients and their corresponding adjacent normal tissues were enrolled, and qPCR, western blot analysis and immunohistochemical analyses were performed to investigate DNA-PKcs protein and mRNA expression in LSCC tissues and detect the subcellular localization of DNA-PKcs in LSCC cells. The aim of the present study was to elucidate whether DNA-PKcs served a role in disease development of LSCC.

The overexpression of DNA-PKcs has previously been detected in various types of human cancer (16-19). For instance, Tonotsuka et al (19) detected the expression of DNA-PKcs in esophageal cancer tissue and adjacent normal mucosa in 13 patients with primary esophageal cancer, and examined for quantitative differences in expression levels of DNA-PKcs proteins by western blotting and immunohistochemistry. It was revealed that the expression levels of moderately-differentiated and poorly-differentiated LSCC patients (Table II; P=0.023).

Figure 3. DNA-PKcs expression in adjacent normal mucosa tissue and differently-differentiated LSCC using immunohistochemical analyses (InVision) and H&E staining. (A) Adjacent normal mucosa tissue (H&E). (B) Negative DNA-PKcs expression in adjacent normal mucosa tissue (InVision). (C) Positive DNA-PKcs expression in well-differentiated LSCC (InVision). (D) Well-differentiated LSCC (H&E). (E) Positive DNA-PKcs expression in moderately-differentiated LSCC (InVision). (F) Positive DNA-PKcs expression in poorly-differentiated LSCC (InVision). Magnification, x200. DNA-PKcs, DNA-dependent protein kinase catalytic subunit; LSCC, laryngeal squamous cell carcinoma.

Figure 4. Survival rates of patients with LSCC. (A) Kaplan-Meier analysis showing association of DNA-PKcs expression and survival curves of patients with LSCC (Log-rank test: Chi-square=3.998, P=0.045). (B) Kaplan-Meier analysis showing comparison of survival curves of patients with LSCC of different pathological grades (Log-rank test: Chi-square=26.200, P<0.0001). DNA-PKcs, DNA-dependent protein kinase catalytic subunit; LSCC, laryngeal squamous cell carcinoma.
Using immunohistochemical analysis, DNA-PKcs in 96 patients with LSCC were also evaluated. Immunohistochemical localization of DNA-PKcs in LSCC cells, but was reduced in normal mucosa, which was consistent with the results of previous studies. Western blot analysis showed that the protein expression of DNA-PKcs was very strong, which indicated that DNA-PKcs had high expression in LSCC cells. The results of integral optical density revealed that the expression of DNA-PKcs was significantly different between LSCC and adjacent normal mucosa. The correlation between DNA-PKcs expression and tumor differentiation was examined in patients with uterine cervix or breast cancer using qPCR and western blot analysis, and found that they were significantly lower than those in normal mucosa. The present results disagreed with these findings. The present study also demonstrated that the expression of DNA-PKcs was significantly higher in LSCC than in adjacent normal mucosa.

A previous study by Tonotsuka et al. (19) indicated that DNA-PKCs was predominantly located in the nuclei of tumor tissue and normal mucosa, which was further confirmed by immunofluorescence on whole cells (35). In the present study, InVision immunohistochemistry was performed to detect the subcellular localization and the expression of DNA-PKCs in LSCC tissue and adjacent normal mucosa. The present study demonstrated that DNA-PKcs expression was predominantly located in the nuclei of LSCC cells, but was reduced in normal laryngeal mucosa, which was consistent with the results of Ren et al. (36).

In conclusion, the present results suggested that DNA-PKcs expression was significantly higher in tumor tissues than those in normal mucosa. Furthermore, another previous study conducted extensive profiling of DNA-PKcs mRNA and protein levels across normal and cancerous tissues (31). In contrast to the results of the present study, previous studies have suggested that the expression of DNA-PKcs in gastric cancer and breast cancer was significantly downregulated compared with benign lesions or peritumoral tissues (32,33). However, to the best of our knowledge, the present study was the first to demonstrate that DNA-PKcs expression of tumor tissues in patients with LSCC was significantly higher than that in adjacent normal mucosa, whereas the gene level and the protein level of DNA-PKcs expression were correlated with each other. Western blot analysis showed that the protein expression of DNA-PKcs was very strong, which indicated that DNA-PKcs had high expression in LSCC cells. The results of integral optical density revealed that the expression of DNA-PKcs was significantly different between LSCC and adjacent normal mucosa. The correlation between DNA-PKcs expression at the mRNA level and at the protein level was demonstrated, which indicated that the protein expression may be determined by the transcription of these genes. Someya et al. (34) previously examined the DNA-PKcs expression of patients with uterine, cervix or breast cancer using qPCR and western blot analysis, and found that they were significantly lower than those in normal volunteers. The present results disagreed with these findings. The present study also demonstrated that the expression of DNA-PKcs was significantly higher in LSCC than in adjacent normal mucosa.

Of particular importance, patients with highly-expressed DNA-PKcs had lower survival rates in poorly-differentiated LSCC. Univariate analysis revealed that elevated DNA-PKcs was closely associated with the survival rate in LSCC patients. Multivariate analysis suggested that DNA-PKcs expression was also an independent prognostic factor for poor clinical outcomes of LSCC patients. Taken together, these results suggested that DNA-PKcs may be used as a novel prognostic marker for patients with LSCC.

In conclusion, the present results suggested that DNA-PKcs were higher in tumor tissues than those in normal mucosa.
identify individuals with increased susceptibility to cancer. Furthermore, these results suggested that DNA-PKcs may become a novel prognostic marker for patients with LSCC.

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