Up-regulation of DNA-dependent protein kinase correlates with radiation resistance in oral squamous cell carcinoma

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DNA-PK is a nuclear protein with serine/threonine kinase activity and forms a complex consisting of the DNA-PKcs and a heterodimer of Ku70 and Ku80 proteins. Recent laboratory experiments have demonstrated that the DNA-PK complex formation is one of the major pathways by which mammalian cells respond to DNA double-strand breaks induced by ionizing radiation. In this study, we evaluated the relationship between expression levels of DNA-PKcs, Ku70 and Ku80 proteins and radiation sensitivity in oral squamous cell carcinoma (OSCC) cell lines and in OSCC patients treated with preoperative radiation therapy. The OSCC cell lines greatly differed in their response to irradiation, as assessed by a standard colony formation assay. However, the expression levels of the DNA-PK complex proteins were all similar, and there was no association between the magnitude of their expression and the tumor radiation sensitivity. Expression of DNA-PK complex proteins increased after radiation treatment, and the increased values correlated with the tumor radiation resistance. Expression of DNA-PKcs and Ku70 after irradiation was increased in the surviving cells of OSCC tissues irradiated preoperatively. These results suggest that up-regulation of DNA-PK complex protein, especially DNA-PKcs, after radiation treatment correlates to radiation resistance. DNA-PKcs might be a molecular target for a novel radiation sensitization therapy of OSCC. (Cancer Science 2003; 94: 894–900)

The DNA-PK complex is one of the major pathways by which cells respond to DNA double-strand breaks (DSBs). The DNA-PK complex consists of a heterodimer comprising 70- and 80-kDa proteins termed Ku and a 465-kDa serine/threonine protein kinase catalytic subunit termed DNA-PKcs.[1)] The Ku (p70/p80) component functions as an activator of the catalytic subunit, and also represents the major double-stranded DNA binding protein.[1, 2)] DNA-PK plays an important role in the repair of DSBs and in V(D)J recombination.[3)] Tumor cell lines defective in the expression of either Ku or DNA-PKcs exhibit marked radiation sensitivity. Cells lacking DNA-PK activity because of defects in DNA-PK components, such as human malignant glioma M059J cells and cells derived from scid mice, show hypersensitivity to ionizing radiation.[3–7)] These previous laboratory findings suggested that DNA-PK is a candidate as a predictor of cellular radiation sensitivity. There is, however, little information on the expression of DNA-PK in primary human tumors and the correlation, if any, with radiation sensitivity, though the results are not definitive.[8–11)] Therefore, the aim of this study is to evaluate the relationship between expression levels of DNA-PK complex proteins and radiation sensitivity.

Materials and Methods

Cell culture. All SCC cell lines were grown in Ham/F12: DMEM (1:1) supplemented with 10% fetal bovine serum, 24 μg/ml adenine, 0.4 μg/ml hydrocortisone and 50 units/ml penicillin and streptomycin. The HSC2, HSC3 and HSC 4 cell lines were provided by Japanese Collection Research Bioresources. The SCC15, SCC25, SCC66 and SCC111 cell lines were provided by Dr. David T. W. Wong, Division of Oral Biology & Medicine, Dental Research Institute, UCLA School of Medicine.

Patients' characteristics. Forty-two oral squamous cell carcinomas were analyzed in this study from patients who underwent preoperative external radiation therapy before surgery at the Department of Oral and Maxillofacial Surgery, Ehime University, School of Medicine, after having obtained informed consent. Of the 42 patients, 23 were male and 19 were female. Their ages ranged from 29 to 84 years, and the average age was 63.5 years. Tumor extent was classified according to the TNM system by UICC.[12)] There were 12 cases of stage II, 17 cases of stage III and 13 cases of stage IV. All cases were M0. Irradiation was performed five times a week with 2 Gy and the mean total dose was 32.9 Gy (26–40 Gy). Twenty of 42 cases received platinum-based chemotherapy as a sensitizer. All patients underwent surgery after an interval of 2 to 3 weeks. Cases were selected for this study only when tumor specimens were available from both before and after radiation therapy.

Clonogenic survival assay. Eight hundred cells were plated in 35-mm tissue culture dishes in triplicate and irradiated 48 h later in various single radiation doses using a linear accelerator (MBR.1520R, Hitachi Medico, Tokyo) at a source-to-target distance of 50 cm. The medium was changed 24 h after irradiation and incubation was continued for approximately 10 days to allow colonies to form. The colonies were fixed, stained with a solution of crystal violet and counted. The surviving fractions were calculated as the ratio of plating efficiencies for treated and untreated cells. These experiments were repeated at least three times. The linear quadratic model[13)] was used to fit the cell survival data to the following equation: ln [surviving fraction (SF)]$=-\alpha D-\beta D^2$, where D represents radiation dose (Gy) and α and β are fitted to the individual data. The parameters α and β were calculated for each curve.

Western blotting analysis. Primary antibodies against DNA-PK, Ku70, Ku 80 and actin were obtained from Santa Cruz Biotech (Santa Cruz, CA).

Human oral squamous cell carcinoma (OSCC) cell line pellets ($1\times10^6-2\times10^6$ cells/pellet) were lysed for 30 min in buffer (1×PBS, 1% Nondiet P-40, 0.5% sodium deoxycholate, 0.1% SDS) containing protease inhibitors: 5 μg/ml each aprotonin and pepstatin A, 1 m*M* benzamidine and 50 μg/ml phenylmethylsulfonyl fluoride. Following sonication and incubation on ice for 30 min, the supernatant was collected. Protein concentrations were determined with a DC protein assay kit (BioRad Laboratories, Hercules, CA). The lysate was centrifuged and

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protein (25 μg) was subjected to electrophoresis on Tris-glycine SDS polyacrylamide gel. The gel was blotted onto PVDF membrane (BioRad Laboratories), and nonspecific binding sites were blocked using milk. Incubation with primary antibodies was carried out overnight at 4°C in Tris-buffered saline. The membrane was washed, and proteins were detected by enhanced chemiluminescence (Amersham Corp., Arlington Heights, IL) according to manufacturer's instructions. Equal loading of extracts was confirmed by measuring actin expression, and expression levels were quantified using a densitometric system normalized to the expression level of actin.

To examine the effect of irradiation on the expression of DNA-PKcs, Ku70 and Ku80, OSCC cells were irradiated with 4 Gy. Control and radiation-treated OSCC cells were collected and lysed after 12 h.

Tumor response assessment. World Health Organization (WHO) standard criteria were used to classify the tumor response.[14] A response was termed complete (CR), if there was no evidence of visible or palpable tumor on gross inspection. A response was partial (PR), if there was a greater than 50% decrease in the products of the longest tumor dimension and the perpendicular diameter and no increase in the extent of any other known disease. Tumor regression to less than 50% of the initial tumor size was termed stable disease unless the product of the two diameters showed a greater than 25% increase over the initial product, in which case the response was categorized as progression.

Immunohistochemistry. Specimens were fixed in 10% formalin and embedded in paraffin. Serial sections with a 4 μm thickness from each specimen were made for immunohistochemistry. Immunohistochemical detection of DNA-PK, Ku70, Ku80 was performed by using polyclonal antibodies at a 1:100 dilution. Tissue sections were deparaffinized and dehydrated in graded alcohols. The sections were treated for 30 min with absolute methanol, including 0.3% H_2O_2, to inhibit endogenous peroxidase activity, then incubated with trypsin (DIFCO, Franklin Lakes, NJ) and 1.5% normal horse serum diluted 1:75 in Tris-buffered saline (145 mmol/NaCl, 20 mmol/liter Tris, pH=7.6) for 10 min, respectively. The specimens were incubated with primary antibody at 4°C for 16 h. Bound antibody was detected using the Envision system (Dako, Kyoto). Diaminobenzidine (1 mg/ml) in the presence of 0.03% hydrogen peroxidase was used to visualize any bound peroxidase and sections were counterstained with methyl green. Negative controls were obtained by omission of the primary antibody. The immunoreactivity of the DNA-PK complex proteins staining was scored according to the percentage of positively stained tumor cell nuclei. At least 10 high-powered tumor fields were counted.

Statistical analysis. All calculations were performed using the statistical computer program Statview 5.1 (Avacus, Austin, TX). The Pearson's correlation coefficient analysis was performed on all data in order to determine the overall correlation coefficient between radiation sensitivity and DNA-PK proteins complex expression. Mean values of DNA-PK complex before and after irradiation were compared using the Mann-Whitney *U* tests. The significance level was set at $P<0.05$ for each analysis.

Results

Relationship between DNA-PK complex protein and radiation sensitivity of OSCC cell lines. The radiation sensitivity of the human OSCC cell lines was variable. SCC25 and SCC66 were more radiosensitive than the other cell lines. On the other hand, SCC111, SCC15 and HSC2 did not response to irradiation (Fig. 1). The radiation sensitivity of the OSCC cell lines is summarized in Table 1. The values of D_{10} ranged from 3.3 Gy for SCC25 to 10.3 Gy for SCC111. The seven OSCC cell lines

Table 1. Radiation survival parameters for oral cancer cell lines

Cell lines	SF_2	D_{10}
HSC2	0.66	7.7
HSC3	0.52	5.8
HSC4	0.46	5.2
SCC15	0.88	9.2
SCC25	0.19	3.3
SCC66	0.49	4.9
SCC111	0.98	10.3

SF_2, surviving fraction at 2 Gy; D_{10}, dose to give *SF* of 10%.

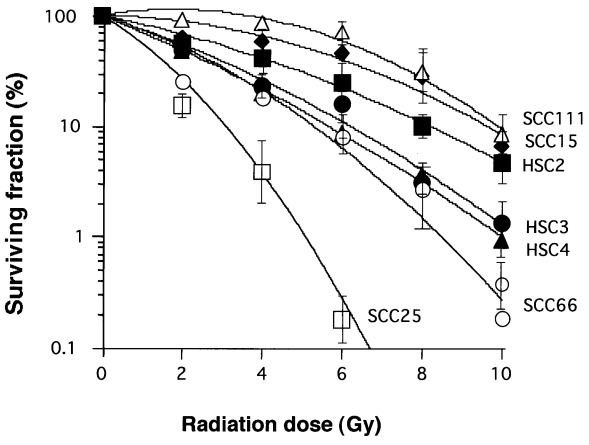

Fig. 1. Clonogenic survival of seven OSCC cell lines after irradiation. Triplicate cultures of each cell line were exposed to the indicated dose of radiation and cultured for 10 days. The surviving fraction was determined as described in "Materials and Methods."

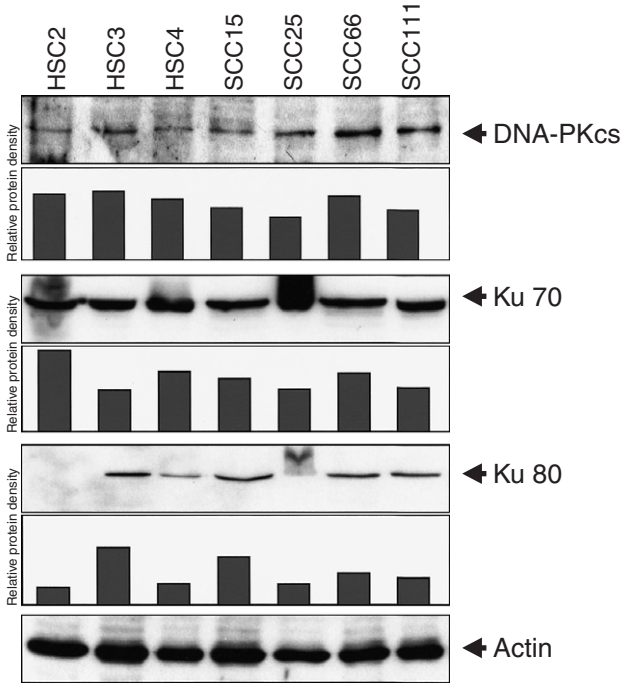

Fig. 2. Expression of DNA-PK complex proteins in seven OSCC cell lines (western blotting). Relative densitometric values were evaluated with NIH 1.62.

were examined for DNA-PKcs, Ku70 and Ku80 protein expression by using western blot analysis. The expression of these proteins was similar among the tumors (Fig. 2).

To examine whether there exists a relationship between *in vitro* radiation response and DNA-PK complex protein expression, the correlation between the D_{10}s of these OSCC cell lines and the protein expression levels was determined. As shown in Fig. 3, there was no significant correlation between the magnitude of expression of DNA-PK complex proteins and the radiation sensitivity of the OSCC cell lines (Fig. 3).

To examine the effect of irradiation, OSCC cells were irradiated with a dose of 4 Gy. The expression levels of DNA-PKcs and Ku70 increased with radiation treatment. However, the Ku80 expression levels did not increase significantly (Fig. 4). The correlation between D_{10}s of OSCC cell lines and the differences of expression levels of DNA-PK complex proteins following irradiation was determined. As shown in Fig. 5, there was a significant ($R^{\wedge 2}$=0.59, P=0.0403) correlation between the differences of DNA-PKcs expression following irradiation and the radiation resistance of OSCC cells, though there was no significant relationship for Ku70 and Ku80 (Fig. 5).

Immunohistological expression of DNA-PK complex and clinical effects of preoperative radiation therapy. The clinical parameters and pathological response to radiation therapy are shown in Table 2. A total of 12 patients had a major objective response and there was a partial response in 25 of these patients (Table 2). All DNA-PK complex proteins were localized in the nucleus of tumor specimens from oral SCC patients before preoperative radiation therapy (Fig. 6). The correlation between the expression levels of DNA-PKcs, Ku70 and Ku80 and the response is shown in Table 3. There was no correlation between the responses to preoperative radiation therapy and the percentage of tumor cells positively stained for DNA-PKcs, Ku70 and Ku80 in the present study (Table 3).

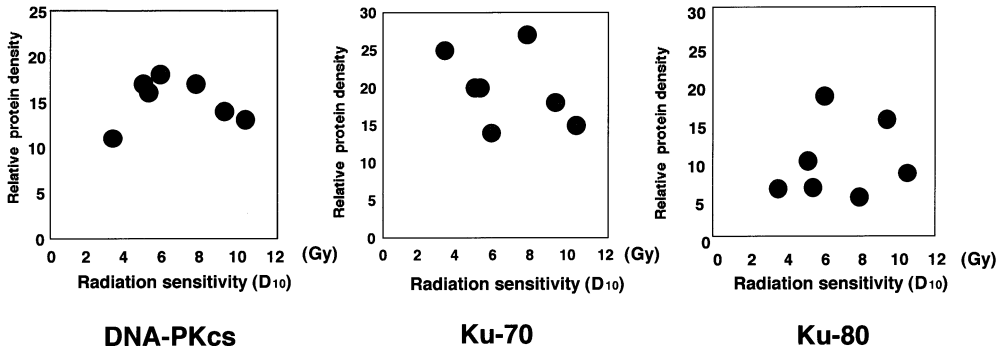

Fig. 3. Relationships between expression of DNA-PK complex proteins and radiation sensitivity (D_{10}s) of OSCC cell lines. There was no correlation between expression of DNA-PKcs, Ku70 and Ku80 and radiation sensitivity.

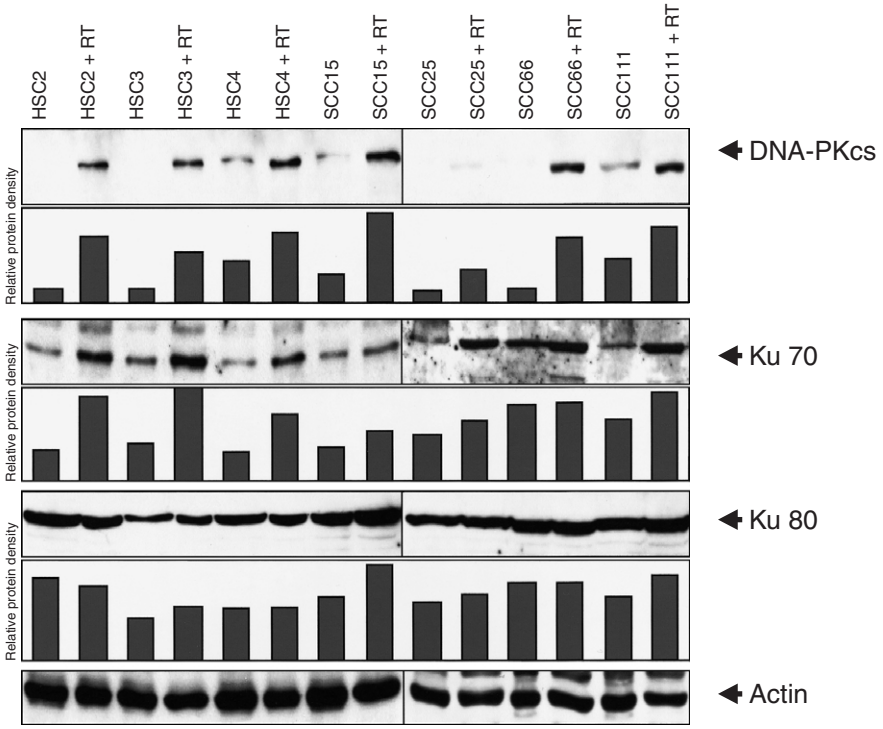

Fig. 4. Expression of DNA-PKcs, Ku70 and Ku80 in OSCC cell lines with and without irradiation. In almost all cell lines, the expression levels of DNA-PKcs, Ku70 increased with irradiation. The expression levels of Ku80 did not increase significantly.

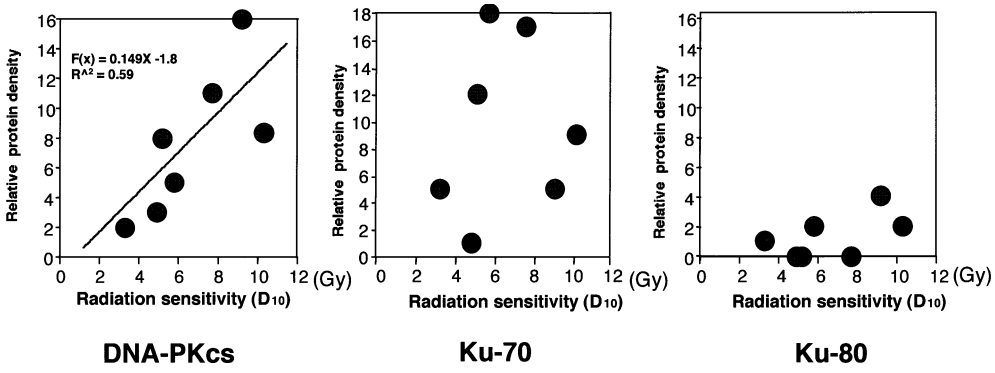

Fig. 5. Relationship between the differences of DNA PKcs, Ku70, Ku80 expression with irradiation and the radiation sensitivity of OSCC cell lines. The correlation and Pearson product moment correlation coefficient for DNA-PKcs and radiation sensitivity was $F(x)=0.149x-1.8$. $R\text{^}2=0$.

Table 2. Description of clinical parameter

Parameter	Group	*n*
Sex	Male	23
	Female	19
Age	29–84 (average±SD: 63.5±13.2)	
Clinical T stage[1)]	II	12
	III	17
	IV	13
Radiation dose	26–40 Gy (average±SD: 32.9±4.7)	
Clinical response[2)]	Complete	12
	Partial	25
	Stable	5

1) UICC, TNM classification of malignant tumors (1997).
2) WHO criteria, WHO handbook for reporting of cancer treatment (1979).

DNA-PKcs expression was observed in the tumor nuclei, and the percentage of positive cells ranged from 20.1 to 68.9% (mean±SD: 40.0±6.6%) in the biopsied specimens before preoperative radiation therapy. The expression in the operative specimens after therapy was significantly increased and ranged from 31.5 to 84.7% (mean±SD: 63.1±12.8%) (Fig. 6A, Fig.7). Ku70 expression was also increased in the surviving cells after radiation treatment (Fig. 6B). The positive percentage ranged from 40.1 to 78.9% (mean±SD: 59.3±8.6%) in the specimens before irradiation and 45.1 to 89.9% (mean±SD: 66.1±11.1%) in the specimens after therapy (Fig. 6B, Fig. 7). In contrast, Ku80 expression was similar in the specimens before and after radiation therapy. The Ku80 positive percentage ranged from 28.9 to 75.4% (mean±SD: 46.1±10.7%) and 30.2 to 80.5% (mean±SD: 50.6±10.3%) in the specimens before and after therapy (Fig. 6C, Fig. 7). In the operative specimens, the percentages of DNA-PKcs and Ku70 positive cells were significantly higher than those of the specimens before irradiation ($P<0.01$, DNA-PKcs, $P<0.05$, Ku70; Fig. 7).

Discussion

Radiation therapy is an acceptable treatment modality for patients with early stage OSCC, and preoperative radiation therapy is often effective to reduce the area of tumor infiltration. It can improve the overall survival rates and maintain oral morphology and function. However, the reaction of the carcinoma to irradiation varies in each patient. To choose the proper therapy, as well as to avoid untoward side effects, a useful method of predicting radiotherapeutic effectiveness must be established.

DSBs can be potentially lethal for cells, because they can lead to cell death if left unpaired. Cells have evolved sophisticated mechanisms of DNA repair. Among these mechanisms, nonhomologous end-joining represents one of the major pathways through which cells of higher eukaryotes repair DSBs.[15)] This mechanism involves various components, including the DNA-PK complex, which is comprised of DNA-PKcs and Ku subunits. DNA-PKcs and Ku subunits appear to play an important role as demonstrated by the fact that they are involved in three of the four complementation groups of mammalian cell mutants that are specifically defective in DSBs repair.[3, 16, 17)] Previous studies have shown a variation in the activity of DNA-PK complex proteins, and a possible role for DNA-PK activity in regulating tumor treatment response to chemotherapy and radiotherapy has been proposed.[18)] There are some reports indicating that the expression of DNA-PK correlates with radiation sensitivity.[9, 19)]

In the current study, we evaluated the relationship between expression of DNA-PK complex proteins and the response to radiation therapy of the OSCC cell lines, but found no significant relationship. These results are consistent with a report that head and neck cancers showed no relation between DNA-PKcs and Ku (p70/p80) expression and radiation sensitivity.[20)] The relationship between immunohistochemical detection of DNA-PK complex and the responses to radiation therapy in OSCC patients was also evaluated. In accordance with the *in vitro* results, there was no significant correlation between the level of expression of DNA-PKcs, Ku70 and Ku80 in OSCC tumors and the radiation sensitivity.

As defects in either the expression of DNA-PKcs or Ku lead to a high radiosensitivity, a correlation between tumor cell radiosensitivity and expression of DNA-PK complex proteins might be expected. However, our results suggested that DNA-PK complex might not be a predictor for radiation sensitivity. A similar study on esophageal cancer cell lines also found no relationship between DNA-PKcs expression and radiation sensitivity, though DNA-PK activity and/or Ku70 expression was significantly associated with radiation sensitivity. Western blot analysis revealed a significant correlation between DNA-PK and Ku70 expression, though there was no relation between DNA-PK activity and Ku80 expression. It was suggested that the regulation of DNA-PK activity involves Ku70.[21)] The observed lack of correlation may be related to the existence of plural radiation-induced DNA damage repair systems[16)] and the existence of DNA-PK-independent pathways of DSB repair.[22)] For example, high cellular radiation sensitivity is observed when DNA-PK is present in ATM-deficient cells.[3, 23)] It may be

that the expression of several DNA repair enzymes should be studied.

The expression levels of DNA-PKcs and Ku70 in OSCC cell lines were increased by 4 Gy radiation treatment, and the differences of DNA-PKcs expression with irradiation were related to radiation resistance. Similar findings were observed in the experiments with OSCC specimens of cancer patients. In OSCC tumor specimens, the proportion of DNA-PKcs and/or Ku70 positive cells increased in the surviving cells after preoperative radiation therapy. In the evaluation of OSCC tumor specimens after radiation therapy, various factors such as the period after radiation therapy or platinum-based chemotherapy could influence the DNA-PK and/or Ku70 expression. Though such factors might have affected the results, data presented in the

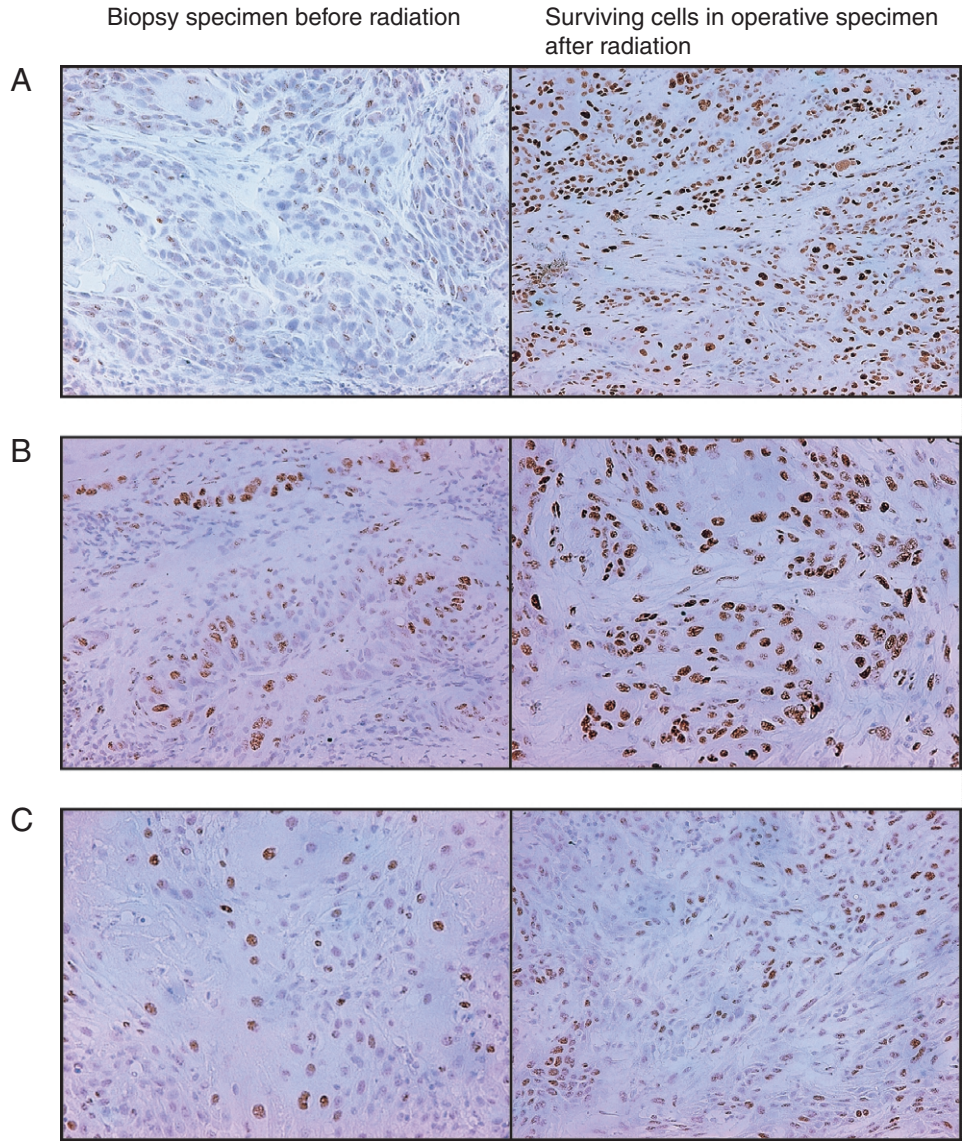

Fig. 6. Immunohistochemical staining of DNA-PKcs (A), Ku70 (B) and Ku80 (C) in OSCC. Positive staining is seen in the nuclei of cancer cells and the percentage of positive cells was increased with increase in the fraction of surviving cells after radiation therapy.

Table 3. Correlation between clinical response and DNA-PKcs, Ku70 and Ku80 expression in biopsy material preoperative radiation therapy

Clinical response[1)] of radiosensitivity	Labeling index of DNA-PKcs, Ku70 and Ku80		
	DNA-Pkcs	Ku70	Ku80
Complete (n=12)	37.9±11.0%	57.6±9.9%	45.5±13.3%
Partial (n=25)	39.4±6.1%	58.8±9.3%	46.1±10.5%
Stable (n=5)	43.2±8.7%	61.7±5.1%	40.4±10.2%

1) WHO criteria, WHO handbook for reporting of cancer treatment (1979).

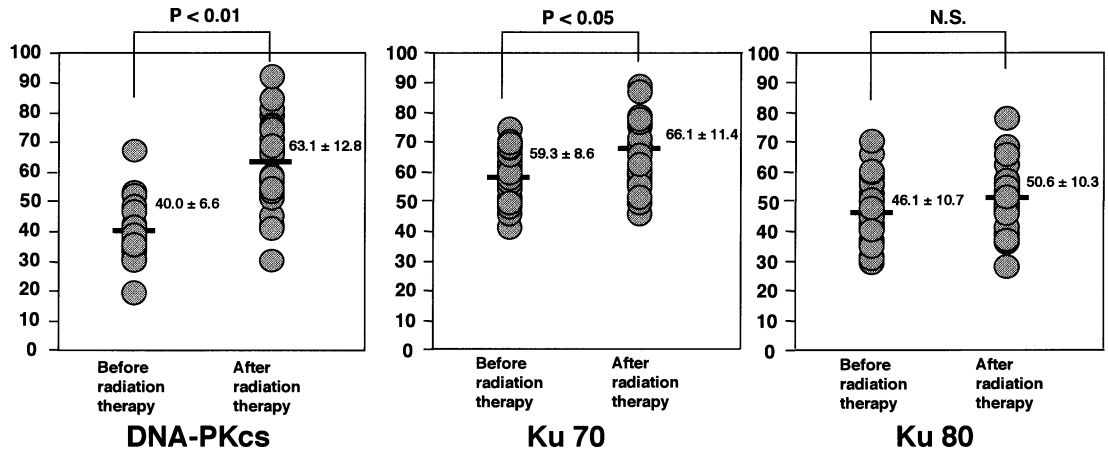

Fig. 7. Percentage of positive cells for DNA-PKcs, Ku70, Ku80 before and after radiation therapy in OSCC specimens. After irradiation, most of the surviving cells in OSCC specimens expressed DNA-PK complex proteins.

current study show that DNA-PKcs and Ku-70 expression was increased by irradiation *in vitro* and in the tumor specimens. Stronati *et al*. reported that the Ku activity increased after exposure to ionizing radiation in a dose-dependent manner in bladder cells. They also observed a decrease in phosphorylated Ku expression in the cytoplasm and a parallel increase in the nucleus after exposure to radiation.[24)]

Our results and these findings suggest that, when DSBs are generated by radiation, DNA-PKcs and Ku70 might increase and up-regulate the DNA-PK activity in OSCC. The up-regulation of DNA-PK activity plays a key role in DSBs repair after radiation therapy.

Both Ku-70- and Ku-80-deficient cells have been shown to have reduced DNA-PK activity.[25, 26)] However, DNA-PKcs protein can be detected in Ku80 mutants.[16)] Also the DNA-PKcs is capable of binding to DNA in the absence of Ku to stabilize its binding to DNA ends.[10)] Recently, Collis *et al*. demonstrated that the small inhibitory RNAs targeting DNA-PKcs gave rise to radiation sensitivity of ~1.4 compared with untransfected and control vector-transfected cells.[27)] Therefore, further study of the change in DNA-PK activity or its components, especially DNA-PKcs, with radiation treatment could be of great importance in understanding the mechanisms involved and confirm the potential utility of these genes as targets for radiation sensitizing agents.

In conclusion, this study found no relationship between the expression levels of DNA-PK complex proteins and the radiation sensitivity in OSCC. However, the expression of DNA-PKcs increased significantly after radiotherapy. Therefore, DNA-PKcs expression and/or DNA-PK activity appear to play a key role in DSBs repair after radiation therapy.

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