Heterogeneous expression of DNA-dependent protein kinase in esophageal cancer and normal epithelium

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Received February 10, 2006; Accepted April 13, 2006

Abstract. Esophageal cancer tissues and adjacent normal mucosae in 13 patients with primary esophageal cancer were examined for quantitative differences in DNA-dependent protein kinase (DNA-PK) activity and for expressions of Ku70, Ku80 and DNA-PKcs proteins by Western blotting and immunohistochemistry. The tumor tissues showed higher DNA-PK activity than the normal mucosae. Protein levels of Ku70, Ku80 and DNA-PKcs correlated with DNA-PK activities in the tumor tissues. Immunohistochemical analysis revealed that Ku70, Ku80 and DNA-PKcs located predominantly in the nuclei in both the tumor tissues and normal mucosae. In the normal epithelium, Ku70, Ku80 and DNA-PKcs were expressed only in the nuclei of the basal cell layers and not in those of the lumenal cell layers. In the tumor tissues, the expressions of DNA-PK proteins showed intratumoral heterogeneity. The different portions in the same tumor showed different expression levels of DNA-PK proteins, and even each tumor cell showed different expression levels. These results suggest that cell differentiation and tumor progression affect cellular DNA-PK protein levels and its activity. Furthermore, the intratumoral heterogeneity of DNA-PK protein expression in esophageal cancer cells/tissues also suggests the difficulty in prediction of radio- or chemosensitivity of the tumor through estimation of DNA-PK activity/protein levels in tumor specimens.

Introduction

DNA-dependent protein kinase (DNA-PK) is a nuclear protein with serine/threonine kinase activity and it composed of the catalytic subunit of DNA-PK (DNA-PKcs) and a heterodimer of Ku70 and Ku80 (1). DNA-PK plays a crucial role in the repair of DNA double-strand breaks (DSBs) induced by ionizing radiation and chemotherapeutic agents (1). Cells lacking DNA-PK activity because of defects in the DNA-PK components show hypersensitivity to ionizing radiation (2-6).

There have been many reports on the examination whether DNA-PK activity correlates with radiation sensitivity and whether it can be a parameter indicating the sensitivity to radiotherapy and/or chemotherapy. Suppression of DNA-PK activity by a phosphatidylinositol 3-kinase inhibitor wortmannin, antisense Ku70/DNA-PKcs, or small inhibitory RNA for DNA-PKcs sensitized cells to ionizing radiation (7-10). Polischouk et al reported that levels of DNA-PK activity associate with the proficiency in rejoining of DNA double-strand breaks (11). These results indicate that levels of DNA-PK activity correlates with cellular sensitivity to ionizing radiation in the cells under the same genetic background.

Relationship between radiation sensitivity and DNA-PK activity/protein levels under different genetic backgrounds has been investigated in cultured cell lines or tissue specimens obtained from patients with cancer (11-20). The results reported were contradictory and it is still unclear whether DNA-PK can be a parameter indicating radiation sensitivity under different genetic backgrounds.

To predict curability of the tumor after radiotherapy or chemotherapy, the sensitivities in both tumor tissue and adjacent normal tissue should be assessed. We previously examined the DNA-PK activities in tumor tissues and adjacent normal tissues in patient with colorectal cancer and found that the tumor tissues showed higher DNA-PK activity than the adjacent normal tissues in 11 out of 12 patients, which suggests poor curability of the tumors after radiation therapy alone under the condition that the DNA-PK activity correlates with radiation sensitivity (21). In the present study, we examined DNA-PK activities and protein levels of the tumor tissues and the adjacent normal mucosae in patients with esophageal cancer by the standard kinase activity assay, Western blotting and immunohistochemistry. The results revealed that DNA-PK activity was higher in esophageal
KCl, 10 mM NaCl, 1.1 mM MgCl2, 1 mM EDTA, 1 mM EGTA, 1 mM PMSF, 1 mM DTT, 1 μg/ml pepstatin, 1 μg/ml antipain], and then frozen in liquid nitrogen and thawed at 30°C three times. After a 60-min incubation at 4°C, the suspension was adjusted to 0.4 M KCl by adding 3.5 M KCl, incubated for 30 min at 4°C, and centrifuged for 10 min at 15,000 rpm. The supernatant was designated as the whole-cell extract. Protein concentrations were determined with the Bio-Rad protein assay (Bio-Rad, Hercules, CA).

**Materials and methods**

**Tissue specimens.** All esophageal tumors and adjacent normal tissues were obtained at the time of surgery at Tohoku University Hospital from 1999 to 2000. Informed consent was received from all patients. The patients received neither radiotherapy nor chemotherapy before surgery. Characteristics of the patients are shown in Table I.

**Cells.** LM217 is an SV40 transformed human fibroblast cell line derived from HS27 (22). LM217 was used as control in measurement of DNA-PK activity and Western blotting.

**Whole-cell and tissue extracts.** Whole-cell extracts and tissue extracts were prepared by a modification of the methods of Finnie et al and Dignam et al (23,24). The samples were washed twice with Tris-buffered saline [2 mM Tris (pH 7.2), 150 mM NaCl], homogenized using a hand-operated homogenizer (Eppendorf, Hamburg, Germany), then suspended in 100 μl of a low-salt buffer [10 mM HEPES (pH 7.2), 25 mM KCl, 10 mM NaCl, 1.1 mM MgCl2, 1 mM EDTA, 1 mM EGTA, 1 mM PMSF, 1 mM DTT, 1 μg/ml leupeptin, 1 μg/ml antipain], and then frozen in liquid nitrogen and thawed at 30°C three times. After a 60-min incubation at 4°C, the suspension was adjusted to 0.4 M KCl by adding 3.5 M KCl, incubated for 30 min at 4°C, and centrifuged for 10 min at 15,000 rpm. The supernatant was designated as the whole-cell extract (25). Protein concentrations were determined with the Bio-Rad protein assay (Bio-Rad, Hercules, CA).

**DNA-PK activity.** DNA-PK activity was assayed as previously described, with a synthetic peptide (EPPLSQEAFAD LWKK) (7). The whole-cell or tissue extracts were incubated in 20 μl of kinase buffer [20 mM HEPES-NaOH (pH 7.2), 100 mM KCl, 5 mM MgCl2, 1 mM DTT, 0.5 mM NaF, 0.5 mM β-glycerophosphate, 0.2 mM ATP, 10 μCi/ml [γ-32P]ATP in the presence of 0.01 mg/ml sonicated salmon sperm DNA and 0.5 mg/ml substrate peptide] at 37°C for 15 min. The final protein concentration in the reaction mixture was 37.5 μg/ml. The reactions were stopped by the addition of 20 μl of 30% acetic acid and the mixtures were spotted onto P81 paper disks (Whatman International Ltd., Maidstone, UK). The disks were washed 4 times in 15% acetic acid. Radioactivity in the paper disks was measured in a liquid scintillation counter.

**Western blotting.** Whole cell extracts or tissue extracts were lysed in the electrophoresis sample buffer [62.5 mM Tris (pH 6.8), 2% SDS, 5% glycerol, 0.003% bromphenol blue, 1% β-mercaptoethanol] and boiled for 5 min. The lysate was resolved by electrophoresis using a gradient gel (Daiichi Pure Chemicals Co., Ltd., Tokyo, Japan), and was electrophoretically transferred to polyvinylidene difluoride membranes (Bio-Rad). The membranes were then probed with anti-Ku70 antibody, anti-Ku80 antibody, anti-DNA-PKcs antibody Ab-4 (Cocktail) (NeoMarkers, Fremont, CA) or Anti-GAPDH antibody (Trevigen, Inc., Gaithersburg, MD). The anti-Ku70 and anti-Ku80 antibodies used were raised in our laboratory as described, with a synthetic peptide (EPPLSQEAFAD LWKK) (7). The whole-cell or tissue extracts were incubated in 20 μl of kinase buffer [20 mM HEPES-NaOH (pH 7.2), 100 mM KCl, 5 mM MgCl2, 1 mM DTT, 0.5 mM NaF, 0.5 mM β-glycerophosphate, 0.2 mM ATP, 10 μCi/ml [γ-32P]ATP in the presence of 0.01 mg/ml sonicated salmon sperm DNA and 0.5 mg/ml substrate peptide] at 37°C for 15 min. The final protein concentration in the reaction mixture was 37.5 μg/ml. The reactions were stopped by the addition of 20 μl of 30% acetic acid and the mixtures were spotted onto P81 paper disks (Whatman International Ltd., Maidstone, UK). The disks were washed 4 times in 15% acetic acid. Radioactivity in the paper disks was measured in a liquid scintillation counter.

**Immunohistochemistry for DNA-PK proteins.** Formalin-fixed and paraffin-embedded tissue specimens were deparaffinized,
cut to 2-μ sections, and stained by the labeled streptavidin biotin (LSAB) technique. Briefly, the sections were incubated with 3% hydrogen peroxide in methanol, and then incubated with 1% bovine serum for 30 min for blocking. The sections were incubated with primary antibody overnight at room temperature, and incubated with biotinylated secondary antibody (Nichirei Biosciences Inc., Tokyo, Japan) for 30 min at room temperature. Then, streptavidin/biotin complex was applied for 30 min (Nichirei Biosciences Inc.), followed by a 30 min incubation in 3,3'-diaminobenzidine substrate, yielding a brown reaction product. Sections were counterstained with hematoxylin and mounted under a coverslip.

**Evaluation.** The intensity of staining in immunohistochemistry was evaluated by the expression score reported by Rigas et al (26). The intensity of staining was rated according to the following scale: 2 = intense brown staining, 1 = light brown staining, 0 = no staining. In each sample, the percentage of cells expressing each protein was determined. To obtain a numerical assessment of the expression of each protein, we calculated the multiple of the intensity of staining by the percentage of cells expressing a protein for each sample. In tumor tissues, we assessed the expression score at the growing edge of the carcinoma, which is defined as the invasive tip, because tumor growth largely depends on the proliferative kinetics at the invasive tip (27). In normal tissues, the expression score was assessed at the normal mucosae. Each expression score was expressed as mean ± SD of the data from three different tissue extracts used in the measurement of DNA-PK activity. In 5 patients, DNA-PK activity was significantly higher in the tumor tissue compared with the normal mucosa (Fig. 1A).

**Results**

**DNA-PK activity of tumor tissues and normal mucosae.** We examined DNA-PK activities of tumor tissues and adjacent normal mucosae in 13 patients with esophageal cancer. In 5 patients, DNA-PK activity was significantly higher in the tumor tissue compared with the normal mucosa (Fig. 1A). Average value of the DNA-PK activities of tumor tissues and normal mucosae in the 13 patients was significantly higher than that of the normal mucosae (Fig. 1B).

**Expression of DNA-PK proteins in tumor tissues and normal mucosae.** Next, we examined DNA-PK protein levels by Western blotting in order to investigate whether the variety of DNA-PK activities observed in tumor tissues and normal mucosae depended on the DNA-PK protein levels. For the Western blotting, we used the same tissue extracts as used in the assessments of DNA-PK activity. Western blotting was
conducted using the tissue extracts from the 5 patients whose had enough tissue extracts for the Western blot analysis (Fig. 2A). In 2 out of the 5 patients, levels of Ku70, Ku80 and DNA-PKcs proteins were significantly higher in the tumor tissue compared with the normal mucosa (Fig. 2B-D). Average value of Ku70 protein levels in the 5 patients was significantly higher in the tumor tissue (Fig. 2E). Significant correlation was observed between DNA-PK activity and protein levels of Ku70, Ku80 and DNA-PKcs in tumor tissues of the 5 patients (Table IIB). Ku70 and Ku80 protein levels correlated with DNA-PKcs protein level in the tumor tissues (Table IIB). Ku70 protein level correlated with Ku80 protein level in the normal tissues (Table IIA).

Immunohistochemical analysis in normal mucosae. Esophageal epithelium consists of non-keratinized stratified squamous cells as shown in Fig. 3A. Stainings for Ku70, Ku80 and DNA-PKcs were predominantly nuclear and they showed a similar pattern (Fig. 3B-E). In the epithelium, Ku70, Ku80 and DNA-PKcs were expressed exclusively in the nuclei of the basal cell layers and not in those of the luminal cell layers (Fig. 3B-D). The DNA-PK proteins were expressed in almost all the nuclei in the middle-basal cell layers of the epithelium, whereas they were not expressed in some nuclei in the most basal cell layers (Fig. 3B-D). In lamina propria and muscularis mucosa, the DNA-PK proteins were expressed in about half of the nuclei (Fig. 3B-D).

Immunohistochemical analysis in tumor tissues. In tumor tissues, stainings for Ku70, Ku80 and DNA-PKcs were also predominantly nuclear and they showed a similar pattern in each tumor (Figs. 4 and 5). The intensity of staining was heterogeneous in the tumor tissues (Figs. 4B-D and 5B-D). The different portions in the same tumor showed different expression levels of DNA-PK proteins, and even each tumor cell showed different expression levels (Figs. 4F-H and 5F-H). These heterogeneous staining patterns of the DNA-PK proteins could be observed in all the tumors examined.

A semi-quantitative assessment of DNA-PK proteins. To assess the expression of DNA-PK proteins semi-quantitatively in immunohistochemical examination, the intensity of staining was evaluated by the expression score described in Materials and methods. The expression score was significantly higher in the tumor tissue than in the normal mucosa in 7 patients for Ku70, 4 patients for Ku80 and 6 patients for DNA-PKcs (Fig. 6A-C). In 2 patients, the expression score for DNA-PKcs was lower in the tumor tissue than in the normal mucosa (Fig. 6C). The average values of expression scores for Ku70 and Ku80 in 13 patients were significantly higher in the tumor tissue than in the normal mucosa (Fig. 6D).

Discussion

Prediction of radio- and chemo-sensitivity of normal and tumor tissues before the treatment will provide crucial information to find the best treatment method for each patient with cancer. In most of the cells, cell survival after X-irradiation depends on the yield of DNA DSBs and the repair of them. Non-homologous end-joining (NHEJ) and homologous recombination (HR) are the two major repair mechanisms for DSBs, and NHEJ plays the most important role in mammalian cells (28). Radiation sensitivity can be possibly predicted through the quantitative evaluation of DNA-PK as a key enzyme for NHEJ because DNA-PK activity has relevance to cellular sensitivity to ionizing radiation and DNA-PK protein levels correlate with DNA-PK activity (7-11). In the present study, the immuno-histochemical analysis revealed the intratumoral heterogeneity of DNA-PK protein expression (Figs. 4 and 5). The different portions in the same tumor showed different expression levels of DNA-PK proteins, and even each tumor cell showed different expression levels (Figs. 4 and 5). This heterogeneous expression of DNA-PK proteins in esophageal cancer tissues suggests the difficulty in prediction of curability of the tumors after radiotherapy or chemotherapy through evaluation of DNA-PK protein levels in the tumor specimens because the curability will reflect the DNA-PK protein levels in the cells that express DNA-PK most abundantly in the tumor under the condition that radiation sensitivity correlates with DNA-PK activity.

In the present study, DNA-PK activity correlated with protein levels of Ku70, Ku80 and DNA-PKcs, and protein levels of Ku70 and Ku80 correlated with the levels of DNA-PKcs in tumor tissues (Table II). These results correspond with previous reports (16,18,20,21). Promoter regions of Ku70, Ku80 and DNA-PKcs contain consensus Sp1 recognition elements and therefore these genes are supposed to be regulated by the same transcriptional factor, Sp1 (21,29,30).

Table II. Correlation coefficient.

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<thead>
<tr>
<th></th>
<th>Ku70</th>
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<tr>
<td>DNA-PK activity</td>
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<tr>
<td>Ku70</td>
<td>0.323</td>
<td>-0.491</td>
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<tr>
<td>Ku80</td>
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<td>0.816</td>
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<td>DNA-PKcs</td>
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A, Correlation coefficients in normal tissues

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<tr>
<td>DNA-PK activity</td>
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<tr>
<td>Ku70</td>
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<td>0.832</td>
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B, Correlation coefficients in tumor tissues

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<tr>
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<td>Ku80</td>
<td>0.000284</td>
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CC, correlation coefficient. Bold values indicating P<0.05.
Sp1 binding sites are located in the promoter regions of a number of growth-regulated genes including insulin-like growth factor-binding protein 2 and vascular/endothelial growth factor (31,32). Suppression of Sp1 by dominant negative Sp1 and Sp1-site-decoy oligonucleotides induces cell growth arrest (32,33). These results suggest involvement of Sp1 in growth-regulation. In the present study, DNA-PK proteins were highly expressed in the nuclei of the basal cell layers of the normal epithelium and were not expressed in luminal cell layers (Fig. 3), which may reflect the growth of the stem cells located in the basal cell layers and the growth arrest of the differentiated cells in the luminal cell layers.

In the present study, the different portions in the same tumor showed different expression levels of DNA-PK proteins, and even each tumor cell showed different expression levels (Figs. 4 and 5). In esophageal cancer, intratumoral heterogeneity has been reported in expressions of many proteins including transcription factor Ets-1, cyclin B1, cyclin D1, retinoblastoma protein, cyclooxygenase-2, GAGE, NY-ESO-1, MAGE-A, SSX, E-cadherin and α-catein (34-40).

Figure 3. The expression of DNA-PK proteins in esophageal mucosae from patient No. 7. (A) Staining with hematoxylin and eosin. (B) Immunostaining for Ku70. (C) Immunostaining for Ku80. (D) Immunostaining for DNA-PKcs. Original magnification x100.

Figure 4. The expression of DNA-PK proteins in esophageal cancer tissues from patient No. 7. (A and E) Staining with hematoxylin and eosin. (B and F) Immunostaining for Ku70. (C and G) Immunostaining for Ku80. (D and H) Immunostaining for DNA-PKcs. Original magnification x16 (A-D) and x400 (E-H).

Figure 5. The expression of DNA-PK proteins in esophageal cancer tissues from patient No. 13. (A and E) Staining with hematoxylin and eosin. (B and F) Immunostaining for Ku70. (C and G) Immunostaining for Ku80. (D and H) Immunostaining for DNA-PKcs. Original magnification x16 (A-D) and x400 (E-H).
It has been reported that esophageal cancer is genetically heterogeneous and it consists of various sub-clones with different status of p53 gene as a result of tumor progression (41-43). This genetic heterogeneity in esophageal cancer may underlie the heterogeneous expression of the proteins mentioned above and that of DNA-PK proteins as reported in tumor tissues compared with the adjacent normal mucosae, and DNA-PK protein levels correlated with DNA-PK activity. Upregulation of Sp1 in the process of tumor progression may increase the DNA-PK activity/protein levels of the tumor cells, as shown in Figs. 1, 2 and 6, and cause the acquisition of radioresistant phenotype.

In summary, DNA-PK activity was higher in the esophageal tumor tissues compared with the adjacent normal mucosae, and DNA-PK protein levels correlated with DNA-PK activity. Expression of DNA-PK proteins showed intratumoral heterogeneity in esophageal cancer tissues, making difficult the prediction of curability of the tumors after radiotherapy or chemotherapy by estimation of DNA-PK activity/protein levels in tumor specimens.

References