Immunohistochemical analysis of Ku70/86 expression of breast cancer tissues

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Abstract. DNA-dependent protein kinase (DNA-PK) has an important role in DNA double-strand break repair. We previously demonstrated the association of DNA-PK activity in peripheral blood lymphocytes (PBL) with incidence of chromosomal aberrations and risk of cancer. In this study, we examined the expression of Ku70 and Ku86 in breast cancer tissue and normal breast tissue with immunohistochemistry. We also measured the DNA-PK activity in PBL of the same patient. One hundred and ten breast cancer patients were included in this study. The expression of Ku70, and Ku86 in normal mammary epithelial cells and breast cancer cells obtained from surgical specimens was immunohistochemically examined. DNA-PK activity of PBL was measured by DNA-pull-down assay. The expression of Ku70 and that of Ku86 tended to parallel each other in normal and cancer tissues. There was also a relationship in the expression of Ku70 and Ku86 between cancer tissues and normal tissues in the same samples. Lower expression of Ku70 or Ku86 tended to be associated with higher malignant nuclear grade of cancer cells and higher frequency of axillary lymph node metastasis. The staining score of Ku70 or Ku86 of normal mammary epithelial cells or breast cancer cells had no significant relationship with DNA-PK activity of PBL. In conclusion, breast cancer cells inherited the characteristics of expression of Ku proteins from original mammary epithelial cells. The staining score of Ku70 or Ku86 of normal or cancer cells had no significant relationship with DNA-PK activity of PBL. This may be due to limitations in the assay sensitivity of immunohistochemistry.

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Introduction

Repair of various types of DNA damage is critical for cell survival. DNA double-strand break (DSB) is believed to be one of the most serious types of damage induced by DNA damaging agents such as ionizing irradiation (1), and if unrepaired or repaired incorrectly, it can lead to cell death during mitosis (2) or accumulation of damage, which in turn induces higher chromosomal instability and neoplastic transformation. DSBs may be caused by ionizing radiation but can also arise during replication, for example when a replication fork passes through a region containing a single-strand break (3).

In DNA DSB repair, at least two major repair mechanisms, homologous recombination (HR) and non-homologous end-joining (NHEJ) have been reported (4). In NHEJ pathway, DSBs are directly, or after processing of the DNA ends, re-joined at an appropriate chromosomal end and DNA-dependent protein kinase (DNA-PK) plays an important role in DNA DSBs repair by NHEJ throughout the cell cycle (5). DNA-PK is a serine/threonine kinase, which is composed of DNA-PK catalytic subunit (DNA-PKcs) and heterodimer of Ku70 and Ku86. DNA-PK binds DSBs in DNA, and phosphorylates and activates DNA-binding protein, including XRCC4 and DNA ligase IV, p53, and several transcription factors. Subsequently, Ligase IV repairs DNA DSB (6). In this way, DNA-dependent protein kinase (DNA-PK) has an important role in DNA DSB repair.

In a previous study, we demonstrated that DNA-PK activities of peripheral blood lymphocytes (PBL) in patients with breast cancer were significantly lower than those in normal volunteers. There was a relationship between DNA-PK activity and expression of Ku70, Ku86, and DNA-PKcs in RT-PCR. A similar tendency in Western blot assay was seen. The frequency of chromosome aberrations such as dicentric chromosomes and excess fragments increased as the DNA-PK activity decreased. We concluded that DNA-PK activity in PBL is associated with risk of breast.

In this study, we examined the expression of Ku70 and Ku86 in breast cancer tissue and normal breast tissue with immunohistochemistry. We also measured the DNA-PK activity in PBL of the same patient to examine the relationship between the DNA-PK activity in PBL and the expression of

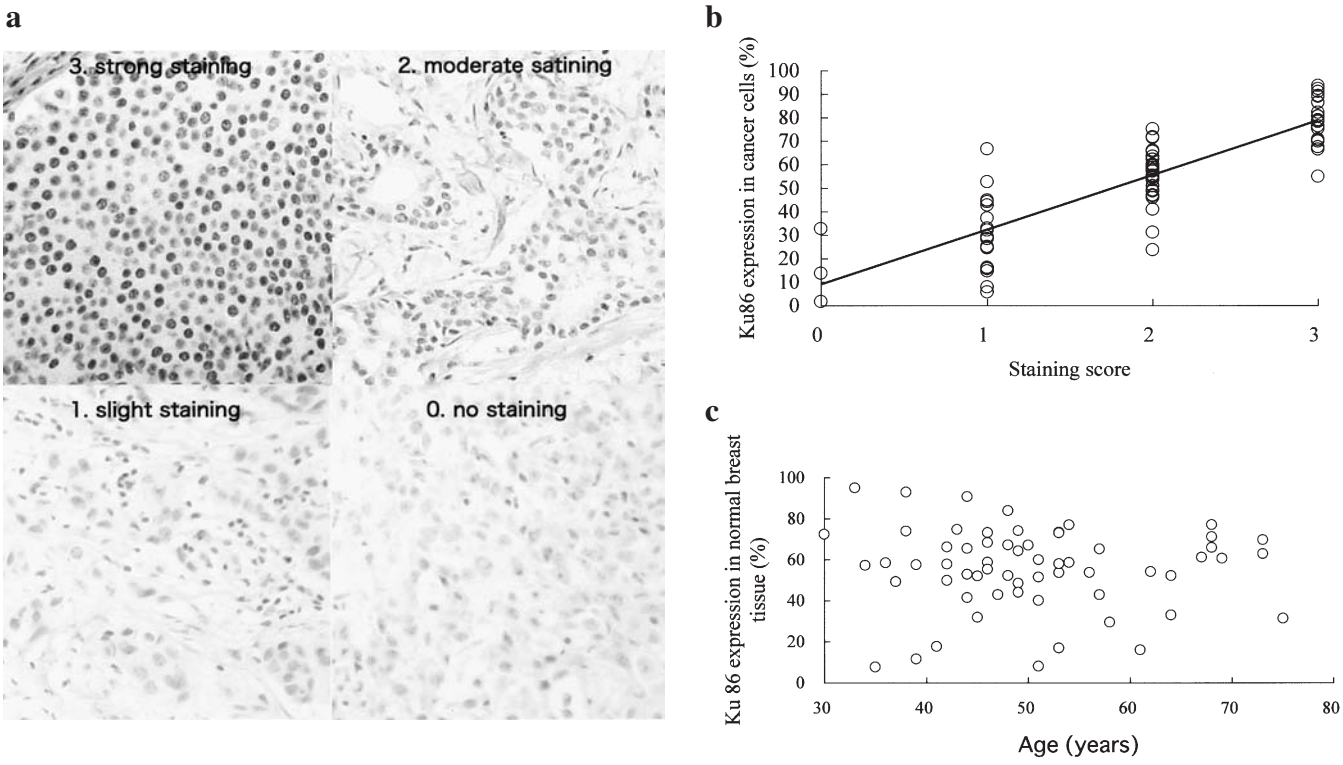

Figure 1. (a) Expression of Ku86 antigen in breast cancer tissues according to the intensity of staining: 0, no staining; 1, slight staining; 2, moderate staining; 3, strong staining. Original magnification, x200. (b) The relationship of two different methods for evaluation of Ku 86 staining, such as the intensity of staining and the percentage of Ku86-positive cells among all the counted tumor cells. (c) The relationship of age and the expression of Ku86.

Ku70 and Ku86 in breast cancer tissues and breast normal tissues.

Patients and methods

Selection eligibility. All subjects were Japanese. One hundred and ten sporadic breast cancer patients who had undergone breast conserving surgery and were due to receive post-operative radiotherapy to conserved breast at Sapporo Medical University, who had neither history of other cancers nor familial breast cancer history were enrolled in this study. The study was approved by the appropriate Committees for Human Rights in Research in our hospital and written informed consent was obtained from each subject. Exclusion criteria included chemotherapy, radiation therapy, or current use of immunosuppressive medications.

Pathological evaluation of the patients. All breast cancer patients had breast conserving surgery and axillary lymph node resection and pathological diagnosis of non-invasive or invasive ductal cancer was confirmed. The nuclear grade (as histological grade of malignancy) in breast cancer was evaluated by combining nuclear atypia and mitotic counts according to the Japanese breast cancer classification (7). Nuclear grade was divided into 3 groups; grade 1 for low-risk, 2 for intermediate-risk and 3 for high-risk malignancy, respectively.

Immunohistochemical staining of Ku70/86 in normal breast and cancer tissues. Formalin-fixed, paraffin-embedded sections of resected specimens from surgery were used. Immunohistochemical staining was carried out with methods previously described (8). Anti-Ku70 or -Ku86 rabbit polyclonal antisera, which have been described elsewhere (8), were used.

Biopsy samples of some patients were scanty and were excluded from this analysis. In total, 106 patients for Ku70 in normal breast tissues, 108 patients for Ku70 in cancer tissues, 108 patients for Ku86 in normal breast tissues, and all patients for Ku70 in breast cancer tissues were analyzed.

Specimens were viewed at x10 magnification and given a score according to the intensity of Ku70/86 (0, no staining; 1, slight staining; 2, moderate staining; 3, strong staining) (Fig. 1a). Edge effects and stromal area and lymphatic ducts were ignored. Ku70/86 expression in normal mammary epithelia and cancer tissues was apparent as brown staining.

Blood collection and PBL separation. Peripheral blood was collected with a sterile heparinized tube from all individuals. Peripheral blood lymphocytes (PBLs) were separated with lymphoprep (Nycomed Pharma AS), centrifuged at 1500 rpm (300 x g) for 30 min at 4˚C, and washed twice with phosphate-buffered saline.

PBL lysis, protein extraction, and DNA-PK assay. Protein extraction and DNA-PK assay were performed as described in our previous report (9,10). Briefly, PBL was thawed with high-salt buffer and the suspension was lysed by three rounds of freeze-thaw cycle and clarified by centrifugation. Protein concentration was assayed using a BCA protein assay kit (Pierce) with bovine serum albumin as the standard. The

Table I. The comparison of expression of Ku70 and Ku86.

		Ku86	
	Ku70	0+1	2+3
Breast tissue	0+1	12.3%	17.0%
	2+3	21.7%	49.1%
Cancer tissue	0+1	9.3%	13.0%
	2+3	13.9%	63.9%

Table II. The comparison of expression of Ku70 and Ku86 between normal mammary tissues and breast cancer tissues.

		Cancer	
	Normal	0+1	2+3
Ku70	0+1	13.2%	20.8%
	2+3	10.4%	55.7%
Ku86	0+1	12.1%	16.8%
	2+3	10.3%	60.7%

Table III. The relationship between Ku70 or Ku86 staining and nuclear grade of breast cancer cells.

	Nuclear grade	
	1	2+3
Ku70 staining		
None	5	4
Slight	10	6
Moderate	22	9
Strong	41	11
Ku86 staining		
None	2	1
Slight	15	6
Moderate	32	15
Strong	31	8

lysate was mixed with kinase assay buffer, synthetic peptide hp53-S15 (sequence: EPPLSQEAFADLWKK; synthesized in Sawady Biotechnology), and with or without sonicated salmon sperm DNA. This reaction mixture was incubated at 37˚C for 10 min. The reaction was stopped by the addition of 30% acetic acid and absorbed onto a phosphocellulose filter disc. The filter discs were washed in 15% acetic acid and in 99% ethanol and the remaining radioactivity was counted in a liquid scintillation counter. The net phosphorylation of hp53-S15 was calculated as phosphate incorporation in reaction with DNA minus that in reaction without DNA, divided by the specific radioactivity of ATP.

Table IV. The relationship between Ku70 or Ku86 staining and axillary lymph node metastasis.

	Lymph node metastasis	
	(-)	(+)
Ku70 staining		
None	7	2
Slight	14	2
Moderate	25	6
Strong	47	5
Ku86 staining		
None	3	0
Slight	17	4
Moderate	40	7
Strong	35	4

Results

The staining of Ku70 and Ku86 was nuclear with none of the normal epithelial cells or malignant cells exhibiting cytoplasmic or membrane immunoreactivity. The staining was diffuse throughout the nucleus of cells (Fig. 1a).

There are two scoring methods of immunostaining, in which one is according to the intensity of staining and the other is according to the percentage of positive cells. Fig. 1b demonstrates the relationship between the intensity of Ku86 of tumor cells and the percentage of Ku86-positive cells among all the counted tumor cells. There was a significant relationship between them (p=0.001), indicating that the two grading methods of immunohistological staining were inter-related. No relationship was found between age and expression of Ku86 (Fig. 1c).

We compared the expression of Ku70 with that of Ku86 in normal mammary epithelial cells and breast cancer cells. Table I indicates that the expression of Ku70 and that of Ku86 tended to parallel each other in breast cancer tissues (p=0.015).

In order to examine if the expression of Ku70 and Ku86 in normal mammary epithelial cells is related with that of breast cancer cells, we compared the expression of these proteins between normal tissues and breast cancer tissues in the same sample (Table II). There was a relationship in the expression of Ku70 (p=0.01) and Ku86 (p=0.02) between the cancer tissues and the normal tissues in the same samples.

Table III demonstrates the relationship between Ku70 or Ku86 staining and nuclear grade of breast cancer cells. As the staining grade of Ku70 or Ku86 decreased, nuclear grade of cancer cells tended to increase, indicating that the lower expression of Ku70 or Ku86 might be related with a higher malignant phenotype of breast cancer cells.

Table IV demonstrates the relationship between Ku70 or Ku86 staining and axillary lymph node metastasis. As the

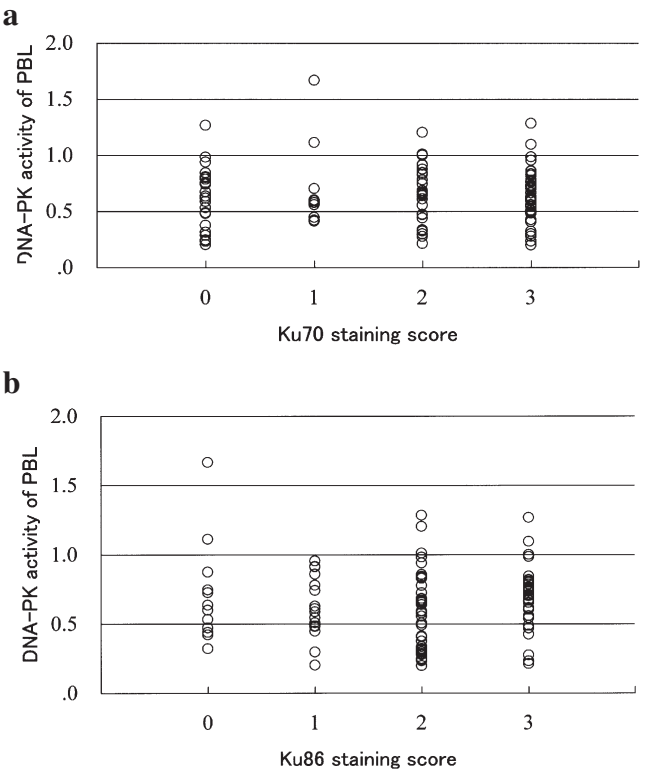

Figure 2. The relationship between the staining score of Ku70 (a) or Ku86 (b) of normal mammary epithelial cells and DNA-PK activity of PBL.

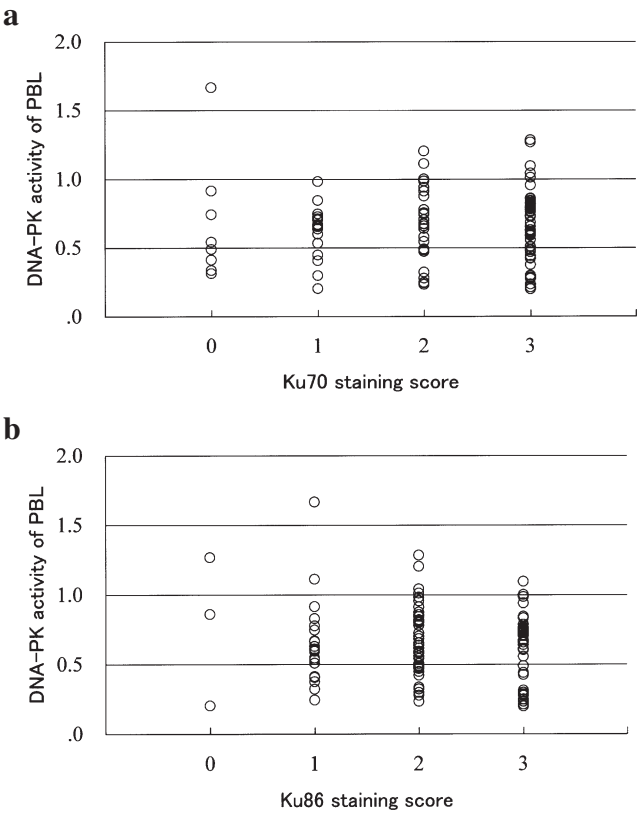

Figure 3. The relationship between the staining score of Ku70 (a) or Ku86 (b) of breast cancer cells and DNA-PK activity of PBL.

staining of Ku70 or Ku86 decreased, frequency of axillary lymph node metastasis tended to increase, indicating that the lower expression of Ku70 might be related with a higher axillary lymph node metastasis.

Fig. 2 demonstrates the relationship between the staining score of Ku70 or Ku86 of normal mammary epithelial cells and DNA-PK activity of PBL. The staining score of Ku70 or Ku86 of normal mammary epithelial cells had no significant relationship with DNA-PK activity of PBL.

Fig. 3 demonstrates the relationship between the staining score of Ku70 or Ku86 of breast cancer cells and DNA-PK activity of PBL. The staining score of Ku70 or Ku86 of breast cancer cells had no significant relationship with DNA-PK activity of PBL.

Discussion

Table I demonstrates that the expression of Ku70 and that of Ku86 tended to parallel each other in breast cancer tissue, indicating that there was a concerted expression of Ku70 and Ku86 in breast cancer tissue. These results agreed with other reports (11,12). Hosoi *et al* reported that Ku70 and Ku86 have consensus Sp1 recognition elements in their promoter region. DNA-PK activity and protein- and mRNA-levels of Ku70 and Ku80 were elevated in tumor tissues in patients with colorectal cancer because of elevated Sp1 protein levels in tumor tissues (13). We also demonstrated that the transcription factor E2F1, which plays an important role in cell cycle progression, exhibited a strong correlation with the DNA-PK activity and may regulate the concerted expression of genes that were related with DNA-PK activity (14).

Table II demonstrates that there was a relationship in the expression of Ku70 and Ku86 between the cancer tissues and the normal tissues in the same samples, indicating that breast cancer cells inherited the characteristics of expression of Ku proteins from original mammary epithelial cells.

Tables III and IV demonstrate that lower expression of Ku70 or Ku86 tended to be associated with higher malignant nuclear grade of cancer cells and higher frequency of axillary lymph node metastasis. In this way, our results demonstrated that breast cancer cells with a lower expression of Ku70 or Ku86 might have aggressive cancer phenotypes such as a higher nuclear grade and positive axillary lymph node metastasis. Aggressive cancer phenotypes are a manifestation of many different genetic alterations that promote rapid proliferation and metastasis (15). Genetic instability prompted the loss or activation of a number of critical genes, such as those involved in cell proliferation, differentiation, and apoptosis (16-19). Repair of various types of DNA damage is critical for genomic instability. DNA double-strand break (DSB) is believed to be one of the most serious types of damage induced by DNA damaging agents (1). Genes involved in DNA DSB repair play an important role in the maintenance of genomic stability (20). Previously, we demonstrated that DNA-PK activity is associated with chromosomal instability (9).

The staining score of Ku70 or Ku86 of normal mammary epithelial cells (Fig. 2) or breast cancer cells (Fig. 3) had no significant relationship with DNA-PK activity of PBL. The reason for this is unknown. The expression of Ku70 and Ku86 in breast mammary epithelial cells may be different from that in PBL. However, Auckley *et al* reported a tight correlation

between DNA-PK activity in PBL and bronchial epithelial cells (a progenitor cell for lung cancer) that were obtained by bronchoscopy, suggesting that PBL can be used as a surrogate cell type for other kinds of cells (21). In this study, we used immunohistochemical staining to detect expression of Ku70 and Ku86. There are limitations in assay sensitivity by immunohistochemistry and a subtle difference in expression patterns of Ku70 and Ku86 that may influence the DNA-PK activity of cells may not be detected.

In summary, breast cancer cells inherited the characteristics of expression of Ku proteins from original mammary epithelial cells. The staining score of Ku70 or Ku86 of normal or cancer cells had no significant relationship with DNA-PK activity of PBL. This may be due to limitations in the assay sensitivity of immunohistochemistry.

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