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# Gene Expression Associated with DNA-Dependent Protein Kinase Activity under Normoxia, Hypoxia, and Reoxygenation

Tadashi TSUCHIMOTO[1], Koh-ichi SAKATA[1]*, Masanori SOMEYA[1], Hiroyuki YAMAMOTO[2], Ryoichi HIRAYAMA[3], Yoshihisa MATSUMOTO[4], Yoshiya FURUSAWA[3] and Masato HAREYAMA[1]

**DNA-PK/Gene expression/Hypoxia/Reoxygenation.**

In order to elucidate the mechanism of concerted expression of various proteins related to DNA-PK activity, we examined the relationship of the expression of these proteins with that of a transcription factor Sp1. We also explored whether the expression of genes related to DNA-PK activity and Sp1 changed after hypoxia and reoxygenation. RT-PCR was employed to measure expression of various proteins related to DNA-PK activity in peripheral blood cells (PBLs) of normal healthy volunteers and cancer patients. M059K and DLD1 cells were made hypoxic with the hypoxia chamber, and the expression of various proteins in the cells was examined. DNA-PK activity was measured using synthetic peptide substrate mimicking the sequence around Ser15 of human p53. Expression of examined genes whose expression was related to DNA-PK activity was significantly correlated with expression of Sp1. The expression of most genes lost its relationship with the expression of Sp1 after hypoxic exposure and reoxygenation. DNA-PK activity tended to decrease after hypoxic exposure and to recover after reoxygenation. Sp1 may control expression of genes related to DNA-PK activity. Expression of those genes deviated from the control Sp1 under hypoxia and reoxygenation. The control mechanism for expression of genes related to DNA-PK activity under hypoxia and reoxygenation may be different from that under aerobic conditions.

## INTRODUCTION

DNA is the principal target for the biologic effects of radiation. Among various types of DNA damages induced by radiation, DNA double-strand break (DSB) is believed to be one of, at least, the most serious types of damage induced by ionizing irradiation[1)] and, unless repaired properly, can lead to cell death during mitosis.[2)] The repair of DNA DSBs influences the radiosensitivity of tumors and normal tissues in clinical radiotherapy.[1)]

In DNA DSB repair, at least two major repair mechanisms, homologous recombination (HR) and non-homologous end-joining (NHEJ), have been reported.[3)] In the NHEJ pathway, which is the major mechanism in mammalian cells, DSBs are rejoined directly, or after processing of the DNA ends, at an appropriate chromosomal end. Here, the DNA-dependent protein kinase (DNA-PK) plays an important role in DNA DSBs repair by NHEJ throughout the cell cycle.[4)] DNA-PK is a serine/threonine kinase which is composed of a DNA-PK catalytic subunit (DNA-PKcs) and heterodimers of Ku70 and Ku86. DNA-PK binds DSBs in DNA, phosphorylates and activates DNA-binding proteins, including XRCC4, DNA ligase IV, p53, and several transcription factors. Subsequently, Ligase IV joins DNA DSB.[5)] In this way, DNA-PK plays an important role in DNA DSB repair. DNA-PK activity was related to the expression of Ku70, Ku86, and DNA-PKcs in peripheral blood lymphocytes (PBLs) of normal volunteers and cancer patients with various levels of DNA-PK activity.[6)] We also demonstrated using cDNA array technology that over 30 genes were correlated with DNA-PK activity in PBLs. Most genes that were related to DNA-PK activity were involved in cell cycle regulation.[7)]

The radiation resistance of a cell under low oxygen conditions, known as the oxygen effect, is critical to radiotherapy. Radio-chemical reactions are generally believed to

*Corresponding author: Phone: +81-11-611-2111,
Fax: +81-11-613-9920,
E-mail: sakatako@sapmed.ac.jp

[1]Department of Radiology, Sapporo Medical University, School of Medicine, Hokkaido, Japan; [2]First Department of Internal Medicine, Department of Radiology, Sapporo Medical University, School of Medicine, S1W16, Chuo-Ku, Sapporo, 060-8543, Japan; [3]Research Center for Charged Particle Therapy, National Institute of Radiological Sciences, Anagawa 4-9-1, Inage, Chiba 263-8555, Japan; [4]Tokyo Institute of Technology, Research Laboratory for Nuclear Reactors, N1-30 2-12-1 Ookayama, Meguro-ku, Tokyo 152-855, Japan.

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be the fundamental mechanisms underlying oxygen effects.[8] It is also reported that the repair of DNA DSBs is an important factor in the mechanism of the oxygen effect.[9]

In this study, in order to elucidate the mechanism of concerted expression of various proteins related to DNA-PK activity, we examined the relationship of the expression of these proteins with that of a transcription factor Sp1. We paid attention to the role of Sp1 in the concerted expression of proteins with DNA-PK activity since significant correlations between Sp1 protein levels and mRNA levels of Ku70, Ku86, and DNA-PKcs were already reported.[10] We found that the expression of all examined genes whose expression was related to DNA-PK activity was significantly correlated with expression of Sp1 in PBLs. Next, we explored whether the expression of genes related to DNA-PK activity and Sp1 changed after hypoxia and reoxygenation. We also measured DNA-PK activity after hypoxia and reoxygenation and examined the relationship with the expression of DNA-PK.

## MATERIALS AND METHODS

### *Blood collection and PBL separation*

All subjects were Japanese. They consisted of seven cancer-free normal healthy volunteers and 20 cancer patients: 17 with sporadic breast cancer, one with hypopharyngeal cancer, and two with uterine cervix cancer. Exclusion criteria included a history of other cancers, chemotherapy, radiation therapy, or current use of immunosuppressive medications. The study was approved by the appropriate Committee for Human Rights in Research in our hospital and written informed consent was obtained from each subject. Twenty ml of peripheral blood was collected from all patients with a sterile heparinized tube. Peripheral blood lymphocytes (PBLs) were separated with lymphoprep (Nycomed Pharma AS, Oslo, Norway), centrifuged at 1500 rpm (300xg) for 30 min at 4°C, and washed twice with Phosphate-Buffer Saline (PBS).

### *Cell culture, hypoxic procedures*

In addition to PBL from human subjects, we also used established tumor cell lines. M059K cell is derived from a biopsy specimen from a human glioma.[11] The DLD1 cell is a human colon carcinoma cell line. We used the hypoxia chamber that Furusawa *et al.* used to bring the cells under a state of hypoxia.[12] Mixed gass of 95% $N_2$ and 5% $Co_2$ were introduced to the hypoxia chamber for one hour. Then, the chamber was sealed and placed in an incubator for 24 or 48 h. After incubation of 24 or 48 h, the glass dishes were taken out of the chamber. For reoxygenation, glass dishes were placed for further 6 or 24 h.

### *Reverse-transcription PCR (RT-PCR)*

Semiquantitative reverse transcription-polymerase chain reaction (RT-PCR) was carried out as previously described.[13] Briefly, total RNA was extracted using TRIzol (Invitrogen), after which a 1 μg sample was reverse transcribed using SuperscriptIII (Invitrogen). The following primer pairs were used for RT-PCR: DNAPKcs F-TCCAGACCCGTACCCAGGAA and R-CCGGTCAGCCAATCAAATGAG, Ku70 F-GATGCCTCCAAGGCTATGTTTGA and R-TTCTCGGTACCATAGAACACCACAG, Ku86 F-AAGAGCAGCCACATAGTCTCTGAAC and R-CCTCAGCTGTGACAGAACTTCA, Cyclin E1 F-TTTGCAGGATCCAGATGAAGA and R-CACAGACTGCATTATTGTCCCAAG, CDC25B F-GACCACCGAGAGCTGATTGGA and R-TTGATGTGCCCGCCTTCATA, MCM5 F-CAAATGCAAATGCGTGGACTTC and R-GCCCATGATGGTAACCCTGTTC, XRCC3 F-CAGCCAGATCTTCATCGAGCAC and R-GAGGCCTGGCTGTCAAATTCA, HSP90AA1 F-GGACCAGAAATCCCGACGATATTA and R-AGCACGTCGTGGGACAAATAGA, ACTB (actin-beta) F-ATTGCCGACAGGATGCAGA and R-GAGTACTTGCGCTCAGGAGGA, SP1 F-CGGATGAGCTACAGAGGCACAA and R-TCCTCATGAAGCGCTTAGGACAC.

The integrity of the cDNA was confirmed by amplifying glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and actin-beta (ACTB) as previously described.[14] Samples of the amplified product were then subjected to 2.5% Nusieve gel electrophoresis and stained with ethidium bromide. Real-time PCR was carried out using a CYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA) on an ABI Prism 7000 as previously described.[15] The accumulation of PCR product was measured in real time as an increase in fluorescent signals and analyzed using ABI Prism 7000 SDS Software (Applied Biosystems). Standard curves relating the initial template copy number to the fluorescence and amplification cycle were generated using the amplified PCR product as a template, and were then used to calculate the mRNA copy number in each sample. The ratios of the intensities of the target genes to those of actin beta signals were considered to be a relative measure of the target genes' mRNA level in each sample. Measurements were carried out twice and average values were employed.

### *DNA-PK activity assay*

Protein extraction and DNA-PK activity assay was done as described in our previous report.[6] Briefly, cells were thawed with high salt buffer and the suspension 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 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. Measurements were carried out three times and average values and standard deviation were employed.

### *siRNA transfections*

Stealth RNA™ siRNA oligonucleotides for Sp1 were purchased from Invitogen (Carlsbad, CA, USA). DLD1 cells were plated in antibiotic-free medium for 24 h prior to transfection. The Sp1 siRNA, or a scrambled siRNA control (Stealth RNA™ Negative Control Duplexes), was transfected using Lipofectamine RNAiMAX (Invitrogen) according to the manufacturer's instructions. Forty eight h after transfection, fresh medium was added. Depletion of Sp1 by siRNA was confirmed by RT-PCR after the siRNA transfection.

### *γH2AX focus*

After M059K cells received hypoxic treatment in the hypoxia chamber for 24 h, cells in the hypoxia chamber were irradiated with various doses of X-rays. Next, cells were taken out of the chamber and incubated for 24 h. Then, the number of instances of X-ray radiation-induced histone H2AX focus (γH2AX focus) formation in cells was measured using the following procedures. Cells diluted to appropriate numbers were grown on a glass slide and fixed with cold methanol for 20 min, rinsed with cold acetone for 10 seconds, and then air-dried. Anti-γH2AX (Upstate) antibodies were used as the primary antibody. Alexa-488-conjugated anti-rabbit IgG (Molecular Probes) were used for visualization of foci with anti-γH2AX antibody. Slides were mounted with antifade reagent (Mounting medium, DAKO). γH2AX foci were observed with an Olympus fluorescent microscope under $10 \times 100$ oil immersion. For quantification

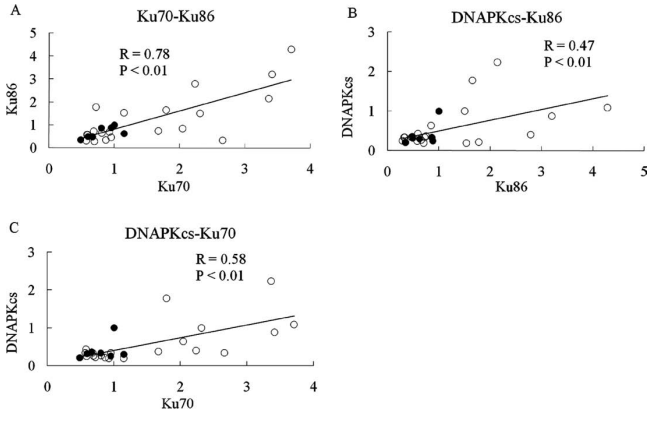


**Fig. 1.** The relationship between expression of Ku70 and Ku86 (A), Ku86 and DNA-PKcs (B), and Ku70 and DNA-PKcs genes (C) in PBLs, measured with RT-PCR. Values of a horizontal and vertical axis show the Ct value relative to β-actin. Open circles: healthy volunteers; closed circles: cancer patients.

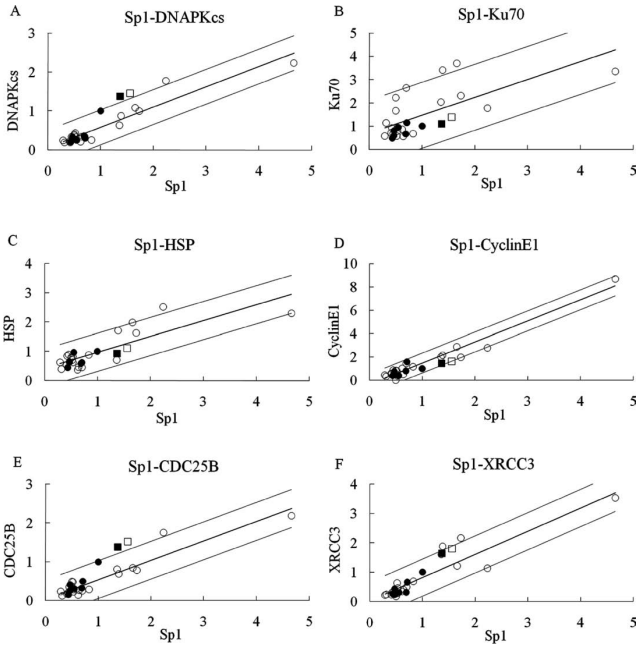


**Fig. 2.** The relationship between expression of Sp1 and various genes in PBLs, measured with RT-PCR. Values of a horizontal and vertical axis show the Ct value relative to β-actin. Open circles: healthy volunteers; closed circles: cancer patients; an open square: DLD-1 cells; a closed square: M059K cells. The thick is regression line and thin lines show the boundary of 95% confidence intervals. Both line were made of data of PBLs.

**Table 1.** Relationship between expression of Sp1 and various proteins in PBLs.*

| Gene name | Accession no. | correlation coefficient | P value |
|---|---|---|---|
| DNAPKcs | U63630.3 | 0.94 | <0.01 |
| Ku86 | AC008123.9 | 0.44 | <0.01 |
| Ku70 | NT_011520.2 | 0.67 | <0.01 |
| HSP90 | M27024.1 | 0.82 | <0.01 |
| CyclinE1 | AC008798.6 | 0.97 | <0.01 |
| CDC25B | AL109804.37 | 0.93 | <0.01 |
| XRCC3 | AF037222.1 | 0.92 | <0.01 |
| MCM5 | Z82244.1 | 0.93 | <0.01 |
| CyclinD3 | AL160163.5 | 0.51 | <0.01 |
| Rb2 | AC007342.3 | 0.90 | <0.01 |
| RAN | AC004602.1 | 0.93 | <0.01 |
| RAB | AL021069.1 | 0.72 | <0.01 |
| GZMB | AL136018.2 | 0.57 | <0.01 |

*The correlation coefficients and P values in the results of Fig. 2 are shown.

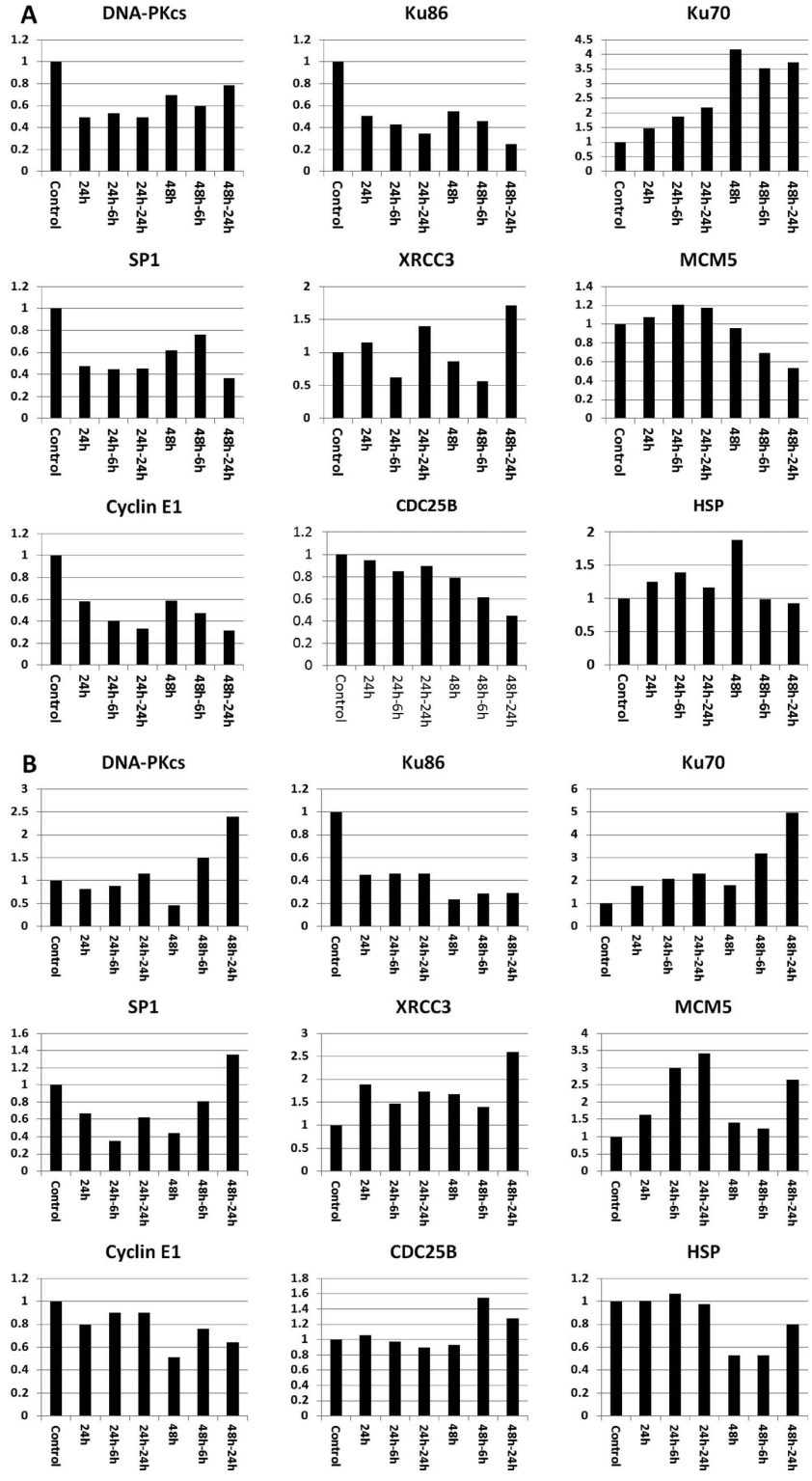


**Fig. 3.** Changes in expression of various genes in M059K (A) and DLD1 (B) cells, when these cells were treated with hypoxic and reoxygenation procedures. The same genes measured in Fig. 2 were examined with RT-PCR. Values of a vertical axis show the delta-delta Ct value relative to β-actin. "control" indicates cells that had no hypoxic treatment. "24 h" indicates cells that were placed in the hypoxic chamber for 24 h and RT-PCR measurements began just after cells were taken out of the chamber. "48 h–24 h" indicates cells that were placed in the hypoxic chamber for 48 h and RT-PCR measurements began after reoxygenation for 24 h. The change in expression was considered to be significant when the decrease of mRNA was less than 0.5 times as compared with the value of control or the increase of mRNA was more than 2 times as compared with the value of control.

of foci, clear and easily distinguished dots of certain brightness were counted as positive foci. The number of γH2AX foci was counted in 100 cells of the sample at each time point by visual inspection, and the average number of foci per cell was calculated. Since γH2AX foci became gradually larger and more distinguishable, here we used the average number of γH2AX foci at 24 h after irradiation for γH2AX foci quantification. The T test was used in order to examine the significance of differences between control and 24 h hypoxic treatment.

## RESULTS

Figure 1 shows significant correlation between the expression of Ku70 and Ku86, Ku70 and DNA-PKcs, and Ku86 and DNA-PKcs genes in PBLs, indicating that the mRNA expression of DNA-PKcs, Ku86 and Ku70 may be coordinated. The expression of Ku70 and Ku86 was especially well correlated (R = 0.78). Cancer patients had lower expression of Ku 86, Ku70 and DNA-PKcs than normal volunteers. However, relationships in expression among those genes were similar in both normal volunteers and cancer patients. Figure 2 and Table 1 demonstrate that expression of all examined genes was significantly related to expression of Sp1 in PBLs. In this analysis, we selected genes whose expression was associated with DNA-PK activity in PBLs with cDNA array technology.[7)]

Figure 2 also shows the expression of various genes in M059K cells and DLD1 cells under normoxia. The expression of SP1 and other genes in DLD1 cells was greater than that in M059K cells although the difference between them was small. Ninety five % confidence interval lines in Fig. 2 were made of data of PBLs. Data of M059K cells and DLD1 cells were within 95% confidence intervals or near 95% confidence interval lines, suggesting that the relationship between Sp1 and other genes in M059K cells and DLD1 cells was similar as that in PBLs. Cancer patients had lower expression of SP1 and various proteins related with DNA-PK activity than normal volunteers. However, relationships in expression among those genes were similar between normal volunteers and cancer patients.

Figure 3 shows changes in expression of various genes of M059K and DLD1 cells when these cells were treated with various durations of hypoxia and reoxygenation. In this analysis, we selected the same proteins as we analyzed in Fig. 1, Fig. 2, and Table 1, which had a significant relationship with Sp1 expression in Table 1. The change in expression after hypoxia and reoxygenation was considered to be significant when the decrease of mRNA was less than 0.5 times, or the increase of mRNA was more than 2 times, the value of control.[16)] The expression of most genes lost its relationship with the expression of Sp1 after hypoxia and reoxygenation in M059 cells and DLD-1 cells, although Ku86 and CyclinE1 maintained a significant relationship with Sp1 in M059K cells (Table 2). Expression of Ku70 significantly increased after hypoxia and reoxygenation in M059K and DLD1 cells (Fig. 3). Conversely, expression of DNA-PKcs, Ku86 and Sp1 decreased after hypoxia in M059K and DLD1 cells. Expression of DNA-PKcs, Ku86, and Sp1 did not recover after reoxygenation in M059K cells,

**Table 2.** Relationship between expression of Sp1 and various proteins in M059K and DLD-1 cells.*

| Gene name | Accession no. | correlation coefficient | P value | correlation coefficient | P value |
|---|---|---|---|---|---|
| | | **M059K** | | **DLD-1** | |
| DNAPKcs | U63630.3 | 0.65 | <0.12 | 0.84 | <0.02 |
| Ku86 | AC008123.9 | 0.88 | <0.01 | 0.18 | <0.70 |
| Ku70 | NT_011520.2 | 0.24 | <0.60 | 0.61 | <0.14 |
| HSP90 | M27024.1 | 0.15 | <0.75 | 0.05 | <0.91 |
| CyclinE1 | AC008798.6 | 0.86 | <0.01 | 0.04 | <0.94 |
| CDC25B | AL109804.37 | 0.34 | <0.45 | 0.50 | <0.25 |
| XRCC3 | AF037222.1 | 0.46 | <0.30 | 0.42 | <0.35 |
| MCM5 | Z82244.1 | 0.04 | <0.94 | 0.13 | <0.78 |
| CyclinD3 | AL160163.5 | 0.20 | <0.67 | 0.34 | <0.45 |
| Rb2 | AC007342.3 | 0.84 | <0.02 | 0.31 | <0.50 |
| RAN | AC004602.1 | 0.78 | <0.04 | 0.14 | <0.76 |
| RAB | AL021069.1 | 0.75 | <0.05 | 0.16 | <0.72 |
| GZMB | AL136018.2 | 0.08 | <0.86 | 0.34 | <0.45 |

*The correlation coefficients and P values in the results of Figs. 3A and 3B are shown.

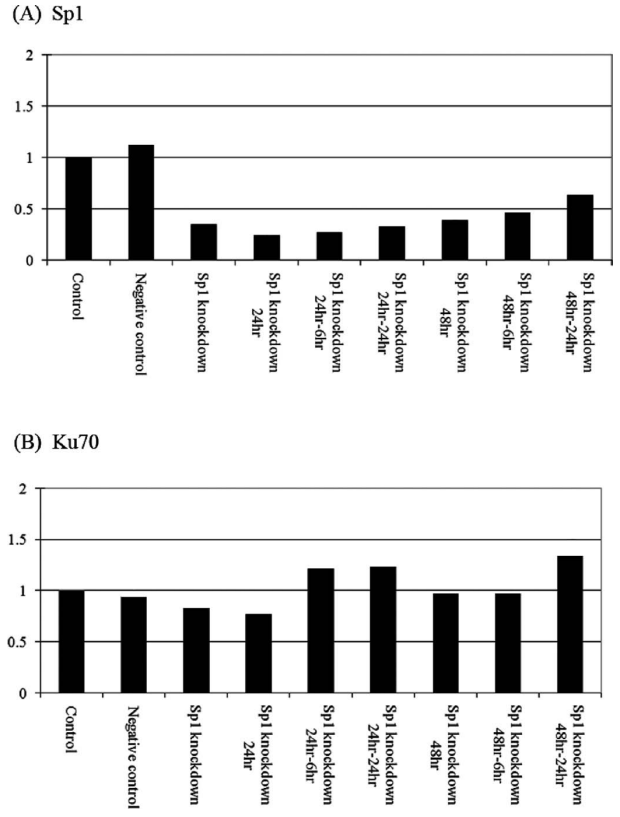


**Fig. 4.** (A) The expression of SP1 in DLD1 cells was suppressed by the specific siRNA transfection. (B) The expression of Ku70 in DLD1 cells whose expression of SP1 was knocked down. Experiments were repeated two times and average values are shown.

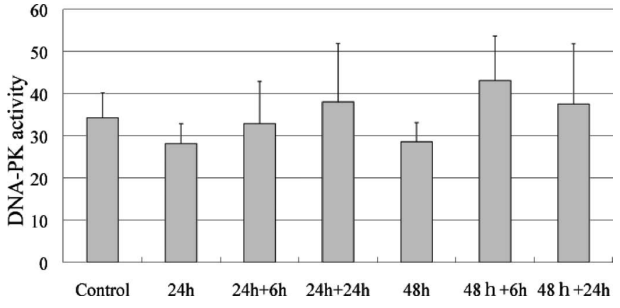


**Fig. 5.** Changes in DNA-PK activity in M059K cells when these cells were treated with hypoxic and reoxygenation procedures. "24 h" indicates M059K cells that were placed in the hypoxic chamber for 24 h and DNA-PK activity measurements began just after cells were taken out of the chamber. "48 h + 24 h" indicates M059K cells that were placed in the hypoxic chamber for 48 h and DNA-PK activity measurements began after reoxygenation for 24 h. Measurements were repeated three times and average values and standard deviation were expressed.

whereas that of DNA-PKcs and Sp1 recovered after reoxygenation in DLD1 cells.

Figure 4 demonstrates that the expression of Sp1 was suppressed by specific siRNA transfection and its expression was less than control after hypoxia and reoxygenation. The expression of Ku70 in DLD1 cells whose expression of Sp1 was subject to knockdown increased after hypoxia and reoxygenation.

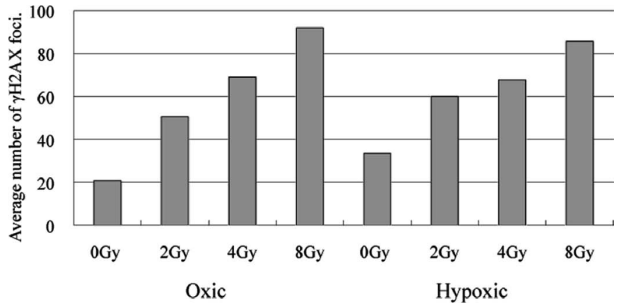


**Fig. 6.** Number of γH2AX foci in M059K cells at 24 h after irradiation with or without 24 h-hypoxic treatment. M059K cells were placed in the hypoxic chamber for 24 h and exposed to various doses of X rays. Cells were taken out of the chamber and incubated for further 24 h. Then, the number of γH2AX foci was counted.

Figure 5 demonstrates changes in DNA-PK activity in M059K cells when these cells were subjected to the same hypoxia and reoxygenation conditions as in Fig. 3. DNA-PK activity tended to decrease after hypoxic exposure and recover after reoxygenation. However, the range of change was not significant and within standard errors compared with control (no hypoxic exposure).

Figure 6 shows residual γH2AX foci at 24 h after various doses of irradiation in M059K cells that were treated with chronic hypoxia (24 h). There were no significant differences in residual γH2AX focus numbers between control and 24 hour-hypoxia treatment groups ($p = 0.473$).

## DISCUSSION

The significant correlation between expression of Ku70 and Ku86, Ku70 and DNA-PKcs, and Ku86 and DNA-PKcs genes found in PBLs (Fig. 1) indicates that these DNA-PK genes may be concertedly regulated. The result with PBLs agrees with that of Hosoi *et al*. in which mRNA levels of Ku70, Ku86, and DNA-PKcs were correlated with one another in colorectal cancer cells.[10)]

We previously reported that the expression of various genes, such as DNAPKcs, Ku70, Ku86, HSP90, Cyclin E1, CDC25B and so forth, was correlated with DNA-PK activity in PBLs with cDNA array technology.[7)] Since Sp1 consensus recognition elements in their promoter regions are found in Ku70,[10)] Ku86,[17)] DNA-PKcs,[18)] HSP90,[19)] Cyclin E1,[20)] CDC 25B,[21)] XRCC3,[22)] and MCM5,[23)] Sp1 may control the concerted expression of these genes. The study of Hosoi *et al*. also suggested that the expression of Sp1-influenced mRNA levels of Ku70, Ku86, and DNA-PKcs, together with DNA-PK activity in colorectal cancer cells.[10)] Therefore, we

examined whether the expression of Sp1 was associated with mRNA levels of Ku70, Ku86, and DNA-PKcs in PBLs from cancer patients and normal volunteers who had various levels of DNA-PK activity. The expression of all examined genes was closely related to expression of Sp1 in PBLs (Fig. 2 and Table 1). This result suggested that the expression of Sp1 might control mRNA levels of Ku70, Ku86 and DNA-PKcs and various proteins that are related to DNA-PK activity in PBLs. Cancer patients had lower expression of Ku86, Ku70 and DNA-PKcs than normal volunteers. This result agreed with our previous report showing that DNA-PK activity of PBL in patients with uterine cervix or breast cancer was significantly lower than in normal volunteers.[6] The reason for lower expression of DNA-PK in cancer patients is unknown. However, relationships between Sp1 and the various proteins related to DNA-PK activity were maintained in cancer patients.

Hypoxic regions are known to exist in many human cancers. Hypoxia in tumors can result from two different mechanisms. Chronic hypoxia results from the limited diffusion distance of oxygen through tissue that is respiring, which makes many tumor cells remain hypoxic for long periods of time. In contrast to chronic hypoxia, acute hypoxia is the result of the temporary closing of a tumor blood vessel owing to the malformed vasculature of the tumor, which makes tumor cells intermittently hypoxic because normoxia is restored each time the blood vessels open up again.[8]

There are several reports that describe the relationship between the relatively short duration of hypoxic exposure (acute hypoxia) and protein/mRNA levels of DNA-PK.[24,25] However, as far as we are aware, there are no reports examining the relationship between long duration of hypoxic exposure (chronic hypoxia) and DNA-PK activity. In this study, we examined the influence of chronic hypoxia (24 h or 48 h) and various durations of reoxygenation on DNA-PK in its expression and its activity. The expression of most genes, except for Ku86 and CyclinE1 in M059K cells (Table 2), lost its relationship with that of Sp1 after hypoxia and reoxygenation (Fig. 3), indicating that the expression of most genes related to DNA-PK activity deviated from the control of Sp1 under hypoxic and reoxygenation conditions. This was supported by the results of Fig. 4 demonstrating that the expression of Ku70 increased after hypoxia and reoxygenation although the cells' expression of Sp1 was knocked down.

Among the instances of expression of the various genes that were investigated, the increase in expression of Ku70 was remarkable after hypoxia: an increase observed in both M059K and DLD1 cells. Conversely, expression of DNA-PKcs, Ku86 and Sp1 decreased significantly after hypoxia in both cells. Although the change in DNA-PK activity after hypoxia and reoxygenation was not significant, DNA-PK activity tended to decrease after hypoxic exposure and recover after reoxygenation in M059K cells (Fig. 5). There was a large discrepancy between DNA-PK activity and expression of Ku70 after hypoxia and reoxygenation. However, some similarity might exist between DNA-PK activity and expression of DNA-PKcs. Since there is a very large pool of Ku proteins relative to DNA-PKcs in the cell,[26] DNA-PKcs may influence DNA-PK activity more than do Ku proteins.

Kang *et al*. reported that the mRNA and protein levels of DNA-PKcs, Ku70 and Ku80, and activity of DNA-PK increased after hypoxia and reoxygenation.[24] They used short duration hypoxic exposure (2 h) and reoxygenation (15 or 30 min). These results indicate that the influence of hypoxia on protein/mRNA levels of DNA-PK might be different according to the duration of the hypoxia. Shackelford *et al*. used a rabbit spinal cord model of reversible ischemia to demonstrate the effect of acute injury of the central nervous system on the activity and expression of DNA-PK.[27] The DNA-binding activity of the Ku antigen, analyzed by an electrophoretic mobility shift assay, increased during reperfusion after a short ischemic insult (15 min of occlusion), from which the animals recovered neurological function. However, after severe ischemic injury (60 min of occlusion) and reperfusion that resulted in permanent paraplegia, Ku DNA binding was reduced. After 60 min of occlusion, the amount of DNA-PKcs decreased during reperfusion. This in vivo study suggested that the DNA-binding activity of the Ku antigen and the expression of DNA-PKcs were different according to ischemic (occlusion) duration.

Figure 6 shows residual γH2AX foci at 24 h after various doses of irradiation in M059K cells after chronic hypoxia (24 h). There were no significant differences in residual γH2AX foci between control cells and 24 hour-hypoxia treated cells in both M059K. This result indicated that 24 hour- hypoxia treatment might not significantly change DNA DSB repair.

In summary, expression of all examined genes whose expression was related to DNA-PK activity was significantly correlated with that of Sp1 in PBLs, indicating that Sp1 expression may control expression of those genes. Expression of most genes lost the relationship with expression of Sp1 after hypoxia and reoxygenation, indicating that the expression of most genes related to DNA-PK activity deviated from the control of Sp1 under hypoxic and reoxygenation conditions. DNA-PK activity tended to decrease after hypoxic exposure and recover after reoxygenation. However, the range of change was not significant, and within standard errors. The control mechanism of expression of genes associated with DNA-PK activity under hypoxia and reoxygenation may be different from that under aerobic conditions.

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## REFERENCES

1. Hall EJ (2000) DNA strand breaks and chromosomal aberrations. In Radiobiology for the radiologist. Lippincott Williams & Wilkins, Philadelphia, pp. 16–30.
2. Sachs RK, Chen AM and Brenner DJ (1997) Review: proximity effects in the production of chromosome aberrations by ionizing radiation. Int J Radiat Biol **71**: 1–19.
3. Ferguson DO, *et al* (2000) The nonhomologous end-joining pathway of DNA repair is required for genomic stability and the suppression of translocations. Proc Natl Acad Sci USA **97**: 6630–6633.
4. Lees-Miller SP (1996) The DNA-dependent protein kinase, DNA-PK: 10 years and no ends in sight. Biochem Cell Biol **74**: 503–512.
5. Jeggo PA (1998) Identification of genes involved in repair of DNA double-strand breaks in mammalian cells. Radiat Res **150**: S80–S91.
6. Someya M, *et al* (2006) The association of DNA-dependent protein kinase activity with chrmosomal instability and risk of cancer. Carcinogenesis **27**: 117–122.
7. Sakata K, *et al* (2007) cDNA analysis of gene expression associated with DNA-dependent protein kinase activity. Inter J Oncol **30**: 413–420.
8. Hall EJ (2000) Oxygen effect and reoxygenation. In Radiobiology for the radiologist. Lippincott Williams & Wilkins, Philadelphia, pp. 85–105.
9. Hirayama R, *et al* (2005) Repair kinetics of DNA-DSB induced by X-rays or carbon ions under oxic and hypoxic conditions. J Radiat Res **46**: 325–332.
10. Hosoi Y, *et al* (2004) Up-regulation of DNA-dependent protein kinase activity and Sp1 in colorectal cancer. Inter J Oncol **25**: 461–468.
11. Lees-Miller SP, *et al* (1995) Absence of p350 subunit of DNA-activated protein kinase from a radiosensitive human cell line. Science **267**: 1183–1185.
12. Aoki M, *et al* (2002) Effect of a hypoxic cell sensitizer doranidazole on the radiation-induced apoptosis of mouse L5178Y lymphoma cells. Journal of Radiat Res **43**: 161–166.
13. Taniguchi H, *et al* (2005) Frequent epigenetic inactivation of Wnt inhibitory factor-1 in human gastrointestinal cancers. Oncogene **24**: 7946–7952.
14. Sasaki Y, *et al* (2003) Identification of the interleukin 4 receptor alpha gene as a direct target for p73. Cancer Res **63**: 8145–8152.
15. Maruyama R, *et al* (2006) Comparative genome analysis identifies the vitamin D receptor gene as a direct target of p53-mediated transcriptional activation. Cancer Res **66**: 4574–4583.
16. Audige A, *et al* (2004) HIV-1 does not provoke alteration of cytokine gene expression in lymphoid tissue after acute infection ex vivo. J Immunol **172**: 2687–2696.
17. Ludwig DL, *et al* (1997) Ku80 gene expression is Sp1-dependent and sensitive to CpG methylation within a novel cis element. Gene **199**: 181–194.
18. Fujimoto M, *et al* (1997) Characterization of the promoter region, first ten exons and nine intron-exon boundaries of the DNA-dependent protein kinase catalytic subunit gene, DNA-PKcs (XRCC7). DNA Res **4**: 151–154.
19. Hung JJ, *et al* (2005) Hsp90alpha recruited by Sp1 is important for transcription of 12(S)-lipoxygenase in A431 cells. J Biol Chem **280**: 36283–36292.
20. Kim S, *et al* (2006) Histone deacetylase inhibitor apicidin induces cyclin E expression through Sp1 sites. Biochem Biophys Res Comm **342**: 1168–1173.
21. Korner K, *et al* (2001) Cell cycle regulation of the murine cdc25B promoter: essential role for nuclear factor-Y and a proximal repressor element. J Biol Chem **276**: 9662–9669.
22. Schmutte C, *et al* (1999) Characterization of the human Rad51 genomic locus and examination of tumors with 15q14-15 loss of heterozygosity (LOH). Cancer Res **59**: 4564–4569.
23. Ohtani K, *et al* (1999) Cell growth-regulated expression of mammalian MCM5 and MCM6 genes mediated by the transcription factor E2F. Oncogene **18**: 2299–2309.
24. Kang MJ, *et al* (2008) DNA-dependent protein kinase is involved in heat shock protein-mediated accumulation of hypoxia-inducible factor-1alpha in hypoxic preconditioned HepG2 cells. FEBS J **275**: 5969–5981.
25. Um JH, *et al* (2004) Association of DNA-dependent protein kinase with hypoxia inducible factor-1 and its implication in resistance to anticancer drugs in hypoxic tumor cells. Exp Mol Med **36**: 233–242.
26. Burgman B, *et al* (1997) Heat inactivation of Ku autoantigen: possible role in hyperthermic radiosensitization. Cancer Res **57**: 2847–2850.
27. Shackelford DA, *et al* (1999) Changes in expression of the DNA repair protein complex DNA-dependent protein kinase after ischemia and reperfusion. J Neurosci **19**: 4727–4738.

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