

Local tumor control and DNA-PK activity of peripheral blood lymphocytes in prostate cancer patients receiving radiotherapy

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ABSTRACT

Repair of DNA damage is critical for genomic stability, and DNA-dependent protein kinase (DNA-PK) has an important role in repairing double-strand breaks. We examined whether the DNA-PK activity of peripheral blood lymphocytes (PBLs) was related to biochemical (prostate-specific antigen: PSA) relapse and radiation toxicity in prostate cancer patients who have received radiotherapy. A total of 69 patients with localized adenocarcinoma of the prostate participated in this study. Peripheral blood was collected 2 years or later after radiotherapy and centrifuged, then DNA-PK activity was measured by a filter binding assay. The high DNA-PK activity group had a significantly higher PSA relapse-free survival rate than the low DNA-PK activity group. The 10-year PSA relapse-free survival was 87.0% in the high DNA-PK activity group, whereas it was 52.7% in the low DNA-PK activity group. Multivariate analysis showed the Gleason score and the level of DNA-PK activity were significant predictors of PSA relapse after radiotherapy. In addition, the low DNA-PK activity group tended to have a higher incidence of Grade 1–2 urinary toxicity than the high DNA-PK activity group. Prostate cancer patients with low DNA-PK activity had a higher rate of PSA relapse and a higher incidence of urinary toxicity. DNA-PK activity in PBLs might be a useful marker for predicting PSA relapse and urinary toxicity, possibly contributing to personalized treatment of prostate cancer.

KEYWORDS: prostate cancer, radiotherapy, predictive assay, late urinary toxicity, DNA-PK activity

INTRODUCTION

When radiotherapy is performed for the treatment of localized prostate cancer, a high dose needs to be delivered to the prostate, and unnecessary irradiation to the surrounding normal organs/tissues (e.g. the rectum, urethra and bladder) needs to be avoided. Development of techniques such as 3D conformal radiotherapy (3D-CRT) and intensity-modulated radiation therapy (IMRT) has allowed escalation of the dose delivered to the prostate, enhancing local tumor control without a significant increase of treatment-related toxicity [1–4]. To achieve further improvement, the results of recent studies on prediction of the

outcome of radiotherapy and late radiation-induced toxicity may be used to contribute to the development of personalized treatment [5, 6].

Ionizing radiation is thought to exert its various biological effects by causing various types of damage to DNA, of which DNA double-strand breaks (DSBs) are considered the most critical type. DSBs are repaired through homologous recombination (HR) and non-homologous end-joining (NHEJ) [7, 8]. Whereas HR is a response reconstituting the sequence around the DSB via strand synthesis using the sister chromatid sequence as the template, NHEJ is a response joining two DNA ends in close vicinity to the DSB. NHEJ

can be further classified into canonical (or classical) NHEJ (C-NHEJ) and alternative (or atypical) NHEJ (A-NHEJ). C-NHEJ is thought to be more ordered and more accurate than A-NHEJ and is involved in variable, diversity and joining [V(D)J] recombination in immune systems [7, 8]. The DNA repair mechanism, especially through C-NHEJ, is considered one of the major determinants for differences in intrinsic radiosensitivity and, thus, one of the keys to predicting response to radiation [9].

DNA-dependent protein kinase (DNA-PK) is a serine/threonine kinase that is composed of the DNA-PK catalytic subunit (DNA-PKcs) and a heterodimer of Ku70 and Ku80; it is thought to play an important role in C-NHEJ [7, 9]. The catalytic activity of DNA-PK is shown to be essential for C-NHEJ. DNA-PKcs has been shown to phosphorylate a number of proteins, including itself and XRCC4 [7, 10] in response to DSBs, although further studies are required for thorough understanding of the targets and meaning of phosphorylation in C-NHEJ.

We measured the DNA-PK activity of peripheral blood lymphocytes (PBLs) because it is not productive to evaluate the DNA-PK activity of infinitesimally small tumor biopsy tissues specimens. Auckley *et al.* [11] demonstrated a tight correlation between the DNA-PK activity in PBLs and that in bronchial epithelial cells (progenitor cells for lung cancer) that had been obtained by bronchoscopy, suggesting that PBLs can be used as a surrogate cell type for other kinds of cells. Using PBLs, we have performed several studies seeking to predict prognosis after radiotherapy. We showed that the level of DNA-PK activity in PBLs might be a prognostic indicator for the response of several types of advanced cancer to radiotherapy [12]. In breast cancer patients, the level of DNA-PK activity in PBLs was related to tumor aggressiveness and to the presence of axillary lymph node metastasis [13].

In the present study, to facilitate the development of personalized radiotherapy for prostate cancer, we examined PBL DNA-PK activity in relation to treatment outcome and late urinary toxicity in 69 prostate cancer patients undergoing 3D-CRT or IMRT.

MATERIALS AND METHODS

Between February 2002 and September 2010, 129 patients with localized prostate cancer were treated with definitive radiotherapy with curative intent at our institution. Of these, 20 patients treated with 3D-CRT and 49 patients treated with IMRT were eligible for this study. All patients were confirmed to have adenocarcinoma of the prostate by biopsy. Their risk groups were classified according to the guidelines of the National Comprehensive Cancer Network (NCCN) [14]. Patient characteristics are listed in Table 1.

Radiotherapy treatment

For daily treatment, patients were requested to empty the rectum and urinate at 1 h before radiotherapy and then drink 500 ml of water in order to achieve a consistent state of the bladder and rectum. All radiotherapy involved the delivery of 10 MV X-rays using a linear accelerator. Three-dimensional CRT was performed using a Varian Clinac 2100 (Varian Medical Systems Inc., Palo Alto, CA). The dose was set as 70 Gy at the isocenter and was delivered in 35 fractions. The clinical target volume (CTV) was defined according

Table 1. Patient characteristics

Age	49–79 (69.3)
Follow up (months)	25–163 (70.9)
NCCN risk group	
Low	7
Intermediate	25
High	37
T1c	42
T2a	4
T2b	4
T2c	2
T3a	6
T3b	11
Gleason score	
5–6	14
7	30
8–10	25
PSA (ng/ml)	
<10	28
10–20	17
>20	24
Hypertension	28
Diabetes mellitus	6
Usage of anticoagulants	9
PTV dose	
70 Gy	21
71–75 Gy	6
76 Gy	42
ADT	
neoadjuvant	30
concurrent	28
adjuvant	17

Median values are showed in parentheses. ADT = androgen deprivation therapy.

to risk, as evaluated by the NCCN guidelines. In the low- and intermediate-risk groups, the CTV was defined as the prostate and the proximal portions of the seminal vesicles. In the high-risk group, the CTV included the entire prostate and seminal vesicles. The

planning target volume (PTV) margin was set at 1 cm in all directions for 3D-CRT planning. Rotational conformal radiotherapy was employed. IMRT was delivered with a Primus (Siemens AG, Munchen, Germany). From the start of IMRT, 2D-bone matching with an ExacTrac X-Ray 6D stereotactic image-guided radiation therapy system (BrainLAB AG, Feldkirchen, Germany) was introduced. The dose covering 95% of the target volume (D95) was set as 76 Gy and was delivered in 38 fractions. The CTV was defined according to risk, as evaluated by the NCCN guidelines. In the low-risk group, the CTV was defined as the prostate and the proximal portions of the seminal vesicles. In the intermediate- and high-risk groups, the CTV included the entire prostate and seminal vesicles. The PTV margin was 8 mm in all directions except posteriorly, where it was 5 mm. Seven to nine planar beams were used.

Blood collection and peripheral blood lymphocyte separation

Blood collections were performed at outpatient visits between February 2012 and November 2013, at least 2 years after completion of radiotherapy. A sample of 20 ml of peripheral blood was collected with a sterile heparinized tube from each individual. Peripheral blood lymphocytes (PBLs) were separated with lymphoprep (Nycomed Pharma AS, Oslo, Norway), centrifuged at 1500 r.p.m. for 30 min at 4°C, washed twice with phosphate-buffer saline (PBS) and stored for DNA-PK activity assay.

DNA-PK activity assay

The PBLs were thawed with high salt buffer [20 mMHEPES-KOH (pH 7.9), 400 mM KCl, 1 mM EDTA, 1 mM EGTA, 0.02% Tween-20, 10% Glycerol, 1 mM DTT, 1 mM PMSF and 1 g/ml leupeptin, pepstatin and antipain, respectively], and the suspension was lysed by three rounds of freeze-thaw cycles, i.e. repeated freezing in a liquid nitrogen bath followed by thawing in a water bath at 30°C; they were then clarified by centrifugation at 15 000 r.p.m. for 7 min at 4°C. Protein concentration was assayed using a BCA protein assay kit (Pierce, Rockford, IL, USA), with bovine serum albumin (BSA) as the standard.

The assay procedure for DNA-PK activity has been described in our earlier publication [15]. The PBL cell lysates were diluted to an appropriate protein concentration (0.25 mg/ml) with high salt buffer. An aliquot of 5 µl of the diluted lysate was mixed with 15 µl of kinase assay buffer [contents of 1 kinase assay buffer: 20 mM HEPES-NaOH (pH 7.2), 5 mM MgCl₂, 150 mM KCl, 50 mM [γ-³²P] ATP, 1 mM DTT, 0.5 mM NaF and 0.5 mM sodium glycerophosphate], 0.25 mg/ml synthetic peptide hp53-S15 (sequence: EPPLSQEAFADLWKK; synthesized in Sawady Biotechnology, Tokyo, Japan) with or without 20 ng/ml sonicated salmon sperm DNA. This reaction mixture was incubated at 37°C for 10 min. The reaction was stopped by the addition of 20 µl of 30% acetic acid and absorbed onto a phosphocellulose filter disk (2.3 cm in diameter, Whatman, Maidstone, UK). The filter disks 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. In this study, DNA-PK activity was expressed as amount of ATP used for DNA-dependent phosphorylation of hp53-S15. The same sample was simultaneously run in all experiments as an internal control, and relative DNA-PK activity values were calculated.

Follow-up

After the completion of radiotherapy, follow-up evaluation was performed at 3–6 month intervals for 5 years and every 6 months thereafter. The median follow-up period for this study was 117 months (91–163 months) in the 3D-CRT group and 68.0 months (25–93 months) in the IMRT group. The cut-off date for analysis was November 2015.

PSA relapse was defined according to the Phoenix definition (nadir + 2 ng/ml) [16]. The PSA relapse-free survival was calculated from the date when the treatment started to the time of PSA relapse. Urinary or rectal toxicity was graded according to the National Cancer Institute Common Terminology Criteria for Adverse Events ver. 4.0. All time intervals were measured from the completion of radiotherapy to the onset of urinary or rectal toxicity.

All statistical tests were two-sided. Survival rates of the patients were measured using the Kaplan-Meier method. Statistical significance of survivals was compared by the log rank test. The Cox proportional hazards model was used to identify variables that had an influence on PSA relapse. Receiver operating characteristic curves were generated to determine all cut-off values. All statistical computing was done with BellCurve for Excel 2.00 (BellCurve, Tokyo, Japan).

This study was approved by our institutional review board (Approval No. 25-191), and written informed consent was obtained from all patients.

RESULTS

At the time of analysis, 18 patients had shown PSA relapse and received additional hormone therapy or chemotherapy. One patient died of distant metastasis, and 4 patients died of other cancers (cancer of the bile duct, gallbladder, lung and bladder, in one case each).

Late urinary toxicity was seen in 15 patients. Among them, 11 patients had Grade 1 toxicity (transient or mild hematuria) and 4 patients had Grade 2 toxicity (ureteral stenosis requiring balloon dilatation).

Late rectal toxicity occurred in 36 patients. Of these, 17 patients had Grade 1 toxicity (transient or mild rectal bleeding treated with steroid suppositories) and 19 patients had Grade 2 toxicity (rectal bleeding requiring ≥1 session of argon plasma laser coagulation therapy).

Figure 1 displays PSA relapse-free survival stratified by the NCCN risk group. The 5-year PSA relapse-free survival rates of the low-risk, intermediate-risk and high-risk groups were 100%, 83.8% and 81.1%, respectively, while the 10-year PSA relapse-free survival rates were 66.7%, 75.4% and 61.2%, respectively.

Table 2 compares treatment characteristics between the groups with or without PSA relapse. None of the parameters showed a statistically significant difference between the patients with and without PSA relapse.

Figure 2A shows the relationship between PBL DNA-PK activity and PSA relapse. When we divided the patients into two groups

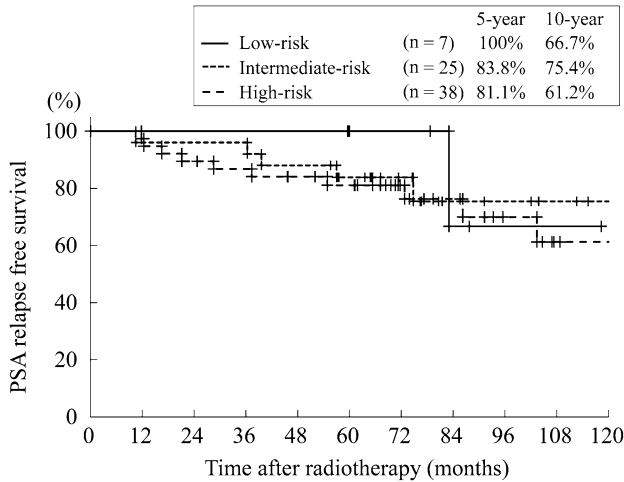


Fig. 1. PSA relapse-free survival after radiotherapy stratified by NCCN risk group.

using a cut-off value of 0.27, the high PBL DNA-PK group had a significantly higher PSA relapse-free survival rate than the low PBL DNA-PK group (Fig. 2B, $P = 0.013$). That is, 5-year and 10-year PSA relapse-free survival was respectively 91.8% and 87.0% in the high PBL DNA-PK group, whereas it was respectively 73.9% and 52.7% in the low PBL DNA-PK group.

Table 3 displays the results of multivariate analysis of factors predicting PSA relapse. The Gleason score (5–7 vs 8–10) and PBL DNA-PK activity (high vs low) were significant predictors of PSA relapse. Other variables were not significant, including the mean PTV dose, pretreatment PSA level, and adjuvant hormone therapy.

Figure 3 shows the relationship between PBL DNA-PK activity and the NCCN risk group. Although it was not statistically significant, there was a trend for lower average PBL DNA-PK activity to be associated with a higher NCCN risk.

Figure 4A shows PSA relapse-free survival stratified by the Gleason score (5–7 vs 8–10). The low Gleason score group had a higher PSA relapse-free survival than the high Gleason score group, but the difference had borderline significance ($P = 0.069$). When we examined the prediction of PSA relapse using the Gleason score combined with PBL DNA-PK, patients with Gleason score 8–10 and low PBL DNA-PK activity had a significantly higher PSA relapse rate than patients with Gleason score 5–7 and high PBL DNA-PK activity (Fig. 4B, $P = 0.003$).

Figure 5A displays the relationship between PBL DNA-PK activity and urinary toxicity. When the patients were divided into two groups using a cut-off value of 0.36, the low PBL DNA-PK activity group had a higher incidence of Grade 1–2 urinary toxicity than the high PBL DNA-PK activity group, but the difference was only of borderline significance (Fig. 5C, $P = 0.068$).

There was no significant relationship between rectal toxicity and the level of PBL DNA-PK activity (Fig. 5B).

DISCUSSION

The present study demonstrated that after radiotherapy for localized prostate cancer, patients with low PBL DNA-PK activity had a

Table 2. Relationship between treatment parameters and PSA relapse

	PSA relapse		P value
	No (n = 51)	Yes (n = 18)	
Age	49–79 (69.8)	61–79 (68.0)	
PTV dose			
70 Gy	12	9	
71–75 Gy	5	1	
76 Gy	34	8	0.059
NCCN risk group			
Low	6	1	
Intermediate	19	6	
High	26	11	0.397
T1c	32	10	
T2a–c	6	4	
T3a–b	13	4	0.764
Gleason score			
5–6	12	2	
7	22	8	
8–10	17	8	0.254
PSA (ng/ml)			
<10	21	7	
10–20	12	5	
>20	18	6	0.982
ADT			
neoadjuvant	23	7	0.784
concurrent	22	6	0.581
adjuvant	14	3	0.749
DNA-PK activity	0.43 ± 0.32	0.32 ± 0.24	0.195

Median values are showed in parentheses. ADT = androgen deprivation therapy.

significantly higher PSA relapse rate than patients with high PBL DNA-PK activity (Figs 2B and 4B). We previously reported that among patients with breast cancer, uterine cervical cancer and head-and-neck cancer, low PBL DNA-PK activity was associated with a higher incidence of distant metastasis and a worse prognosis than high PBL DNA-PK activity [12]. Therefore, low DNA-PK activity in PBLs may be a potential marker of a worse prognosis for breast

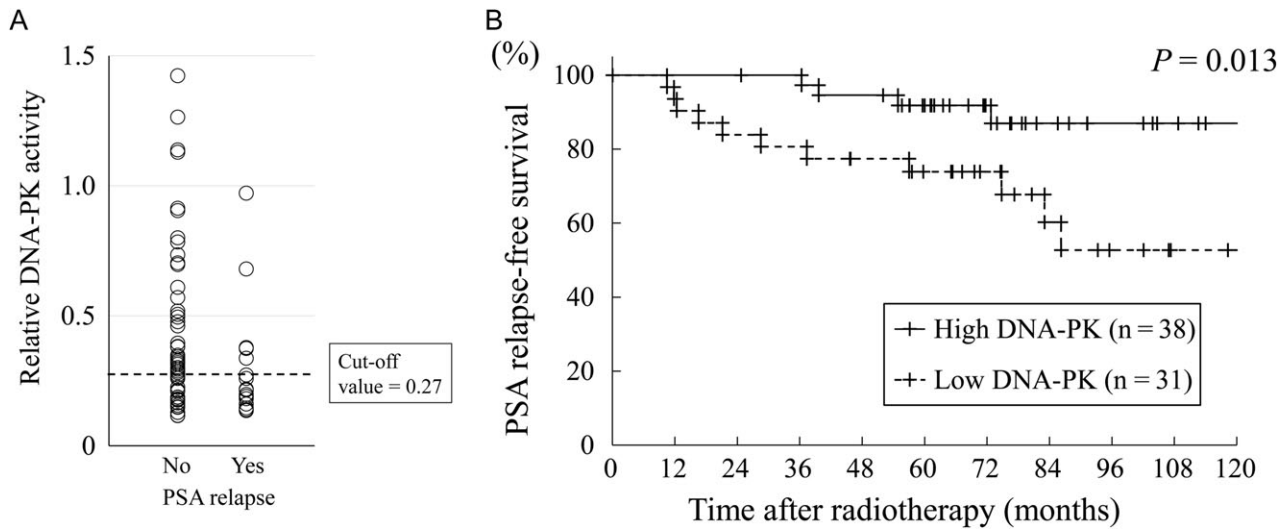


Fig. 2. (A) Relationship between relative PBL DNA-PK activity and PSA relapse. Dotted line showed the cut-off value of 0.27. (B) PSA relapse-free survival after radiotherapy stratified by PBL DNA-PK activity at the cut-off value shown in (A) (high PBL DNA-PK group vs low PBL DNA-PK group).

Table 3. Multivariate analysis of predictive factors for PSA relapse

Variable	Hazard ratio	95% CI	P value
Gleason score			
5–7 vs 8–10	3.774	1.160–12.29	0.027
PTV dose			
70 Gy vs >70 Gy	0.751	0.243–2.321	0.619
PSA			
<10 vs 10–20 vs >20	1.439	0.765–2.704	0.259
Adjuvant ADT			
No vs yes	0.402	0.093–1.735	0.222
DNA-PK			
High vs low	3.695	1.171–11.66	0.026

cancer, cervical cancer and head-and-neck cancer due to the associated higher frequency of distant metastasis.

In the current study, only one of 69 patients (1.4%) with PSA relapse had distant metastasis and regional lymph node metastasis, indicating that PSA relapse was overwhelmingly due to local failure. The detailed reasons for why low PBL DNA-PK activity was associated with radioresistance of prostate cancer are not very well understood. Figure 3 shows the relationship between PBL DNA-PK activity and the NCCN risk group, indicating that DNA-PK activity tended to decrease as the NCCN risk increased. Since the NCCN risk is an important prognostic factor for patients receiving

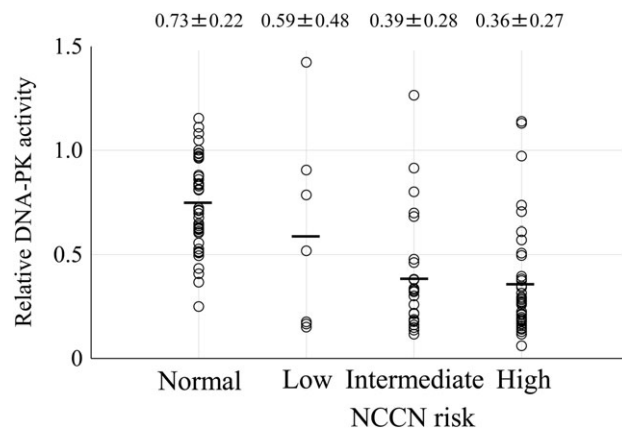


Fig. 3. Relationship between relative PBL DNA-PK activity and NCCN risk. PBL DNA-PK activity of normal healthy volunteers is cited from our previous report [15].

radiotherapy, patients with low PBL DNA-PK activity could have had a worse prognosis due to their more advanced NCCN risk.

Lower level of DNA-PK activity could profoundly affect DNA DSB repair, resulting in the perpetuation of chromosomal damage. We have previously demonstrated that low DNA-PK activity is related to chromosomal instability [15]. Aggressive features of cancer, such as a higher NCCN risk classification, result from many different genetic alterations that promote rapid tumor cell proliferation and metastasis [17]. Genetic instability can lead to loss or activation of a number of critical genes involved in cell proliferation, differentiation, and apoptosis [18–21]. Low PBL DNA-PK activity might be associated with phenotypic features of tumor aggressiveness, such as a higher risk of PSA relapse of prostate cancer.

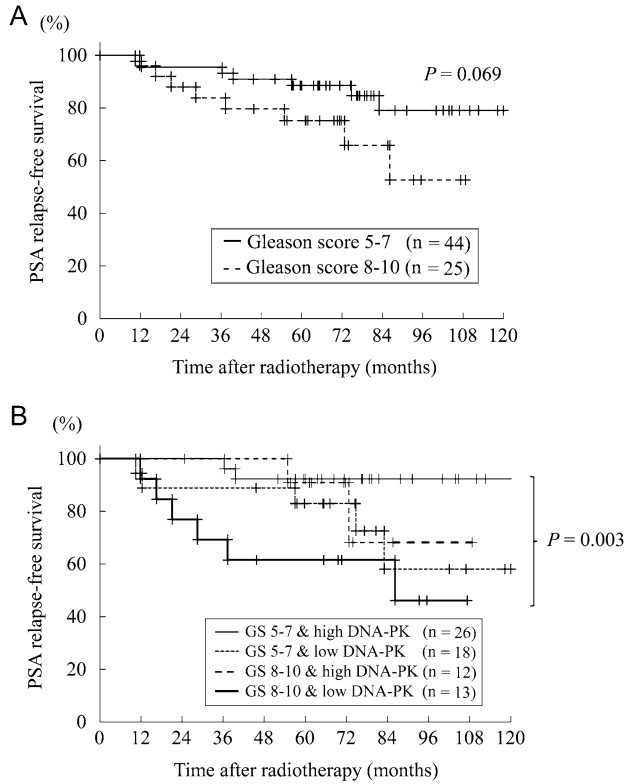


Fig. 4. (A) PSA relapse-free survival after radiotherapy stratified by Gleason score (GS) (5–7 vs 8–10). **(B)** PSA relapse-free survival after radiotherapy stratified by GS (5–7 vs 8–10) and PBL DNA-PK activity at the cut-off value shown in Fig. 2A.

We found a borderline relationship between PBL DNA-PK activity and urinary toxicity (Fig. 5A and C). That is, the low PBL DNA-PK activity group tended to have a higher incidence of Grade 1–2 urinary toxicity than the high PBL DNA-PK activity group (Fig. 5C). In contrast, there was no relationship between PBL DNA-PK activity and rectal toxicity (Fig. 5B). We previously reported that Grade 2–3 rectal bleeding after radiotherapy for prostate cancer was well correlated with the combination of low Ku80 expression and radiation-induced miR-99a expression in PBLs [22]. The miR-99 family of miRNAs targets SWI/SNF chromatin remodeling factor SNF2H/SMARCA5. Remodeling of chromatin seems to be crucial during the early stages of the NHEJ pathway to facilitate chromatin relaxation and allow repair proteins to access the sites of DNA DSBs [23]. Introduction of the miR-99 family of miRNAs into cells reduces the rate and overall efficiency of repair by both HR and NHEJ. Taken together, these results indicate that both urinary and rectal toxicity of radiotherapy for prostate cancer are related to the repair capacity for DNA DSBs, suggesting that measurement of the activity or expression of proteins involved with NHEJ could be a promising approach to the prediction of urinary toxicity and rectal toxicity.

In conclusion, prostate cancer patients with low PBL DNA-PK activity had a higher rate of PSA relapse and higher incidence of

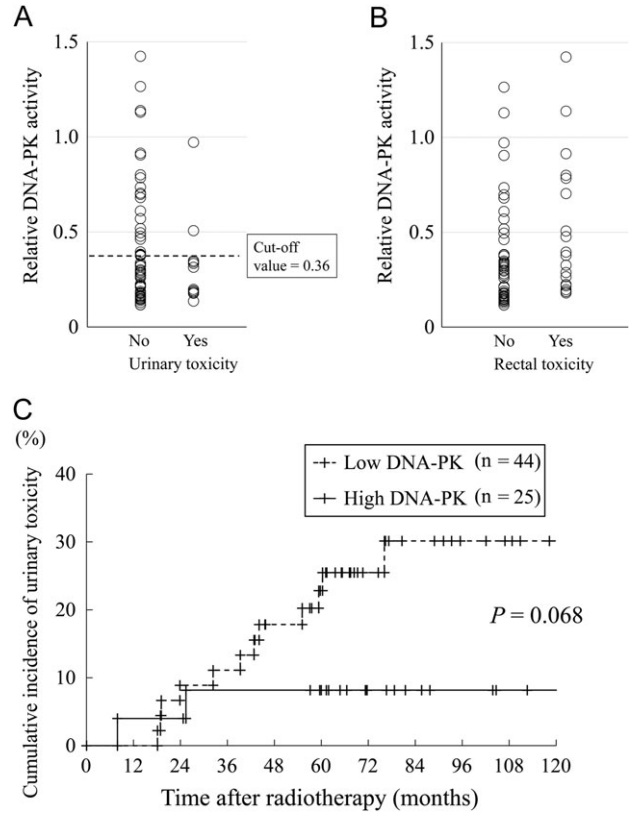


Fig. 5. (A) Relationship between relative PBL DNA-PK activity and Grade 1–2 urinary toxicity. Dotted line shows the cut-off value of 0.36. **(B)** Relationship between relative PBL DNA-PK activity and Grade 2–3 rectal toxicity. **(C)** Cumulative incidence of urinary toxicity stratified by PBL DNA-PK activity at the cut-off value shown in (A) (high PBL DNA-PK group vs low PBL DNA-PK group).

urinary toxicity after radiotherapy compared with patients with high PBL DNA-PK activity. Accordingly, the level of DNA-PK activity in PBLs might be a marker that could contribute to personalized treatment of prostate cancer.

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CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest.

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