Abstract. DNA double-strand break (DSB) is one of the most deleterious lesions induced by DNA damaging agents. DSB repair pathway is implicated in maintaining genomic integrity via suppression of genetic instability and neoplastic transformation. DNA-dependent protein kinase (DNA-PK) has a pivotal role in DNA DSB repair. The Nijmegen breakage syndrome protein (NBS1), essential for DSB repair, re-localizes into subnuclear structures upon induction of DNA damage by ionizing radiation, forming so-called ionizing radiation-induced foci (IRIF), which is visualized by immunostaining. We measured DNA-PK activity and the number of persistent NBS1 IRIF per nucleus 24 h after irradiation of peripheral blood lymphocytes (PBL) from patients with sporadic breast cancer. Chromosomal aberrations were examined by cytogenetic methods. We examined the relationship between these measurements and clinical characteristics of patients such as tumor size, lymph node metastasis and nuclear grade of cancer cells. A higher number of NBS1 IRIF or lower DNA-PK activity correlated with higher chromosome instability. Patients whose PBL had lower DNA-PK or higher NBS1 IRIF had aggressive cancer phenotypes such as a larger tumor, higher nuclear grade and positive axillary lymph node metastasis. The combination of DNA-PK activity and NBS1 IRIF were useful for predicting lymph node metastasis. The ability of DSB repair in PBL is related to aggressive breast cancer phenotypes. Axillary lymph node dissection can be avoided by examining DNA-PK activity and NBS1 IRIF of PBL, which can contribute to improving the quality of life of breast cancer patients.

Introduction

One of the hallmarks of malignant transformation is genomic instability, which promotes a wide range of mutations, such as chromosome deletions, gene amplifications, translocations and polyploidy. The presence of genomic instability in cells is known to play an important role in multistage carcinogenesis (1) and genes involved in the maintenance of genomic stability can be considered as cancer suppressor genes. Repair of various types of DNA damage is critical for genomic instability. Of these, DNA double-strand break (DSB) is believed to be one of the most serious damage induced by DNA damaging agents (2).

In DNA DSB repair, non-homologous end-joining (NHEJ) is one major mechanism (3). In the NHEJ pathway, DSBs are directly, or after processing of the DNA ends, rejoined at an appropriate chromosomal end and DNA-dependent protein kinase (DNA-PK) plays an important role in DNA DSB repair by NHEJ throughout the cell cycle (4). 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, phosphorylates and activates a DNA-binding protein, such as XRCC4 and DNA ligase IV, p53 and several transcription factors. Subsequently, Ligase IV repairs DNA DSB (5).

The Nijmegen breakage syndrome protein (NBS1), is a component of a protein complex containing Mre11 and Rad50. Hypomorphic mutations in Nbs1 are known to lead to Nijmegen breakage syndrome in humans, known as a radiation hypersensitive disease (6). This complex plays a role in many processes involved in maintaining genomic stability (7). Though its precise function is still unclear, the NBS1 complex is known to be involved in cell cycle checkpoint activation and repair of DSBs (8). The NBS1 complex plays a pivotal role in preventing genomic instability during DNA replication and DNA DSB repair (6).
hRAD50 complex re-localize into subnuclear structures upon the induction of DNA damage by ionizing radiation, the so-called ionizing radiation-induced foci (IRIF) (9,10). These IRIF are known to form at the site of DNA damage (11,12). IRIF can be visualized indirectly through immunostaining with antibodies against the protein of interest, or directly through expression of the protein tagged to a fluorescent protein. The higher fraction of NBS1 IRIF remaining after irradiation may be related to more DNA DSBs unrepaired, indicating a lower ability to repair DNA DSBs. Unrepaired DNA ends might contribute to the development of chromosomal translocations by acting as transposable elements (13,14).

We have previously reported that DNA-PK activity and the number of persistent NBS1 IRIF in peripheral blood lymphocytes (PBLs) is associated with chromosomal instability and the risk of sporadic breast cancer (15,16).

In this study, we measured DNA-PK activity and NBS1 IRIF remaining after irradiation of PBL obtained from patients with sporadic breast cancer. We examined the relationship between these measurements and pathological characteristics of these patients such as tumor size, axillary lymph node metastasis and nuclear grade of cancer cells in invasive breast cancer patients in order to predict clinical malignancy with these measurements.

Patients and methods

Selection eligibility. All subjects were Japanese. Ninety-six of sporadic breast cancer patients underwent breast conserving surgery and are planning to receive postoperative radiotherapy to conserved breast at Sapporo Medical University. Those who had neither a history of another cancer 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 each patient gave their written informed consent. Exclusion criteria included previous or current use of chemotherapy, radiation therapy, or immunosuppressive medications.

Pathological evaluation of the patients. All breast cancer patients who had breast conserving surgery and axillary lymph node resection and pathological diagnosis of invasive ductal cancer were confirmed. Size of the tumor, expression of hormone receptors and lymph node metastasis were also examined. Nuclear grade (such as histological grade of malignancy) in breast cancer was evaluated by combining nuclear atypia and mitotic counts according to the Japanese breast cancer classification (17). Nuclear grade was divided into 3 groups: grade 1 for low-risk, 2 for intermediate risk and 3 for high risk malignancy, respectively.

Blood collection and PBL separation. Peripheral blood (20 ml) was collected with a sterile heparinized tube from each individual before radiotherapy began. Peripheral blood lymphocytes (PBLs) were separated with lymphoprep (Nycomed Pharma AS), centrifuged at 1500 rpm (300 x g) for 30 min at 4°C, washed twice with phosphate-buffer saline.

PBL lysis, protein extraction, DNA-PK assay. Protein extraction and DNA-PK assay was done as described in our previous report (16). PBL was thawed with high salt buffer and the suspension was lysed by three rounds of freeze-thaw cycle and clarified by centrifugation at 15,000 rpm (18,000 x g) for 7 min at 4°C. Protein concentration was assayed using a BCA protein assay kit (Pierce) with bovine serum albumin as the standard. The PBL cell lysates were diluted to 0.25 mg/ml with high salt buffer. The lysate was mixed with kinase assay buffer, synthetic peptide hp53-S15 (sequence: EppSQEAFL), 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 (2.3 cm in diameter, Whatman). 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.

Immunofluorescent staining for radiation-induced NBS1 foci in PBLs. Ionizing radiation-induced NBS1 foci formation in PBLs were also measured in 46 out of 96 patients. Separated PBLs were incubated with RPMI-1640 medium (Sigma Aldrich), supplemented with 20% fetal calf serum. 4 Gy were irradiated with 120kV X-ray at 1.17 Gy/min. PBLs were applied on slide glass with centrifugation in 1, 4 and 24 h after irradiation, respectively. PBLs diluted to appropriate numbers were grown on a glass slide and fixed with cold methanol for 20 min, rinsed with cold acetone for 10 sec and then air-dried. Anti-NBS1 (Novus Biologicals) were used as the primary antibody. Alexa-488-conjugated anti-rabbit IgG (Molecular Probes) were used for visualization of foci with anti-NBS1 antibody. Slides were mounted with antifade reagent (Mounting medium, Dako).

NBS1 foci were observed with an Olympus fluorescent microscope under 10x100 oil immersion. For quantification of foci, clear and easily distinguishable dots of certain brightness were counted as positive foci. The number of NBS1 foci was counted in 200 cells of the sample at each time point by visual inspection and average number of foci per cell was calculated. A representative example and time course were indicated in Fig. 1A and B. Since NBS1 foci were gradually larger and more distinguishable, here we used average number of NBS1 foci at 24 h after irradiation for NBS1 foci quantification.

Chromosomal aberrations in PBLs. Spontaneous chromosomal aberration in PBLs were observed by Giemsa staining in 30 patients. The procedure was described previously (15). Two hundred metaphase cells from each individual were analyzed and the numbers of dicentric chromosomes and chromosome breaks were counted. Chromosome breaks not accompanying a dicentric chromosome were recorded as excess fragments.

Statistical methods. The unpaired t-test was used to compare DNA-PK activity between groups. All statistical tests were two-sided. Multiple regression analysis was used to clarify significant variables which correlate with lymph node metastasis. All statistical computing was done with StatView version 4.58 (Abacus Concepts).
Results

DNA-PK activity in PBL. The DNA-PK activity of PBL in patients with invasive breast cancer was 8.7±4.5 pmol.

Kinetics of NBS1 IRIF formation in PBL. Fig. 1A shows representative photographs of NBS1 IRIF of PBLs. There was no obvious foci detected for unirradiated control of any subject. As an internal control, PBLs from the same individual were simultaneously studied in the first 20 experiments and the number of NBS1 IRIF of internal control had a good correlation in each experiment. The formation of NBS1 IRIF was examined at 1, 4 and 24 h after 4 Gy of X-ray irradiation (Fig. 1B). The number of NBS1 IRIF increased at 1 to 4 h after irradiation, then gradually decreased until 24 h after irradiation. The number of cells with IRIF correlated well with the average number of foci per cell (data not shown). So, we only calculated the average number of foci per cell.

The association of spontaneous chromosome aberration with DNA-PK activity and NBS1 foci. Fig. 1C showed a relationship between spontaneous chromosomal aberration and DNA-PK activity and NBS1 foci. Lower DNA-PK activity and a higher retention of NBS1 foci were associated with a higher frequency of excess fragments (r=-0.548 and r=0.488 for DNA-PK activity and NBS1 foci, respectively).

Relationship between nuclear grade and DNA-PK activity in PBL. The reproducibility and reliability of DNA-PK assay has been described in our earlier publication (15). The sample of the same person was simultaneously run for DNA-PK assays as an internal control and relative DNA-PK activities were calculated for each assay.

By combining the nuclear atypia and mitotic counts, nuclear grades were defined as the sum of scores for the nuclear atypia (1 for low-degree atypia; 2 for intermediate-degree atypia; 3 for high-degree atypia) with the scores for the mitotic counts per 10 high-power fields (x40 objective lens) (1 for 0-4 mitoses; 2 for 5-9 mitoses; 3 for >10 mitoses). The nuclear grade was 1, 2 and 3 when the sum of scores for the nuclear atypia and those for mitotic counts were 2-3, 4 and 5-6, respectively (17).

Fig. 2A shows the comparison of DNA-PK activity of PBL between patients whose tumor cells had nuclear grade 1 and those whose tumor cells had nuclear grade 2+3. Average of relative DNA-PK activity was 0.67±0.28 in nuclear grade 1 and 0.55±0.21 in nuclear grade 2+3. Patients with nuclear grade 2+3 disease had marginally lower DNA-PK activity than those with nuclear grade 1 (P=0.082).

Association between DNA-PK activity and breast cancer stage. Fig. 2B shows the comparison of DNA-PK activity of PBL between patients with T1 tumor and those with T2 tumor. Average of relative DNA-PK activity was 0.67±0.28 in T1 and 0.54±0.12 in T2. Patients with T1 disease had marginally lower DNA-PK activity than those with T2 disease (P=0.093).

Association between DNA-PK activity in PBL and axillary lymph node metastasis. Fig. 2C demonstrates the comparison...
of DNA-PK activity of PBL between patients with axillary lymph node metastasis and those without axillary lymph node metastasis. Average of relative DNA-PK activity was 0.52±0.15 in patients with axillary lymph node metastasis and 0.66±0.28 in patients without axillary lymph node metastasis. Patients with axillary lymph node metastasis had marginally lower DNA-PK activity than those without nuclear axillary lymph node metastasis (P=0.072).

Association between NBS1 foci formation in PBL and axillary lymph node metastasis. Fig. 3 demonstrates the comparison of NBS1 IRIF of PBL at 24 h after irradiation between patients with axillary lymph node metastasis and those without.

Prediction of lymph node metastasis by combination of DNA-PK and NBS1 IRIF. We divided the patients into 2 groups based on the median value of DNA-PK activities and number of NBS1 foci. In the group that had lower DNA-PK activity and higher NBS1, half of the patients (7 out of 14) had lymph node metastasis, whereas no patients had lymph node metastasis in the higher DNA-PK activity and lower NBS1 IRIF group (Fig. 4). Multiple regression analysis shows that lower DNA-PK activity (P=0.009) and the lower number of radiation-induced NBS1 foci (P=0.001) are significant factors that predict lymph node metastasis and that other factors (age, ER status, T stage and nuclear grade) did not correlate with lymph node metastasis (Table I).

Discussion

We previously demonstrated that DNA-PK activity in PBLs of patients with invasive breast cancer or uterine cervix cancer...
was significantly higher than in normal healthy volunteers (15). We also showed that the number of persistent radiation-induced NBS1 foci in PBLs of patients with invasive breast cancer was significantly higher than in normal healthy volunteers (16). Age and smoking had no association with DNA-PK activity or NBS1 foci in PBLs. These results indicate that the number of persistent radiation-induced NBS1 foci and DNA-PK activity in PBL are associated with the risk of breast cancer. In this study, we examined whether there may be a possible link between clinical and pathological characteristics of breast cancer and DNA DSB repair capability measured with NBS1 IRIF and DNA-PK activity.

Results of Fig. 1A and B establish the influence of time after induction of DNA damage by irradiation on NBS1 IRIF. A time-dependent increase in IRIF is seen in all PBLs investigated. In general, the number of NBS1 IRIF peaked at 4 h and decreased to 24 h. The number of IRIF per nucleus varies among patients, indicating that patients had a different ability of DNA DSBs repair since the fraction of NBS1 IRIF remaining after irradiation may be related to DNA DSBs unrepaired (16). The DNA-PK activity of PBL in patients with invasive breast cancer also varied considerably (Fig. 2) (15).

Then, we investigated the relationship among NBS1 IRIF, DNA-PK activity and chromosomal aberrations by cytogenetic methods. We showed an association among NBS1 IRIF, DNA-PK activity and spontaneous yield of excess fragments. A higher number of NBS1 IRIF or lower DNA-PK activity correlated with higher chromosome instability (Fig. 1C). The higher fraction of NBS1 IRIF remaining after irradiation may be related to more DNA DSBs unrepaired, indicating a lower ability to repair DNA DSBs (16). Reduced DNA-PK activity can profoundly affect the ability to repair double-strand breaks, resulting in the perpetuation of chromosome damage. Unresolved signal ends might contribute to the development of translocations by acting as transposable elements (13,14). Our results indicate that the lower DNA-PK activity or the higher fraction of NBS1 IRIF remaining after irradiation is related with chromosomal instability. Repair of various types of DNA damage is critical for genomic integrity. Of these, DNA double-strand break (DSB) is believed to be one of the most serious damage induced by DNA damaging agents (2). Genes involved in DNA DSB repair plays an important role in the maintenance of genomic stability (18).

Table I. Variables that predict axillary lymph node metastasis by multiple regression analysis.

<table>
<thead>
<tr>
<th>Variable</th>
<th>CC</th>
<th>SE</th>
<th>P</th>
<th>Lower bound</th>
<th>Upper bound</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>0.002</td>
<td>0.161</td>
<td>0.978</td>
<td>-0.018</td>
<td>0.019</td>
</tr>
<tr>
<td>Estrogen receptor</td>
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<td>0.078</td>
<td>-1.418</td>
<td>0.084</td>
</tr>
<tr>
<td>T stage</td>
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<td>0.165</td>
<td>0.29</td>
<td>-0.168</td>
<td>0.529</td>
</tr>
<tr>
<td>Nuclear grade</td>
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<td>0.159</td>
<td>0.167</td>
<td>-0.565</td>
<td>0.106</td>
</tr>
<tr>
<td>DNA-PK activity lower/higher group</td>
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<td>0.161</td>
<td>0.009</td>
<td>-0.476</td>
<td>-2.947</td>
</tr>
<tr>
<td>NBS1 foci lower/higher group</td>
<td>0.575</td>
<td>0.144</td>
<td>0.001</td>
<td>0.272</td>
<td>0.879</td>
</tr>
</tbody>
</table>

CC, correlation coefficient; SE, standard error; CI, confidence interval; n=46; R²=0.691; P=0.021.

...the planning of adjuvant therapy as well as an excellent regional disease control (26). Patients with positive axillary nodal disease have a worse prognosis than those with negative nodal disease. So, patients with positive axillary nodal disease receive systemic adjuvant therapy (27). However, axillary lymph node dissection is associated with significant arm morbidity, chronic lymphoedema being the most...
notorious form (26). We demonstrated that the combination of DNA-PK activity and NBS1 foci were useful for the prediction of axillary lymph node metastasis (Fig. 4). In particular, no patients had lymph node metastasis when they had a higher DNA-PK activity and lower NBS1 IRIF. These results indicate that axillary lymph node dissection can be avoided by examining DNA-PK activity and NBS1 IRIF of PBL, which can contribute to improving of quality of life of patients with breast cancer.

In summary, a higher number of NBS1 IRIF or lower DNA-PK activity correlated with higher chromosome instability. Patients whose PBL had lower DNA-PK or higher NBS1 IRIF had aggressive cancer phenotypes such as the higher nuclear grade, a larger tumor and positive axillary lymph node metastasis. This result means that DNA-PK activity correlated with higher chromosome instability (26). Patients whose PBL had lower DNA-PK or higher NBS1 foci can detect lymph node metastasis and may be an independent prognostic factor for early stage invasive breast cancer. Axillary lymph node dissection can be avoided by examining DNA-PK activity and NBS1 IRIF of PBL, which can contribute to improving the quality of life of breast cancer patients.

References


