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Title(English)	Biological Consequences of Ionizing Radiation Exposure and the Role of XRCC4 Protein Phosphorylation in DNA Double-strand Break Repair through Non-homologous End Joining
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論 文 要 旨

THESIS SUMMARY

専攻： Department of	Nuclear Engineering 専攻	申請学位（専攻分野）： Academic Degree Requested	博士 Doctor of Engineering
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要旨（英文 800 語程度）
Thesis Summary（approx.800 English Words ）

This thesis is entitled "Biological Consequence of Ionizing Radiation Exposure and the Role of XRCC4 Protein Phosphorylation in DNA Double-strand Break Repair through Non-homologous End Joining" and consists of 6 chapters as follows.

Chapter 1 is titled "Introduction". Here, the research background and the purpose of this study are described. DNA double-strand break (DSB), which is considered the most harmful type of DNA damage among those induced by radiation, is repaired mainly through two pathways: homologous recombination (HR) and non-homologous end-joining (NHEJ). The core machinery unit of NHEJ consists of Ku70/86 heterodimer, DNA-dependent protein kinase catalytic subunit (DNA-PKcs), and the DNA Ligase IV/XRCC4/XLF complex. There are several lines of evidence indicating that the protein phosphorylating activity of DNA-PKcs is essential for NHEJ but the targets and significance of phosphorylation by DNA-PKcs remain to be clarified. DNA-PK has been shown to phosphorylate XRCC4 *in vitro* and our laboratory also demonstrated that XRCC4 undergoes phosphorylation in living cells in response to treatment with ionizing radiation or a DSB-inducing agent in a manner dependent on DNA-PKcs. Two papers reported the identification of Ser260 as well as Ser320 (or Ser318 in the alternatively spliced form) as the major phosphorylation sites in XRCC4 by purified DNA-PK *in vitro* through mass spectrometry. However, it has not been clear whether these sites are phosphorylated in living cells. Therefore, this study aimed to clarify whether these sites, especially Ser260, are phosphorylated by DNA-PK in living cells in response to radiation or other DNA damaging agents and what is the role of phosphorylation in DSB repair.

Chapter 2 is titled "Experimental Procedures". Here, the materials and methods used in this study are described: cell culture, γ-ray irradiation, sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting.

Chapter 3 is titled "Generation of phosphorylation-specific antibody for XRCC4 Ser260". A rabbit polyclonal antibody against XRCC4 phosphorylated on Ser260 was generated. The antiserum was obtained by immunizing a rabbit with a synthetic peptide, corresponding to XRCC4 254-266 with Ser260 phosphorylated. The phosphorylation-specific antibody was collected from the antiserum by binding to a column conjugated with the above phosphorylated peptide and, subsequently, passing through another column conjugated with unphosphorylated peptide.

In enzyme-linked immunosorbent assay (ELISA), the antibody reacted with Ser260-phosphorylated peptide but not with unphosphorylated peptide or with Ser320-phosphorylated peptide. This antibody reacted with XRCC4 which had been phosphorylated by purified DNA-PK *in vitro* but not with unphosphorylated control. Moreover,

when Ser260 was changed into alanine, this antibody no longer reacted with XRCC4 even after phosphorylation reaction with DNA-PK. These results showed that this antibody specifically recognizes XRCC4 phosphorylated on Ser260.

Chapter 4 is titled "Evaluation of *in cellulo* phosphorylation status of XRCC4 Ser260 and its role in radiation sensitivity". First, the phosphorylation status of XRCC4 Ser260 in cultured cell was evaluated using the antibody generated above. In human cervical carcinoma HeLa cells, XRCC4 Ser260 phosphorylation was mostly unchanged with or without irradiation and in the presence or absence of inhibitors for DNA-PK and a related enzyme ATM. Nevertheless, the phosphorylation of XRCC4 Ser260 increased after irradiation with higher dose, *i.e.*, 30-300 Gy, and this increase was suppressed by DNA-PK inhibitor but not by ATM inhibitor. To confirm the identity of the band, immunoprecipitation using anti-XRCC4 antibody was performed. The band of XRCC4 Ser260 phosphorylation was detected in anti-XRCC4 immunoprecipitant of 30 or 300 Gy γ-irradiated HeLa cells. XRCC4 Furthermore, although Ser260 phosphorylation increased after 30 or 300 Gy γ-irradiation in wild-type TK6, it was mostly diminished in DNA-PKcs$^{-/-}$ cell. These results collectively indicated that XRCC4 Ser260 undergoes phosphorylation by DNA-PK in response to irradiation.

To examine the importance of this phosphorylation site, it was changed into alanine and aspartate. XRCC4^{S260A} and XRCC4^{S260D} mutants could collect the high radiosensitivity of XRCC4-deficient M10 cells to a similar extent to wild-type XRCC4. However, the clonogenic survival of cells expressing XRCC4^{S260A} and XRCC4^{S260D} was slightly but significantly lower than that of cells expressing wild-type XRCC4. In addition, XRCC4^{S260A}-exressing cells displayed significantly greater number of γ-H2AX foci than XRCC4^{WT}-exressing cells 4 h after 1 Gy irradiation and also without irradiation. These results collectively indicated the importance of XRCC4 Ser260 in the DSB repair.

Chapter 5 is titled "Analyses of phosphorylation of XRCC4 Ser320". Whereas a rabbit polyclonal antibody against XRCC4 phosphorylated on Ser320 was recently reported from our laboratory, monoclonal antibodies were newly generated here. Using these antibodies, the phosphorylation status of XRCC4 Ser320 was examined. The results showed that XRCC4 Ser320 undergoes phosphorylation by DNA-PK in response to DNA damage, indicating that XRCC4 Ser320 would serve as an indicator for DNA-PK functionality *in situ*.

Chapter 6 is titled "Conclusion". In this study, a new antibody, which reacts specifically with Ser260-phosphorylated XRCC4, was generated. XRCC4 Ser260 is phosphorylated by DNA-PK in response to irradiation. XRCC4 mutants, in which Ser260 is changed into other amino acids, are incomplete in DSB repair function as shown by cellular radiosensitivity and γ-H2AX foci. These results in the aggregate indicated that the phosphorylation of XRCC4 Ser260 by DNA-PK plays a potential role in DSB repair.