

Methylation of Breast Cancer Susceptibility Gene 1 (*BRCA1*) Predicts Recurrence in Patients With Curatively Resected Stage I Non–Small Cell Lung Cancer

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BACKGROUND: Even after early detection and curative resection of early stage non-small cell lung cancer (NSCLC), a significant fraction of patients develop recurrent disease. Molecular biomarkers that can predict the risk of recurrence thus need to be identified to improve clinical outcomes. **METHODS:** Using the methylation-specific polymerase chain reaction assay, promoter methylation of the breast cancer susceptibility gene 1 (*BRCA1*) was assessed in cancer tissues from 70 patients with curatively resected stage I NSCLC. The clinical relevance of *BRCA1* methylation status was evaluated in terms of outcome of the disease. **RESULTS:** Methylation of the *BRCA1* promoter was detected in 13 of 70 patients (18.6%). Multiple logistic regression analysis revealed that *BRCA1* methylation was an independent risk factor for recurrence ($P = .0197$) and that patients with *BRCA1* methylation demonstrated significantly poorer recurrence-free survival compared to those without ($P = .0139$). Cox's proportional hazard regression analysis revealed that *BRCA1* methylation was an independent risk factor for recurrence-free survival ($P = .0155$). **CONCLUSIONS:** Methylated *BRCA1* can be a potential biomarker that predicts the prognosis after curative resection of stage I NSCLC. Considering that *BRCA1* plays a role in chemotherapy-induced apoptosis, it is plausible that identification of methylated *BRCA1* could provide information that is clinically relevant to tailored adjuvant therapy. *Cancer* 2013;119:792–8. © 2013 American Cancer Society.

KEYWORDS: *BRCA1*, methylation, epigenetics, prognosis, non-small cell lung cancer.

INTRODUCTION

Lung cancer has become the leading cause of cancer deaths in many countries. The treatment strategy chosen for patients with lung cancer is generally guided by tumor–node–metastasis (TNM) classification. Surgery with curative intent is the standard treatment of choice for patients with stage I non–small cell lung cancer (NSCLC). However, even after complete resection of stage I NSCLC, there is a wide spectrum of outcomes.^{1,2} The possibility that molecular biomarkers might better predict biological characteristics and outcomes than TNM classification should be investigated.³

A growing body of evidence indicates that aberrant methylation of cytosine-guanine dinucleotide (CpG) islands in the promoter regions of tumor suppressor genes silences these genes by blocking transcription.^{4–7} Promoter methylation in various tumor suppressor genes has been demonstrated to be involved in the development and/or progression of lung cancer^{3,8–10} and has thus been used as a molecular biomarker to accurately predict the outcome of disease.¹¹

Germline mutations in the breast cancer susceptibility gene 1 (*BRCA1*) elevate the risk of breast and ovarian cancer development;¹² hypermethylation of the *BRCA1* promoter has been demonstrated in sporadic breast and ovarian cancers.^{13,14} It is now known that *BRCA1* is a tumor suppressor gene that encodes a multifunctional protein that is involved in both breast and ovarian cancers.¹⁵ In addition, germline mutations in *BRCA1* are a statistically significant risk factor in the prognosis of pancreatic and cervical cancers.¹⁶ Several studies have investigated the clinical relevance of messenger RNA expression¹⁷ and methylation of the *BRCA1* promoter^{18,19} in lung cancer, with emphasis primarily on responses to chemotherapy.²⁰ The relationship between epigenetic modifications of *BRCA1* and the prognosis of curatively resected stage I NSCLC has not been elucidated.

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In this study, we examined the methylation status of the *BRCA1* promoter in 70 patients with curatively resected stage I NSCLC. Using methylation-specific polymerase chain reaction (PCR), we investigated the possible association of *BRCA1* methylation with the outcome of this disease.

MATERIALS AND METHODS

Study Population

A total of 70 patients who underwent complete resection for stage I NSCLC at National Hospital Organization Kure Medical Center and Chugoku Cancer Center in Japan between June 2005 and March 2009 were enrolled in this study. The end of the follow-up period was defined as December 2011. No patients received any preoperative chemotherapy or radiation therapy. Patients who underwent surgery for Noguchi Type A or B tumors were excluded from this study. The demographic data and clinicopathological features of enrolled patients were collected using an institutional database. Written, informed consent was obtained from all enrolled patients, and the protocol for this study was approved by the institutional review board.

Sample Preparation

Surgically resected lung tissue samples were immediately snap-frozen and subsequently stored in liquid nitrogen. Genomic DNA was extracted from both primary tumors and adjacent normal lung tissue, through use of proteinase K digestion and phenol-chloroform extraction followed by ethanol precipitation, as described.²¹

Bisulfite Modification and Methylation-Specific PCR

For sodium bisulfate modification, DNA was digested using *Bam*HI (New England Biolabs, Ipswich, Mass), and 1 μ g of the digested DNA was denatured in 0.3 N NaOH at 37°C for 15 minutes. The samples underwent 15 cycles of 30-second denaturation at 95°C and 15-minute incubation at 50°C in 3.1 N sodium bisulfite (pH 5.0) and 0.5 mM hydroquinone. The product was desalted with the Wizard DNA cleanup system (Promega), and desulfonated in 0.6 N NaOH. The sample was ethanol-precipitated and dissolved in 20 mL of TE (Tris plus ethylenediamine tetraacetic acid) buffer. Methylation-specific PCR was carried out using 1 μ L of the sodium bisulfite–modified DNA; primers for amplification were specific for methylated or unmethylated sequences, as described.^{22–24} The primer sequence and PCR conditions for *BRCA1* were as follows: unmethylated forward primer (5'-TGGTAGTTTTTGGTTTTTGTGGTAATG), unmethylated reverse primer

(5'-TCAACAAACTCACACCACACAATCA), methylated forward primer (5'-CGGTAGTTTTTTGGTTTTCGTGGTAACG), methylated reverse primer (5'-TCAACGAACTCACGCCGCGCAATCG), for 37 cycles and annealing at 66°C. All procedures were repeated at least 3 times for each sample.

Immunohistochemical Analysis

Paraffin blocks of resected tumors were cut into 5- μ m slices, then processed using standard deparaffinization and rehydration techniques. A monoclonal antibody against *BRCA1* (1:500; Calbiochem, Billerica, Mass) was used as the primary antibody for detecting protein expression. Immunodetection was performed by incubation with a specific biotinylated secondary antibody followed by use of the Vectastain ABC kit (Vector Laboratories, Burlingame, Calif). 3,3'-Diaminobenzidine (BD Biosciences, Franklin Lakes, NJ) was used as the developing reagent followed by a hematoxylin counterstain.

Statistical Analysis

Associations between clinicopathological characteristics and *BRCA1* methylation were determined using the Fisher's test. Logistic regression analysis was performed to estimate the odds ratios of independent factors for recurrence. The effect of promoter methylation on time to death or recurrence was estimated using the Kaplan-Meier method, and the log-rank test was used to analyze differences between groups. Overall survival was calculated from the date of surgery to the date of death or the last follow-up. Recurrence-free survival was calculated from the date of surgery to the date of recurrence or the last follow-up. Cox's proportional hazard regression analysis was used to analyze the hazard ratios of independent factors for survival. Differences were considered statistically significant with a *P* value < .05.

RESULTS

DNA Methylation Profiles of Tumor Tissues in the Evaluation Set

To identify a prognostic marker associated with recurrence, DNA methylation in 14 genes (*p16*, Cyclin-dependent kinase inhibitor 2A; *APC*, Adenomatous polyposis coli; *CDH1*, Cadherin 1; *RARB*, Retinoic acid receptor beta; *BRCA1*, Breast cancer susceptibility; *TIMP3*, Tissue inhibitor of metalloproteinase-3; *RASSF1A*, Ras association domain family member 1; *p27*, Cyclin-dependent kinase inhibitor 1B; *FHIT*, Fragile histidine triad; *MGMT*, O6-methylguanine-DNA methyltransferase; *TERC*, Telomerase RNA component; *MLH1*, Human mutL homolog 1; *GSTP1*, Glutathione

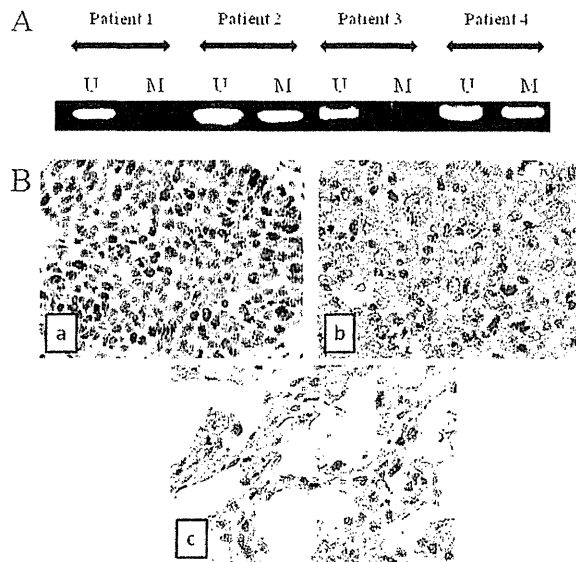


Figure 1. (A) Analysis of *BRCA1* gene methylation is shown in stage I non-small cell lung cancer (NSCLC). Methylation-specific polymerase chain reaction of *BRCA1* was performed using primers specific for unmethylated (U) and methylated (M) forms of *BRCA1* in tumor tissue. *BRCA1* methylation was detected in tumor tissues of patients 2 and 4 (arrow). (B) Immunohistochemical analysis of *BRCA1* proteins is shown in resected NSCLC specimens. Positive immunoreactivity is visible as a brown precipitant in cell nuclei. Representative expression of *BRCA1* protein in the patient without *BRCA1* methylation is shown (panel a), and a concordant lack of immunoreactivity is found in the patient with *BRCA1* methylation (panel b). Normal adjacent lung tissue with expression of *BRCA1* protein is shown as the positive control (panel c). Original magnification, $\times 400$.

S-transferase P 1; and *MASPIN*, Serpin peptidase inhibitor) was analyzed in selected patients, who were matched with respect to sex, age, histology, stage, and differentiation as the evaluation set. In this evaluation set, patients with recurrence showed a significantly higher frequency of *BRCA1* methylation compared with patients without recurrence, and the methylation status in any of the remaining genes did not show statistically significant association with recurrence in this evaluation set (data not shown).

***BRCA1* Methylation in Resected Stage I NSCLC**

Figure 1A shows representative results of methylation-specific PCR for *BRCA1*. The overall frequency of methylation of the *BRCA1* promoter was 18.6% (13 of 70 patients, Table 1). The proportion of patients with methylated *BRCA1* was quite similar to that observed in previous studies.²⁵ Five of 13 patients with *BRCA1* methylation in tumor tissues had *BRCA1* methylation in their matched noncancerous tissues as well (Table 1). Three of these 5 patients developed recurrent tumors

TABLE 1. Comparison of *BRCA1* Methylation Status Between Cancerous and Matched Noncancerous Adjacent Tissue

Status		Cancerous Tissue	Matched Noncancerous Tissue
		(n = 70)	(n = 70)
<i>BRCA1</i>	Methylated	13 (18.6%)	5 ^a
	Unmethylated	57 (81.4%)	65

^aThese 5 patients showed *BRCA1* methylation in both cancerous and matched noncancerous tissues.

(multiple lung metastases were diagnosed at 2 years after surgery in 1 patient; bone metastasis was diagnosed at 7 months after surgery in 1 patient; and brain metastasis was diagnosed at 7 months after surgery in 1 patient).

Images showing representative immunohistochemical staining of *BRCA1* protein are shown in Figure 1B. Staining within nuclei of tumor cells was considered positive. Immunohistochemical analysis confirmed that all tumors with *BRCA1* promoter methylation showed an absence of or marked reduction in *BRCA1* expression; tumor cells without methylation contained considerably more *BRCA1* protein in their nuclei. As described in previous studies, *BRCA1* expression levels were associated with promoter hypermethylation status.^{19,26}

Correlation Between Disease Outcome and *BRCA1* Methylation Status

Demographics and clinical characteristics according to *BRCA1* methylation status are shown in Table 2. There was no statistically significant relationship between *BRCA1* methylation status and any of the clinicopathological features that we analyzed.

Table 3 shows the odds ratio for the risk of recurrence in clinicopathological variables by univariate logistic regression analysis. The degree of differentiation, vessel infiltration, and *BRCA1* methylation status were statistically significant as predictors for recurrence ($P = .0347$, $P = .0300$, and $P = .0369$, respectively). Multiple logistic regression analysis of clinicopathological variables that were significant by univariate analysis revealed that *BRCA1* methylation status was an independent risk factor for recurrence ($P = .0197$, Table 4).

Prognostic Impact of *BRCA1* Methylation

The median follow-up time of patients in this study population was 1350 ± 458 days. Patients without event survived for at least 900 days after surgery by the end of the follow-up period. Three-year survival rates were 89.5% for overall survival and 83.9% for recurrence-free survival.

Recurrence-free survival rates in patients with *BRCA1* methylation were significantly poorer than in those without *BRCA1* methylation ($P = .0139$, Fig. 2). Overall survival did not reach statistical significance between groups assigned by methylation status ($P = .2588$, data not

shown). Cox's proportional hazard regression analysis revealed that *BRCA1* methylation was an independent risk factor for recurrence-free survival ($P = .0155$, Table 5). These data indicate that *BRCA1* methylation could be used as a biomarker for predicting the disease outcome after curative resection of stage I NSCLC. Of the 13 patients with *BRCA1* methylation, 5 developed recurrent tumors. Two of these 5 patients received chemotherapy (paclitaxel and carboplatin in 1 patient; gefitinib in 1 patient) after diagnosis of the recurrent tumors. Two patients were followed without any intensive therapy, and 1 patient received radiation therapy for brain metastasis. Two patients (paclitaxel and carboplatin, 1; best supportive care, 1) survived more than 3 years after the diagnosis of recurrence. In contrast, 7 patients without *BRCA1* methylation developed recurrent tumors. One of these 7 patients survived more than 3 years after surgical resection of pulmonary metastases that had been diagnosed 1 year

TABLE 2. Analysis of Baseline Demographics and Clinical Characteristics of Study Population by *BRCA1* Methylation Status

Characteristic	<i>BRCA1</i>		<i>P</i>
	Methylated (n = 13)	Unmethylated (n = 57)	
Age, y	65.2 ± 2.6	68.7 ± 1.2	.2193
Sex			
Female	5	12	.2801
Male	8	45	
Histology			
AD	10	39	.8204
SQ	2	13	
Others	1	5	
Differentiation			
Well	6	24	.7901
Moderately, poorly	7	33	
Lymphatic duct infiltration			
+	6	17	.3297
-	7	40	
Vessel infiltration			
+	7	20	.2258
-	6	37	
Pathological stage			
IA	8	36	.9132
IB	5	21	
Smoking			
Current, former	9	46	.5141
Never	4	11	
Chronic lung disease			
+	8	24	.2323
-	5	33	
Any prior tumor			
+	0	13	.1074
-	13	44	

Abbreviations: AD, adenocarcinoma; SQ, squamous cell carcinoma.

TABLE 4. Multiple Logistic Regression Analysis of Clinicopathological Variables Found to Be Significant by Univariate Analysis

Variables	OR	95% CI	<i>P</i>
<i>BRCA1</i> methylation status (+/-)	6.3450	1.3503–33.4334	.0197
Differentiation (Well/moderately, poorly)	0.1729	0.0226–0.8019	.0232
Vessel infiltration (+/-)	1.5430	0.3648–6.7321	.5500

Abbreviations: CI, confidence interval; OR, odds ratio.

TABLE 3. Univariate Logistic Regression Analysis of Clinicopathological Variables for Recurrence

Factors		Recurrence		OR	95% CI	<i>P</i>
		+: Event	-: Unevent			
		n = 12	n = 58			
Age > 70 y	Yes/no	7/5	23/35	2.1304	0.6079–7.9791	.2346
Sex	Female/male	2/10	15/43	0.5733	0.0820–2.5028	.4844
Histology	AD/SQ, others	6/6	43/16	0.3488	0.0948–1.2697	.1083
Differentiation	Well/moderately, poorly	2/10	28/30	0.2143	0.0311–0.9030	.0347
Lymphatic duct infiltration	+/-	5/7	18/40	1.5873	0.4207–5.6656	.4820
Vessel infiltration	+/-	8/4	19/39	4.1053	1.1448–17.0146	.0300
Pathologic stage	IA/IB	6/6	38/20	1.8999	0.5310–6.8335	.3177
Smoking	Current, former/never	11/1	44/14	3.4999	0.5970–66.7958	.1861
Chronic lung disease	+/-	8/4	24/34	2.8333	0.7974–11.6248	.1084
Any prior tumor	+/-	3/9	10/48	1.6000	0.3142–6.5550	.5417
<i>BRCA1</i> methylation	+/-	5/7	8/50	4.4643	1.0996–17.8341	.0369

Abbreviations: AD, adenocarcinoma; CI, confidence interval; OR, odds ratio; SQ, squamous cell carcinoma.

after the resection of his primary lung cancer. Four of these 7 patients survived less than 4 months after diagnosis of their recurrent tumors and died of cancer; 2 patients survived less than 6 months, with recurrent tumors by the end of the follow-up period.

Correlation Analysis of Combination Genes With BRCA1

Because the methylation status of *p16*, *APC*, *CDH1*, *RASSF1A*, and *FHIT* were reported to predict the risk of recurrence in curatively resected stage I NSCLC,^{3,8} the association between methylation of these genes and recurrence was assessed in all 70 study patients. Promoter methylation was detected in 20.0% for *p16*, 34.3% for *APC*, 52.9% for *CDH1*, 30.0% for *RASSF1A*, and 27.1% for *FHIT*. Among these genes, *p16* methylation, which was demonstrated to have association with recurrence by Brock et al,³ was not significantly but marginally associated with recurrence in our patients (odds ratio, 3.89; 95% confidence interval (CI), 0.972-15.170; *P* = .0546).

Methylation of the other 4 genes did not show statistically significant association with recurrence. After combination analyses, methylation of either *p16* or *BRCA1* was associated with an increased risk of recurrence (odds ratio, 13.13; 95% CI, 3.052-91.744; *P* = .0003), and significantly poorer recurrence-free survival (*P* = .0004). Methylation of either *FHIT* or *BRCA1* was associated with an increased risk of recurrence (odds ratio, 6.16; 95% CI, 1.628-30.228; *P* = .0067), and significantly poorer recurrence-free survival (*P* = .0070).

DISCUSSION

Hypermethylation of CpG islands in the promoter region of various genes has been reported in lung cancer. Gene silencing through epigenetic alterations has been widely implicated in a variety of pathologic processes, including cancer induction and progression.^{3,8-10} Although many investigators have studied various clinicopathological features that might predict the outcome of curatively resected lung cancer, the clinical relevance of aberrant promoter methylation to the outcome of disease has been investigated by only a few groups. These investigations have identified aberrant methylation of specific genes or cohypermethylation of several genes as risk factors for recurrence. Recent reports have identified *p16*, H-cadherin 13 (*CDH13*), *RASSF1A*, *APC*, and *FHIT* as risk factors for recurrence in patients with stage I NSCLC treated by curative surgery.^{3,8} Results of multivariate analysis in this study indicate that *BRCA1* methylation had significant effects on the outcome of the disease, even in curatively resected stage I NSCLC. Moreover, correlation analysis demonstrated that the combination of *BRCA1* with *p16* or *FHIT* seemed to be the better predictor of the outcome of disease; however, because the cohort of this study was small, future prospective studies using larger sample sizes are needed to investigate how methylation of *BRCA1* contributes to the outcome of curatively resected stage I NSCLC.

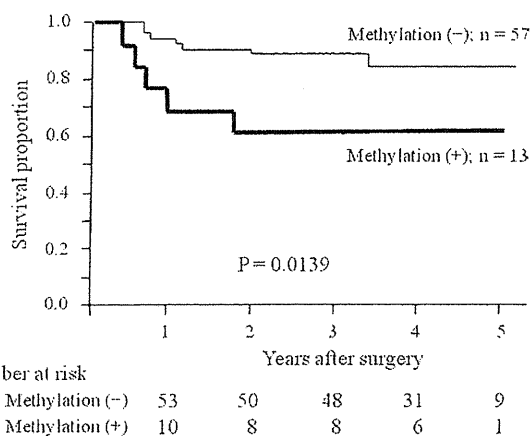


Figure 2. Patient survival is classified by *BRCA1* methylation status. Recurrence-free survival differed significantly between patients with *BRCA1* methylation and those without (*P* = .0139). *P* value was calculated using the log-rank test.

TABLE 5. Cox's Proportional Hazard Regression Analysis of Prognostic Variables Found to Be Significant by Univariate Analysis

Variables		Univariate Analysis			Multivariate Analysis		
		HR	95% CI	<i>P</i>	HR	95% CI	<i>P</i>
<i>BRCA1</i> methylation status	(+/-)	3.6934	1.0923-11.5869	.0366	4.7256	1.3780-15.0518	.0155
Differentiation	(Well/moderately, poorly)	0.2320	0.0356-0.8807	.0302	0.1864	0.0283-0.7219	.0129

Abbreviations: CI, confidence interval; HR, hazard ratio.

BRCA1 was the first breast cancer susceptibility gene to be identified (in 1990).²⁷ The majority of this gene was cloned in 1994 by Miki and colleagues.²⁸ Previous studies indicate that higher *BRCA1* methylation levels in breast cancer correlate with more advanced tumor stages at diagnosis and are associated with a 45% increase in mortality compared with patients who have unmethylated *BRCA1* promoters.²⁹ In our patients, the frequency of *BRCA1* methylation in resected stage II or III NSCLC was 14.6% (data not shown), compared with 18.6% in stage I patients. These data indicate there is no apparent relationship between *BRCA1* methylation level and tumor stage in lung cancer. We demonstrated that methylation of the *BRCA1* promoter gene correlates with lower recurrence-free survival in patients with curatively resected pathological stage I NSCLC; however, *BRCA1* promoter methylation status was not associated with any clinicopathological features, including pathological stage. Interestingly, 5 patients showed *BRCA1* methylation in tumor tissue and adjacent normal lung tissue as well. Three of these 5 patients developed recurrent tumor; however, the characteristics of recurrence status seem to be nonspecific. Although it is not clear whether these 5 patients possessed germline methylation of *BRCA1*, positive unmethylation status was demonstrated in noncancerous tissue, and the degree of methylation in noncancerous tissue was not strong compared with cancerous tissue. Furthermore, epigenetic alteration initiated by exposure to carcinogens in tobacco smoke is one of the main causes of lung cancer, and tobacco smoking plays a significant role in the prognosis of patients with lung cancer.³⁰ Despite these facts, no association between *BRCA1* promoter methylation and smoking status was observed in this study.

Several molecular markers have been identified for use as both prognostic tools and targets for novel therapeutic approaches. Such molecular markers also help to identify patients who would benefit from specific anticancer therapies. A growing body of evidence indicates that *BRCA1* plays a central role in DNA repair and in cell cycle control.^{31,32} A lack of functional *BRCA1* leads to increased sensitivity of tumor cells to molecular damage, suggesting that *BRCA1* could be used as a predictive molecular marker to identify patients who would benefit from specific anticancer therapies. According to the results of several investigations, *BRCA1* confers sensitivity to apoptosis induced by antimicrotubule drugs (eg, paclitaxel and vincristine), but induces resistance to DNA-damaging agents (eg, cisplatin and etoposide) and radiotherapy.^{33–36} Because adjuvant chemotherapy is currently a matter of great debate, particularly regarding its use in

curatively resected NSCLC, *BRCA1* methylation could be a promising molecular marker for predicting not only the disease outcome but also the effectiveness of chemotherapy. In this study, the small sample size limited the assessment of differences in the effects of chemotherapy between patients with methylated *BRCA1* and those without. Further investigations using a larger sample size are needed to determine whether assessment of *BRCA1* methylation status provides clinical information relevant to tailored adjuvant therapy.

In summary, we observed methylation of the *BRCA1* promoter in 13 of 70 (18.6%) cases of curatively resected stage I NSCLC and determined that such methylation was an independent risk factor for tumor recurrence. *BRCA1* methylation status was not associated with any specific clinicopathological features, including pathological stage. These results indicate that *BRCA1* methylation plays an important role in the progression of NSCLC and that *BRCA1* methylation is a promising biomarker that predicts the outcome of disease after curative resection of stage I NSCLC. *BRCA1* has been recognized as a promising genetic determinant of responses to different types of chemotherapy; therefore, further studies using larger samples are warranted to investigate the usefulness of determining a patient's *BRCA1* methylation status. Such studies should be designed with a special emphasis on establishing customized adjuvant treatment strategies for patients with curatively resected stage I NSCLC.

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CONFLICT OF INTEREST DISCLOSURE

The authors made no disclosure.

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Biology Contribution

Influence of Age on the Relative Biological Effectiveness of Carbon Ion Radiation for Induction of Rat Mammary Carcinoma

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Summary

The risk of secondary cancer in children after particle radiation therapy is unclear. Using rats, we show that the period spanning neonatal development and early adulthood, but not fetal development or full adulthood, is associated with susceptibility to mammary carcinogenesis after γ -ray exposure. The effectiveness of a 290-MeV/u carbonion

Purpose: The risk of developing secondary cancer after radiotherapy, especially after treatment of childhood cancers, remains a matter of concern. The high biological effects of carbon-ion radiation have enabled powerful radiotherapy, yet the approach is commonly restricted to the treatment of adults. Susceptibility of the fetus to particle radiation-induced cancer is also unclear. The present study is aimed to investigate the effect of carbon-ion irradiation in childhood on breast carcinogenesis.

Methods and Materials: We irradiated female Sprague-Dawley rats of various ages (embryonic days 3, 13, and 17 and 1, 3, 7, and 15 weeks after birth) with ¹³⁷Cs γ rays or a 290-MeV/u monoenergetic carbonion beam (linear energy transfer, 13 keV/ μ m). All animals were screened weekly for mammary carcinoma by palpation until they were 90 weeks old.

Results: Irradiation of fetal and mature (15-week-old) rats with either radiation source at a dose of 0.2 or 1 Gy did not substantially increase the hazard ratio compared with the nonirradiated group. Dose responses (0.2-2.0 Gy) to γ rays were similar among the groups of rats irradiated 1, 3, and 7 weeks after birth. The effect of carbon ions increased along with the age at the time of irradiation, indicating relative biological effectiveness values of 0.2 (−0.3, 0.7), 1.3 (1.0, 1.6), and 2.8 (1.8, 3.9) (mean and 95% confidence interval) for animals that were 1, 3, and 7 weeks of age, respectively.

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beam (13 keV/μm) in inducing mammary cancer is also age dependent, with the greatest effectiveness seen in early adulthood and the least effectiveness seen for neonates.

Conclusions: Our findings imply that carbonion therapy may be associated with a risk of secondary breast cancer in humans, the extent of which may depend on the age of the patient at the time of irradiation. © 2013 Elsevier Inc.

Introduction

Particle radiation therapy continues to improve cancer treatment by permitting accurate dose localization and strong biological effects attributed to the sharp Bragg peak and high linear energy transfer (LET) (1). The National Institute of Radiological Sciences (NIRS) in Japan, where clinical studies using carbonion radiation from the Heavy-Ion Medical Accelerator in Chiba (HIMAC) have been conducted since 1994, is one of several facilities of its kind in the world (1). Because Bragg peaks are normally too narrow for therapeutic applications, spread-out Bragg peaks (SOBP) have been devised to obtain a broad and uniform dose distribution (2). Fractionated irradiation with a beam with 6-cm SOBP, which is developed from the 290-MeV/u carbon ions and has a LET range of 40 to 90 keV/μm within the SOBP component, has been used to treat several cancer types.

There is increasing concern that medical radiation exposure can increase the risk of cancer, especially in children. Breast tissue is susceptible to radiation-induced carcinogenesis, and epidemiologic studies have suggested a high risk of breast cancer after radiation exposure during childhood (3, 4). Carbonion radiation therapy is currently not applied for the treatment of childhood cancers, partly because of the potential risk of promoting secondary cancer. Previous studies have indicated the high relative biological effectiveness (RBE) of heavy ions, such as carbon ions, for the induction of cancer in mammary glands and other sites (5-8). We previously reported a high RBE for the induction of mammary carcinogenesis in rats irradiated with a 6-cm SOBP carbonion beam at 7 weeks after birth (ie, early adulthood) (5). Because the mammary gland is one of the organs irradiated during radiation therapy for tumors in the chest area, it is likely that healthy mammary tissue will be exposed to the relatively low-LET (13 keV/μm) component of the carbonion beam. Nevertheless, there is no published information from studies conducted in humans or using animal models about the risk of carcinogenesis after carbon-ion irradiation, whether fractionated or single, during childhood.

Apart from clinical issues, evidence suggests high susceptibility of the fetus to radiogenic cancer (9). Indeed, neutrons are reported to impose high cancer risk to fetal mice (10), indicating

a need for basic knowledge on the consequence of fetal exposure to heavy ions. In the present study we used a common rat model of mammary carcinogenesis to investigate the effects of irradiation with 13 keV/μm carbon ions on rats of various ages in comparison with rats irradiated with γ rays.

Methods and Materials

Heavy-ion and γ-ray irradiation

Carbon-ion irradiation was conducted at the HIMAC in NIRS; the 290-MeV/u monoenergetic carbonion beam (LET, 13 keV/μm in the plateau region; diameter, 11 cm) was used. Each rat was placed in a cylinder-shaped, 1-cm-thick polymethylmethacrylate chamber 10 cm deep and with a diameter of 10 cm, which was set in the plateau region of the beam. The absorbed dose of carbon ions was calculated as described previously (2). The duration of irradiation ranged from 1 to 2 minutes, resulting in an average dose rate of 0.1 to 3.0 Gy/min. The γ-irradiation was performed as previously described (5) using a ¹³⁷Cs γ-irradiator at a dose rate of 0.6 Gy/min.

Animal experiments

All animal experiments were approved by the Institutional Animal Care and Use Committee of NIRS. Female Sprague-Dawley rats (Jcl:SD; Clea Japan, Tokyo, Japan) were housed in autoclaved cages and maintained in rooms with controlled temperature (23°C ± 1°C) and humidity (50% ± 5%) under a regular 12-hour light/12-hour dark cycle with a standard laboratory diet (CE-2; Clea Japan) and water provided ad libitum. Pregnant rats (postcoitus days 3, 13, and 17) and postnatal rats (weeks 1, 3, 7, and 15 after birth) were either whole-body irradiated with γ rays or carbon ions at 0.2, 0.5, 1.0, or 2.0 Gy or left unirradiated under pentobarbital-mediated anesthesia (30-35 mg/kg, for reduction of stress during transportation between the breeding and irradiation facilities), as outlined in the experimental plan shown in Table 1. The number of animals per group was 21 to 43, as indicated in

Table 1 Experimental groups

Symbol	Age at irradiation	Stage of development	Dose irradiated (Gy)
0 Gy	Not irradiated	Unirradiated control	0
E3	Embryonic day 3	Fetal, pre-implantation	0.2
E13	Embryonic day 13	Fetal, major organogenesis	0.2, 1.0
E17	Embryonic day 17	Fetal, late stage	0.2, 1.0
1W	1 wk after birth	Neonatal	0.2, 0.5, 1.0, 2.0
3W	3 wk after birth	Prepubertal	0.2, 0.5, 1.0, 2.0
7W	7 wk after birth	Early adulthood	0.2, 0.5, 1.0, 2.0
15W	15 wk after birth	Full adulthood	0.2, 1.0

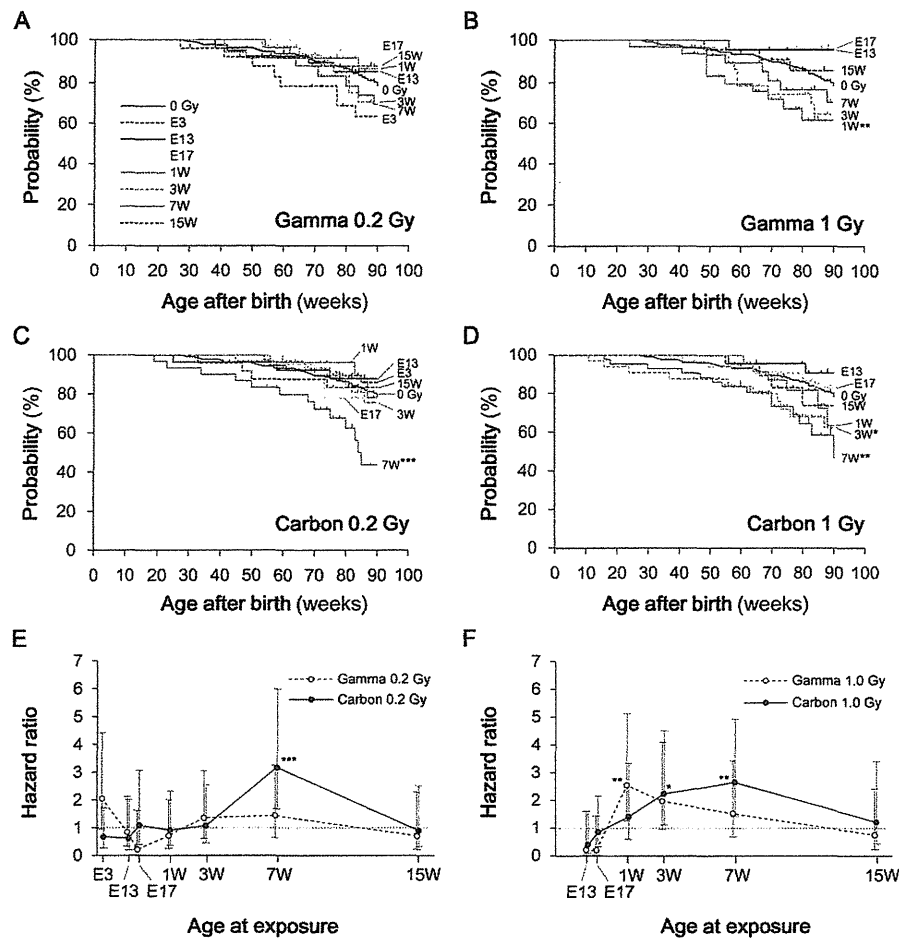


Fig. 1. Mammary carcinogenesis after irradiation with γ rays and carbon ions at various ages between embryonic and adulthood stages. (A-D) Kaplan-Meier plots for carcinoma-free survival of rats left unirradiated (0 Gy; common in A-D) and irradiated with γ rays (Gamma) or carbon ions (Carbon) at embryonic day 3, 13, or 17 (E3, E13, and E17, respectively) or 1, 3, 7, or 15 weeks after birth (1W, 3W, 7W, and 15W, respectively). (E, F) Cox proportional hazard ratio plotted against the age at irradiation. Vertical bars indicate the 95% confidence interval. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ versus 0 Gy by the log-rank test.

Table e1 (available online; www.redjournal.org). Rats not exposed to γ - or carbon-ion irradiation together constituted the control group (217 rats). After maternal irradiation, 6 to 7 pups were raised by a dam. Juvenile rats were weaned at day 21 after birth. During weekly observations, any tumor that was detected by palpation was recorded. Estrous cycles were recorded monthly for 5 consecutive days according to the cytology of vaginal smears ($n = 3$ for each irradiated group; $n = 9$ for the control group). Rats were killed if they showed any signs of general deterioration over the course of the experiment. The experiment was terminated when rats that had survived to an age of 90 weeks were killed by exsanguination under ether anesthesia and then autopsied. Excised tumors were fixed in 10% neutral-buffered formalin, and 2- μ m-thick paraffin-embedded sections were prepared and stained with hematoxylin and eosin for histologic evaluation (11). For the palpable carcinoma-free survival analysis, the first appearance of a palpable carcinoma was estimated retrospectively from both pathology results at autopsy and the tumor palpation history.

Statistics

Cox proportional hazard analysis was performed for the palpable carcinoma-free survival time (ie, time to the first palpation of

carcinoma) using the free statistical software R (<http://www.r-project.org/>). Animal death before the first detection of carcinoma by palpation was classified as censoring. A χ^2 test was performed for comparison of the tumor incidence; Yates' correction was used for analysis where frequency values were < 5 . Dunnett's test was used to assess differences from the control group with respect to the age at the time of cessation of the regular estrous cycle. The measure for statistical significance was $P < 0.05$. The relationship between the dose (in Grays) and hazard ratio was fitted to a linear equation with a y -intercept of 1 by the least-square method. Excess relative risk (ERR) (Gy^{-1}) was defined as the estimated slope of the line. Given that RBE is generally defined as the ratio of doses of 2 radiation species that give an identical biological effect, we defined it as the ratio of ERR values obtained for each of the 2 types of radiation. Standard errors of the ratio values were calculated using the delta method.

Results

Female Sprague-Dawley rats at various stages of development (embryonic days 3, 13, and 17 and 1, 3, 7, and 15 weeks after birth) were irradiated with ^{137}Cs γ rays or 290 MeV/u

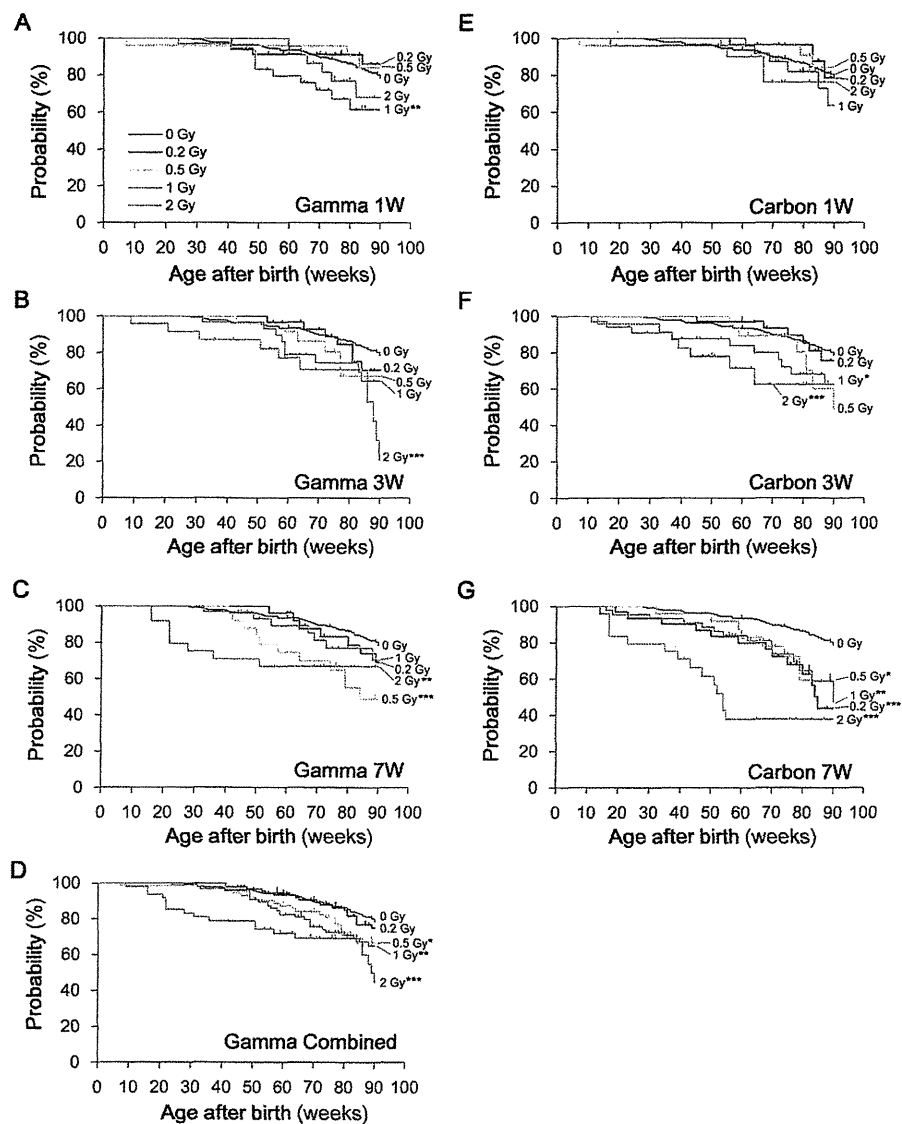


Fig. 2. Kaplan-Meier plots for mammary carcinoma-free survival in rats irradiated with various doses of γ rays or carbon ions at 1, 3, or 7 weeks of age. (A-C) Rats were left unirradiated (0 Gy; common in all panels) or irradiated with 0.2, 0.5, 1.0, or 2.0 Gy of γ rays (Gamma) at 1, 3, or 7 weeks after birth (1W, 3W, and 7W, respectively). (D) Data in A-C were pooled and reanalyzed, except for the group irradiated with 2.0 Gy at 1 week after birth, in which precocious cessation of the estrous cycle occurred. (E-G) Rats were irradiated with carbon ions (Carbon) as in A-C. * $P < .05$; ** $P < .01$; *** $P < .001$ versus 0 Gy by the log-rank test.

monoenergetic carbon ions (with an LET of 13 keV/ μ m) at the doses indicated in Table 1. A group of nonirradiated rats was subjected to the same analysis to enable comparison. All rats were observed weekly for development of palpable mammary tumors until 90 weeks of age (or earlier if general deterioration of an animal required that it be killed), after which the animals were killed. Excised mammary tumors were analyzed histologically and classified as either adenocarcinoma or benign tumors, such as adenoma and fibroadenoma. Although carcinoma constituted a minor fraction of the tumors, its raw incidence at autopsy ranged from 7% to 71% among groups, indicating that carcinoma development was indeed increased in some of the irradiated groups compared with the unirradiated control group (24%; Table e1). However, the observation period (ie, the age at the time of autopsy) also differed between groups (64-89 weeks; Table e1). The timing of autopsy was delayed in one group, and this was

analyzed further (Table e2). In addition, the incidence of lung metastasis of mammary carcinoma did not show clear difference between the 2 radiation species (Table e1), in contrast to our previous observation of high incidence for the SOBP carbon ion beam (5). The results of delivery and development of pups after fetal irradiation are summarized in Table e3; we did not make a selection of pups on the basis of their defects.

To compare the susceptibility to carcinoma induction among the wide range of ages at irradiation, the palpable carcinoma-free survival time was analyzed using the Kaplan-Meier method for those groups in which rats were irradiated with γ rays or carbon ions at 0.2 Gy or 1.0 Gy (Fig. 1A-D). The hazard ratio for the development of palpable carcinoma was calculated according to these data using Cox proportional hazard model (Fig. 1E and F). As a result, γ irradiation at 1 week of age (1.0 Gy) and carbonion irradiation at 3 weeks (1.0 Gy) and 7 weeks (0.2 and 1.0 Gy) of

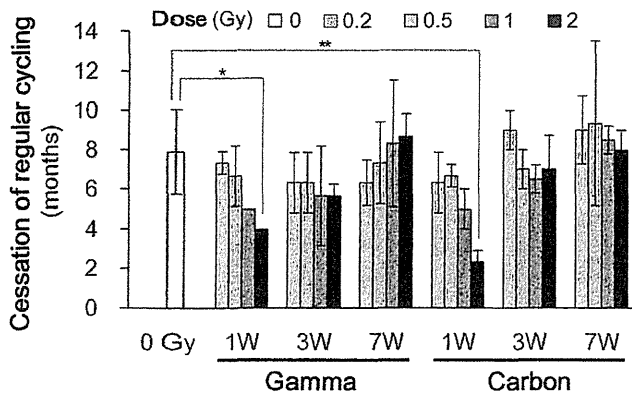


Fig. 3. Age at cessation of the regular estrous cycle. Female rats were irradiated with 0.2 to 2.0 Gy of γ rays (Gamma) or carbon ions (Carbon) at 1, 3, or 7 weeks after birth (1W, 3W, and 7W, respectively) or left unirradiated (0 Gy). For all treatments, the vaginal smear was scored monthly for 5 consecutive days. Bars indicate the mean age at the cessation of regular cycling, with the vertical bars indicating standard deviation. * $P < .05$; ** $P < .01$ versus 0 Gy.

age significantly increased the risk of carcinogenesis. The comparison thus shows that, for rats, the period of susceptibility to radiation-induced mammary carcinogenesis lies between 1 and 7 weeks after birth, with minimal susceptibility after irradiation during either the embryonic or full-adulthood stage.

Dose-effect relationships were then analyzed for experiments involving irradiation with γ rays or carbon ions at 1, 3, and 7 weeks after birth. The palpable carcinoma-free survival data indicated an overall tendency of dose-dependent increases in carcinoma development (Fig. 2A-C and E-G). It seemed, however, that the highest dose (2.0 Gy) of radiation at 1 week of age caused no clear increase in rates of carcinogenesis (Fig. 2A and E). We reasoned that this latter effect might be attributed to the highly effective diminishment of the ovarian follicular pool that occurs after neonatal irradiation of rats (12). Indeed, γ or carbonion irradiation at 1 week of age (2.0 Gy) induced premature cessation of the estrous cycle (Fig. 3). Therefore, these 2 data points were excluded from the analysis of the dose-effect relationship (Fig. 4A and E, open circles). In this analysis, plotting of hazard ratios against dose indicated similar effects of γ irradiation among the groups of rats irradiated between 1 and 7 weeks after birth (Fig. 4A-C). In contrast, the effect of carbon ions was greater for animals irradiated at later stages of development (Fig. 4E-G).

To derive RBE values for carbonion irradiation at different ages, we first calculated the value of ERR (Gy^{-1}) for each combination of radiation and age, as defined by the increase in the hazard ratio with unit dose (Table 2). We tentatively used a linear fitting because the present data, composed of 5 dose groups, were not suitable for detailed analysis of the shape of the dose response, and because linear dose response has been frequently observed for radiation carcinogenesis (4-7). The ERR values of the γ -ray groups fell within close proximity, having mutually overlapping confidence intervals (Table 2). Therefore, we combined these γ -ray groups to form a new set of dose-effect relationship data (Figs. 2D and 4D) to obtain a better estimation of ERR for γ rays (Table 2, bottom row); because significant effects on the estrous cycle were observed, as mentioned above, after 2.0 Gy irradiation at 1 week of age, these data were excluded. The RBE values for

carbonion irradiation were first calculated as the ratio of ERR for carbon ions to ERR of γ rays within each age group, giving the values 0.2, 1.1, and 3.3 for irradiation at 1, 3, and 7 weeks of age, respectively (Table 2, second-to-last column). The use of ERR for the combined γ -ray data as a reference resulted in similar values of 0.2, 1.3, and 2.8, respectively, with smaller confidence intervals (Table 2, rightmost column). Thus, the data suggested lower RBE values for breast cancer risk from carbonion irradiation of neonates and juveniles compared with that of young adults.

Discussion

The present study indicates that postnatal weeks 1 to 7 is the period during which rats are most susceptible to γ -ray induced mammary carcinogenesis. In contrast, irradiation with γ rays during the embryonic and full-adulthood stages does not significantly increase the risk of mammary carcinoma. These results agree with the epidemiologic observation that fetal exposure to the atomic bomb radiation (which mostly consisted of γ rays) is associated with a relatively low risk of solid cancer compared with the risk after exposure during early childhood (13). On the other hand, another study on the atomic bomb survivors indicated that a high excess absolute risk (EAR) of breast cancer is associated with exposure at an early age (4). ERR is, however, independent of the age at the time of exposure, because of the varying background incidence among the groups of different ages at exposure (4). The present observation of susceptibility during childhood and young adulthood but not at full adulthood (15 weeks of age in rat) resembles the EAR result, supporting the idea that EAR is more likely to reflect the biologically relevant period of susceptibility.

The present evidence suggests that induction of mammary carcinoma by carbonion irradiation is less likely in neonates and juveniles than in young adults. The mechanistic basis of this novel observation is difficult to explain. Most prepubertal mammary cells are quiescent in the absence of hormone-induced cell proliferation and, therefore, they may not be proficient in homologous recombination, which operates in the late S or G2 phase of the cell cycle and is required for repairing carbon ion-induced complex DNA damages (14-16). Alternatively, cell cycle delays induced by the particle radiation may affect the proliferation of stem-like cells, which is most active in the prepubertal mammary gland (17), and suppress expansion of initiated cells. We also noted an association between premature cessation of estrous cycling and low susceptibility to mammary carcinogenesis. This may be relevant to the clinical situation whereby a high ovarian dose induces premature menopause and reduces the radiation therapy-associated risk of secondary breast cancer (18), implying modification of cancer risk by dose localization. Ovarian estrogen may be required for secretion of prolactin into circulation or of growth factors in the cancer microenvironment, both of which are known promoting factor for breast cancer (19).

Unlike humans, Sprague-Dawley rats show very high prevalence of mammary carcinoma if monitored over their lifetime, a limitation of the Sprague-Dawley rat model. Nevertheless, use of carbon ions for thoracic tumors of children (eg, osteosarcomas and chordoma) would result in exposure of the mammary tissue to doses and LET commensurate with the experimental situation used here (approximately 1.5 Gy per fraction, approximately 13 keV/ μm). Irradiation of other areas will result in exposure to very low radiation doses from scattered beams (20), which is not tested

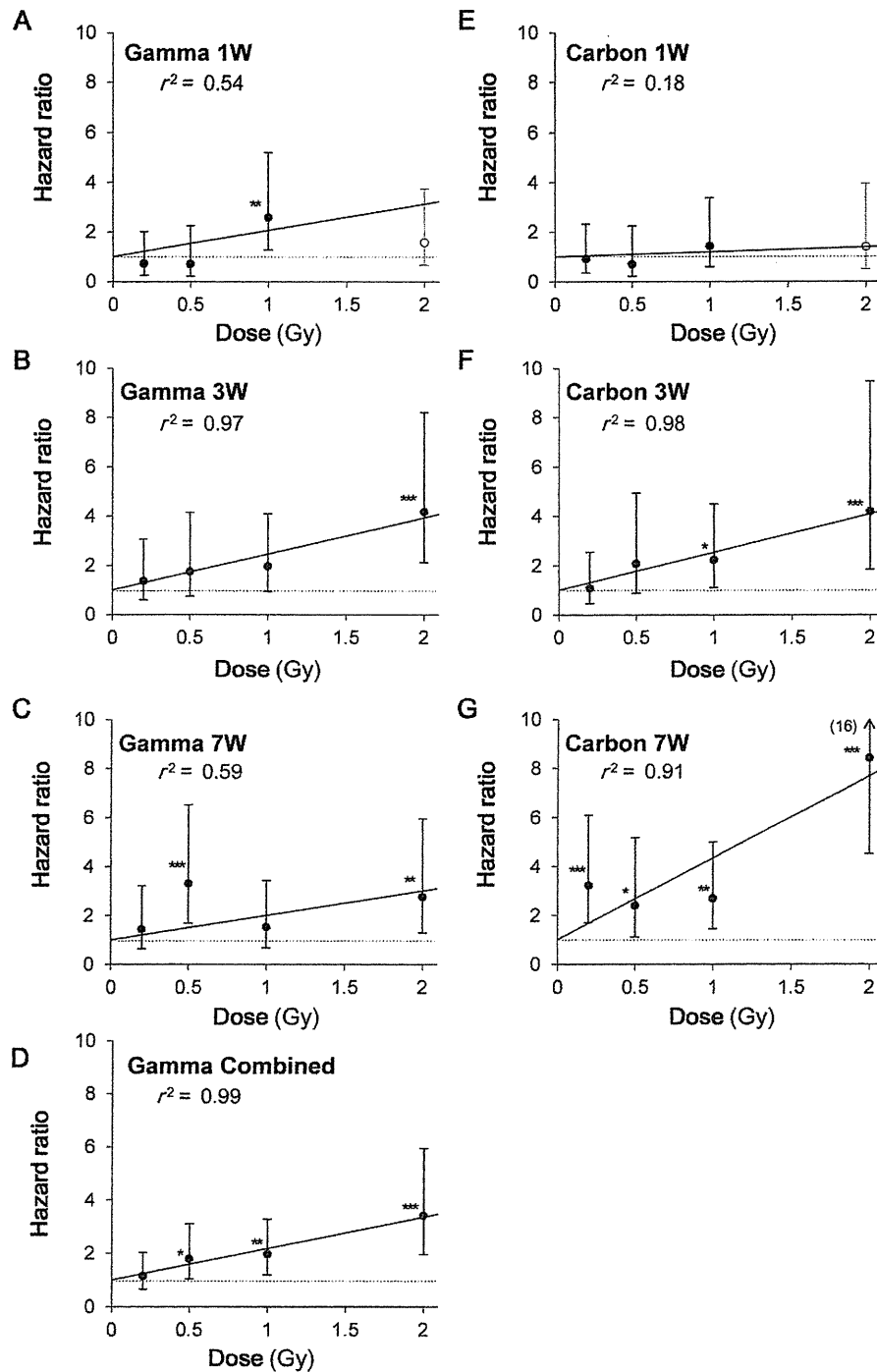


Fig. 4. Dose-effect relationship for induction of rat mammary carcinoma. Female rats were irradiated with 0.2-2.0 Gy of γ rays (A-D) or carbon ions (E-G) 1, 3, or 7 weeks after birth (1W, 3W, and 7W, respectively). The hazard ratio for the development of palpable mammary carcinoma is plotted against the dose. Vertical bars indicate the 95% confidence interval. Linear fitting was performed by the least-squares method, and the r^2 value is indicated in each panel. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ versus 0 Gy by the log-rank test.

in these experiments. The absorbed dose from carbon ions to the normal tissue is generally less than one-third of that used in photon radiation therapies because of the high biological effect and improved dose localization (2); therefore, even for the highest RBE postulated in our study (ie, 2.8), the expected cancer risk would be lower than that anticipated for photon radiation therapies. Because exposure is localized and fractionated in clinical

situations, future studies will be needed to clarify how such factors modify the carcinogenic effect. Investigations are also required regarding the effect on normal tissues at other sites, because the effect will likely depend on tissue type (4). Taken together, the present study provides evidence of a carcinogenic effect of carbon ions that depends on the age of the patient at the time of irradiation.

Table 2 Excess relative risk (ERR) per Gray of γ rays and 290 MeV/u monoenergetic carbon ions and relative biological effectiveness (RBE) of carbon ions for induction of rat mammary carcinoma

Age at exposure	ERR (Gy ⁻¹)*		RBE	
	γ rays	Carbon ions	Separate analysis [†]	Combined analysis [‡]
1 wk	1.1 (−0.3, 2.4) [§]	0.2 (−0.4, 0.8)	0.2 (−0.4, 0.8)	0.2 (−0.3, 0.7)
3 wk	1.5 (1.2, 1.7)	1.5 (1.3, 1.8)	1.1 (0.8, 1.3)	1.3 (1.0, 1.6)
7 wk	1.0 (0.1, 1.9)	3.3 (2.2, 4.5)	3.3 (0.0, 6.7)	2.8 (1.8, 3.9)
All ages combined	1.2 (1.0, 1.3)			

* Defined by least-squares fitting of hazard ratio data to $1 + \text{ERR} \times \text{dose (Gy)}$ as in Figure 4.

[†] Calculated as the ratio of ERR of carbon ions to ERR of γ rays for each age.

[‡] Calculated as the ratio of ERR of carbon ions for each age to ERR of γ rays for all ages combined.

[§] Mean (95% confidence interval).

^{||} Data for 1, 3, and 7 wk were combined and reanalyzed as in Figures 2D and 4D, except for the group irradiated with 2.0 Gy at 1 wk after birth, in which precocious cessation of the estrous cycle occurred.

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Asymmetric nature of two subunits of RAD18, a RING-type ubiquitin ligase E3, in the human RAD6A–RAD18 ternary complex

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ABSTRACT

RAD18, a RING-type ubiquitin ligase (E3) that plays an essential role in post-replication repair, possesses distinct domains named RING, UBZ, SAP and the RAD6-binding domain (R6BD) and forms a dimer. RAD6, an ubiquitin-conjugating enzyme (E2), stably associates with R6BD in the C-terminal portion. In this study, we established a method to distinguish between the two subunits of RAD18 by introduction of different tags, and analyzed mutant complexes. Our results, surprisingly, demonstrate that RAD6A and RAD18 form a ternary complex, RAD6A–(RAD18)₂ and the presence of only one R6BD in the two RAD18 subunits is sufficient for ternary complex formation and the ligase activity. Interestingly, ligase activity of a mutant dimer lacking both R6BDs is not restored even with large amounts of RAD6A added in solution, suggesting a requirement for precise juxtaposition via interaction with R6BD. We further show that mutations in both subunits of either RING or SAP, but not UBZ, strongly reduce ligase activity, although inactivation in only one of two subunits is without effect. These results suggest an asymmetric nature of the two RAD18 subunits in the complex.

INTRODUCTION

Ubiquitin ligases (E3s) catalyze the transfer of ubiquitin from E2 (ubiquitin-conjugating enzyme)–ubiquitin conjugates to lysine residues in target proteins. A subset of E3s contains a RING (really interesting new gene) domain, which binds to E2–ubiquitin conjugates and seems to activate thioester bonds (1,2). Some RING-type E3s are known to form heterodimers such as BRCA1–BARD1

(3–5), Ring1b–Bmi1 (6,7) and MDM2–MDMX (8–10), while others like cIAP2 (11) and RNF4 (12) act as homodimers. The heterodimeric RING-type E3s are composed of active and inactive RING domains, and dimerization enhances the ligase activity, suggesting that the pairing itself is very important for enzyme function (5,6,9).

Through a RAD6-binding domain (R6BD) located in its C-terminal region, the RAD18 RING-type ubiquitin ligase forms a stable complex with a specific E2, RAD6 (13–17). Since RAD6 also contacts the RING domain near the N-terminal of RAD18 for catalytic function (2,15,18), it could interact with two distinct domains of RAD18 simultaneously. Such interactions between an E2 and an E3 are quite unique to RAD6–RAD18. Recently, it has been reported that interaction between R6BD and RAD6 blocks the intrinsic activity of RAD6 in forming ubiquitin chains, ensuring mono-ubiquitination of PCNA (19). Previous studies have also reported that RAD18 forms a dimer and suggested the RAD6–RAD18 complex to be a dimer of RAD6–RAD18 heterodimer (15,20,21), hereafter designated as (RAD6–RAD18)₂.

RAD6 and RAD18 play an essential role in post-replication repair of damaged DNA *via* ubiquitination of proliferating cell nuclear antigen (PCNA) at Lys164 (22,23). The RAD6–RAD18 complex itself catalyzes mono-ubiquitination of PCNA *in vitro* (24–28). The mono-ubiquitinated PCNA appears to enhance lesion bypass replication by stimulation of entry of translesion DNA polymerases at stalled 3'-ends, through interactions between ubiquitin-binding domains of the polymerases and ubiquitin moieties of mono-ubiquitinated PCNA (24,26,29–31). Furthermore, RAD18 features a pol η-binding domain that is important for recruiting pol η to stalled 3'-ends (17,32).

RAD18 has two other domains UBZ (ubiquitin-binding zinc finger) (33–35) and SAP (SAF-A/B, Acinus and

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PIAS) (15,36–38). The former is required for accumulation of RAD18 at damage sites (34,37,39) and has affinity for Ub chains (33,34) suggesting specific binding to damage-associated poly-ubiquitinated proteins (39). The SAP domain possesses DNA-binding activity (15,38), although it appears to be unnecessary for accumulation of RAD18 at damage sites (37,39). Rather, it is crucial for pol η focus formation (37) possibly depending on PCNA ubiquitination, which is attributed to an essential role of the SAP domain in ligase activity (38,39).

Although multiple domains of RAD18 are clearly concerned with ligase activity, it is unknown how the distinct entities of the two RAD18 subunits interact with each other for enzyme function. In the present study, we established a method to analyze the structure and functions of the human RAD6A–RAD18 complex and demonstrated an asymmetric nature of the two RAD18 molecules in the complex.

MATERIALS AND METHODS

Plasmids

Expression plasmids for RAD6A–RAD18, E1, ubiquitin, PCNA and RFC were as described previously (26,40–42). For overproduction of human RAD6A, RAD6A^{His}–RAD18, FLAG–RAD6A^{His}–RAD18, the genes were cloned into pET20b(+) (Novagen) to yield pET–RAD6A and pET–RAD6A/hisRAD18, pET–flagRAD6A/hisRAD18, respectively. To make an expression plasmid compatible with pET plasmids in *Escherichia coli* cells, the entire coding unit of pET–RAD6A/RAD18 (42) was cloned into pACYC Duet1 (Novagen) to yield pAC–RAD6A/RAD18. Expression plasmids for overproduction of RAD6A^{FLAG}–RAD18 and FLAG–RAD6A^{FLAG}–RAD18, pAC–RAD6A/flagRAD18 and pAC–flagRAD6A/flagRAD18 were generated, respectively. In those plasmids, His-tagged sequences were taken from pET15 and all tagged sequences were attached to immediately before the start codons of the respective genes. Expression plasmids in human cells for *RAD6* were cloned in pCMV, and for *RAD18* in pCDNA3 flag and pCAGGS (43).

Proteins

Proteins used in this study were overproduced in *E. coli* cells. During all purification steps, monitoring was done by SDS–PAGE followed by staining with Coomassie Brilliant Blue R-250 (CBB), or western blotting. Protein concentrations were determined by Bio-Rad protein assay using BSA (Bio-Rad) as the standard. PCNA, RFC, E1, ubiquitin and RAD6A–RAD18 complex were purified as described previously (26,40,41). Detailed procedures for purification of recombinant proteins established in this study are described in Supplementary Data.

Sucrose density gradient sedimentation

Sucrose density gradient sedimentation was performed as described earlier (44). Purified RAD6A–RAD18 complexes (1.7 μ g), were sedimented through 2 ml of

10–40% sucrose gradient in buffer A containing 300 mM NaCl by centrifugation at 55 000 rpm for 20 h in a TLS 55 rotor (Beckman) at 4°C and fractions (100 μ l) were collected from the bottom of the tube and analyzed by SDS–PAGE. Gel bands were stained with CBB and quantified using Multi Gauge software Version 3.0 (FUJIFILM). Sedimentation coefficients were determined relative to those of standard proteins sedimented in parallel gradients.

Antibodies

To obtain polyclonal antibodies against RAD18, truncated His-tagged RAD18 proteins (127–255 amino acids) were expressed in BL21 (DE3) (45), purified and used to immunize rabbits. Anti-Penta-His monoclonal (Qiagen, 34660), anti-FLAG M2 monoclonal (Sigma, F3165), anti-RAD6 polyclonal (Abcam, ab31917) and anti-PCNA polyclonal (Santa Cruz, sc-7907) antibodies were purchased.

PCNA-mono-ubiquitination assays

The standard reaction mixture (25 μ l) contained 20 mM HEPES–NaOH (pH 7.5), 50 mM NaCl, 0.2 mg/ml BSA, 1 mM DTT, 10 mM MgCl₂, 1 mM ATP, 100 ng poly(dA)-oligo(dT), PCNA (1 pmol), RFC (350 fmol), E1 (850 fmol), ubiquitin (170 pmol) and the indicated amounts of RAD6A–RAD18 complex. Reaction mixtures were prepared on ice then incubated at 30°C for 10 min. The reactions were terminated by addition of 2 \times SDS sample buffer containing 25 mM EDTA. Ubiquitination of PCNA was assessed by western analysis, detected by an ECL chemiluminescence kit (GE Healthcare Life Science).

RESULTS

Physicochemical properties of the human RAD6A–RAD18 complex

To study the subunit composition of RAD6A–RAD18 complex, we first determined the Stokes' radius and sedimentation coefficient of the purified RAD6A–RAD18 complex (26) by gel filtration and sucrose density gradient centrifugation, respectively (Table 1). The obtained value of Stokes' radius (62 Å) corresponds to an apparent molecular mass of 490 kDa, but that of the

Table 1. Physicochemical properties of the RAD6A–RAD18 complex

Stokes' radius ^a (Å)	Sedimentation coefficient ^b $\times 10^{-13}$ s ($S_{20,w}$)	Molecular mass ^c (kDa)
62	5.0	131

^aDetermined by Superdex 200 gel filtration using the size markers ferritin (61.0 Å), aldolase (48.1 Å), albumin (35.5 Å) ovalbumin (30.5 Å) and ribonuclease A (16.4 Å), and the data were based on A_{280} values monitored during the chromatography.

^bDetermined with ferritin (17.6 S), catalase (11.3 S), aldolase (7.4 S), albumin (4.2 S) and ribonuclease A (1.8 S) as standards, and the data were based on the SDS–PAGE gel profile.

^cEstimated from the Stokes' radius and the sedimentation coefficient assuming a partial specific volume of 0.73 (46).

sedimentation coefficient (5.0S) corresponds to an apparent molecular mass of 70 kDa. The large difference between these two values suggests that the complex does not have a compact globular shape. Employing the method described by Siegel and Monty (46), we estimated the molecular mass of the RAD6A–RAD18 complex to be 131 kDa (Table 1). Since calculated molecular masses of RAD6A and RAD18 are 17.3 and 56.2 kDa, respectively, the total of molecular mass should be 73.5 kDa for RAD6A–RAD18 and 147 kDa for (RAD6A–RAD18)₂. Although the value estimated from the experiments was close to that of (RAD6A–RAD18)₂, we further investigated the stoichiometry of RAD6A and RAD18 in the complex.

Direct evidence that the RAD6A–RAD18 complex contains two RAD18 molecules

To obtain direct evidence that the RAD6A–RAD18 complex contains two molecules of RAD18, we co-expressed both His-tagged *RAD18* and FLAG-tagged *RAD18* genes together with *RAD6A* gene in the same *E. coli* cells. Three different complexes containing His⁶RAD18–His⁶RAD18, FLAG³RAD18–FLAG³RAD18 or His⁶RAD18–FLAG³RAD18 would be expected if we assume that the complex should contain two molecules of RAD18. When the cell lysate was loaded to a Ni-chelating column, we found some FLAG³RAD18 to be absorbed to the column and eluted together with approximately equal amounts of His⁶RAD18 at a lower imidazole concentration (Figure 1A, lane 2), whereas the remainder of the His⁶RAD18 was eluted at a higher imidazole concentration (see Supplementary Materials and Methods section). Since it was expected that the former was His⁶RAD18–FLAG³RAD18 hetero complex and the latter was His⁶RAD18 homo complex, the former was further purified through a heparin column (Figure 1A, lane 3) and analyzed by gel filtration (Figure 1A, lane 4). The elution profile was the same as that of untagged

RAD6A–RAD18 complex (Table 1 and see also Figure 6C), suggesting the tag sequences do not affect the overall structure of the complex. While western blotting with anti-Penta-His and anti-FLAG antibodies specifically detected His⁶RAD18 and FLAG³RAD18 proteins, respectively (Figure 1B), blotting with anti-RAD18 antibodies detected the two proteins equally, thereby indicating that the purified complex contains His⁶RAD18 and FLAG³RAD18 at a 1:1 ratio. To further verify that RAD6A, His⁶RAD18 and FLAG³RAD18 form a complex, a fraction eluted from gel filtration was applied to FLAG-affinity chromatography. The result demonstrated that the three proteins were adsorbed to the anti-FLAG M2 affinity gel and eluted with the FLAG peptide (Figure 1A, lane 5). We thus conclude that the RAD6A–RAD18 complex contains two RAD18 molecules.

Subunit composition of the RAD6A–RAD18 complex is RAD6A–(RAD18)₂

To examine the stoichiometry of RAD6A in the complex, we directly compared amounts of RAD6A in the complex with purified RAD6A protein as a reference. We applied 500 ng of the purified complex, RAD6A–His⁶RAD18–FLAG³RAD18, in parallel with different amounts (65 or 115 ng) of RAD6A monomers to SDS-PAGE. The amounts of RAD6A in the complex should be 65 or 115 ng, if the complex is RAD6A–(RAD18)₂ or (RAD6A–RAD18)₂, respectively (Figure 2A). The results of CBB staining and western blotting showed the amount of RAD6A in the complex to be closer to 65 ng (Figure 2A), thus suggesting that the complex contains RAD6A and RAD18 at the ratio of 1:2. To confirm this, the FLAG-tag was introduced to RAD6A and the FLAG³RAD6A–His⁶RAD18–FLAG³RAD18 complex was purified as described above. We applied 505 ng of the purified complex to SDS-PAGE, in parallel with 500 ng of RAD6A–His⁶RAD18–FLAG³RAD18 as a reference (Figure 2B). Western blotting with anti-RAD6 antibodies confirmed that both complexes contained equivalent amounts of RAD6A (Figure 2B), and blotting with an anti-FLAG antibody clearly demonstrated that the molecular ratio of FLAG³RAD6A and FLAG³RAD18 was 1:1 (Figure 2B), suggesting strongly the ratio of RAD6A to RAD18 to be 1:2.

R6BD is located within amino acid residues 340–395 of RAD18 (Figure 3D) (17,19). Using a deletion mutant consisting of amino acid residues 1–341 of RAD18 (hereafter designated as RAD18^{ΔC1}, Figure 3D), we further examined the stoichiometry of RAD6 and RAD18. When His⁶RAD18^{ΔC1} and FLAG³RAD18^{ΔC1} were co-produced with RAD6A in the same *E. coli* cells, a complex containing His⁶RAD18^{ΔC1} and FLAG³RAD18^{ΔC1} was purified similarly as described above, but it did not contain RAD6A (Figure 2C, lane 3). This result indicates that the R6BD is required for complex formation with RAD6 but is dispensable for dimerization. In contrast, when His⁶RAD18^{ΔC1} was co-produced with FLAG³RAD18 and RAD6A in the same *E. coli* cells, we successfully obtained a RAD6A–His⁶RAD18^{ΔC1}–FLAG³RAD18 ternary complex

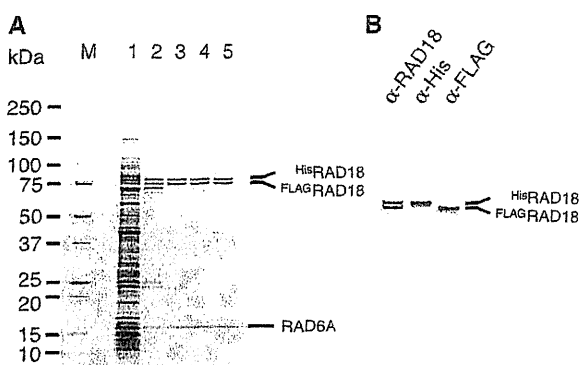


Figure 1. Purification of the RAD6A–His⁶RAD18–FLAG³RAD18 complex. (A) Pooled fractions eluted from respective columns were analyzed by SDS-PAGE followed by staining with CBB. Lane 1, cell lysate; lane 2, Ni-chelating column; lane 3, heparin column; lane 4, gel-filtration column; lane 5, anti-FLAG affinity column. Molecular masses of each marker (lane M) are shown to the left of the gel. (B) Western analysis of the pooled fraction eluted from the gel filtration column. Membranes were probed with the indicated antibodies.

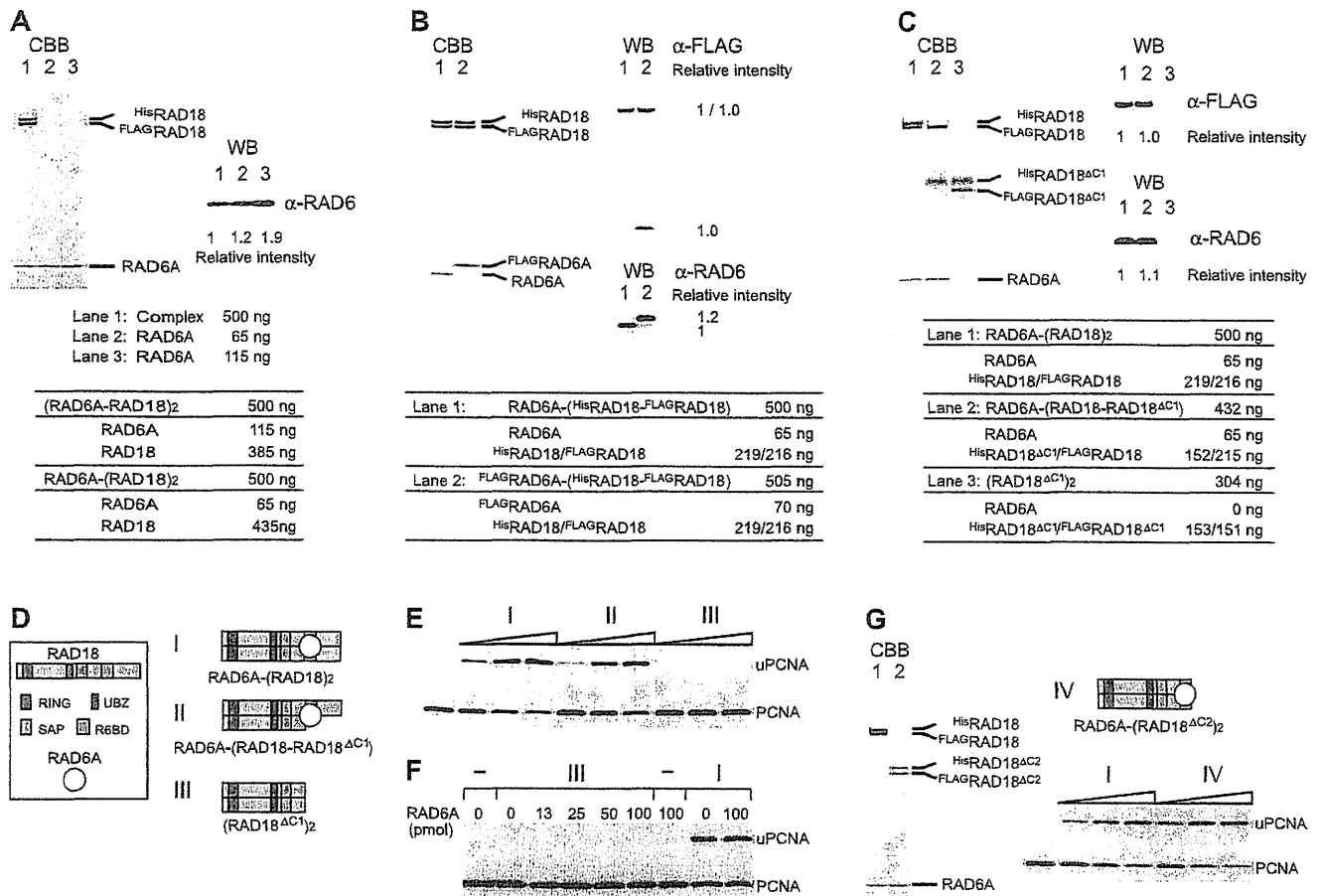


Figure 2. Subunit composition of the RAD6A-RAD18 complex and deletion analysis of RAD18. (A) The indicated amounts of purified RAD6A-RAD18 complex and RAD6A were analyzed by SDS-PAGE followed by staining with CBB and western blotting probed with anti-RAD6 antibodies. Relative chemiluminescence signals detected with a CCD camera are shown. Amounts of each subunit calculated for two different postulated subunit compositions are shown in the table. (B and C) Indicated amounts of purified RAD6A-RAD18 complexes were analyzed by SDS-PAGE followed by staining with CBB and western blotting probed with anti-FLAG and anti-RAD6 antibodies. Relative chemiluminescence signals detected with a CCD camera are shown. Amounts of each subunit calculated for the postulated subunit compositions are shown in the tables. (D) Schematic representations of the structures for respective RAD6A-RAD18 complexes. (E) Ligase activities of the respective RAD6A-RAD18 complexes. Increasing amounts of the complexes (0.5, 1 and 2 pmol) were subjected to standard assays. The reaction products were analyzed by western blotting with anti PCNA antibodies. I, RAD6A-(HisRAD18-FLAGRAD18); II, RAD6A-(HisRAD18^{ΔC1}-FLAGRAD18); III, HisRAD18^{ΔC1}-FLAGRAD18^{ΔC1}. (F) Titration of RAD6A in the reaction with HisRAD18^{ΔC1}-FLAGRAD18^{ΔC1}. Indicated amounts of RAD6A were incubated with 2 pmol of HisRAD18^{ΔC1}-FLAGRAD18^{ΔC1} (III) under standard assay conditions. As control reactions, 1 pmol of RAD6A-(HisRAD18-FLAGRAD18) (I) was incubated in the presence or absence of additional RAD6A. (G) Analysis of complex formation and ligase activity of another C-terminal deletion mutant of RAD18. Purified complexes (3.7 pmol as a trimer) were analyzed by SDS-PAGE followed by staining with CBB. Lane 1, RAD6A-(HisRAD18-FLAGRAD18) (I); lane 2, RAD6A-(HisRAD18^{ΔC2}-FLAGRAD18^{ΔC2}) (IV). Structure of the mutant complex (IV) was represented schematically. Assays were performed as described in (E).

(Figure 2C, lane 2), indicating that only one R6BD in the two RAD18 subunits could form the complex with RAD6A. Again, amounts of each protein in these complexes were compared by CBB staining and western blotting. For Figure 2C, we applied 500 ng of RAD6A-HisRAD18-FLAGRAD18 (lane 1) and 432 ng of the RAD6A-HisRAD18^{ΔC1}-FLAGRAD18 complex (lane 2) to SDS-PAGE. If stoichiometry of each component were 1:1:1 in both complexes, amounts of each FLAGRAD18 and each RAD6A in the two complexes should be identical (Figure 2C). The results of CBB staining and western blotting proved to be in good agreement with our estimations (Figure 2C, lanes 1 and 2), suggesting that the

RAD18 dimer, as well as the RAD18 monomer, is capable of accommodating only one RAD6 molecule (Figure 2D). Very interestingly, the hetero complex RAD6A-(HisRAD18^{ΔC1}-FLAGRAD18) was catalytically active in terms of PCNA ubiquitination (Figure 2E). As expected, HisRAD18^{ΔC1}-FLAGRAD18^{ΔC1} was inactive (Figure 2E), and its ligase activity was hardly restored by addition of excess amounts of RAD6A in solution (Figure 2F). To see whether the defect is due to the loss of function of the C-terminal portion, another truncated RAD18 mutant consisting of amino acid residues 1-388 (hereafter designated as RAD18^{ΔC2}, Figure 3D), was examined. It was expected that RAD18^{ΔC2} could form a

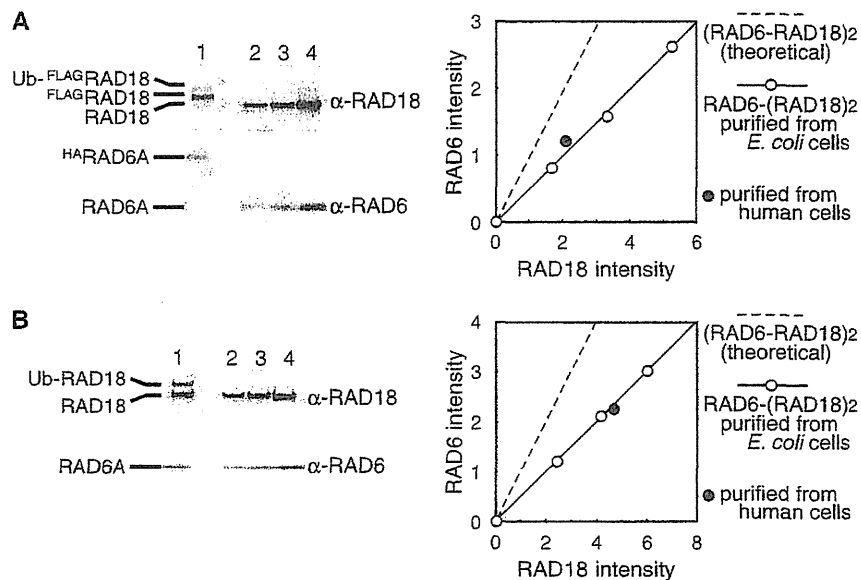


Figure 4. Ternary complex formation of RAD6A and RAD18 *in vivo*. (A) $^{HA}RAD6-^{FLAG}RAD18$ complexes isolated from human cells were analyzed by SDS-PAGE (lane 1) together with $RAD6A-(RAD18)_2$ isolated from *E. coli* (lanes 2–4) followed by western blotting probed with anti-RAD18 and anti-RAD6 antibodies. Relative chemiluminescence signals of $^{HA}RAD6A$ and $^{FLAG}RAD18$ (sum of unmodified $^{FLAG}RAD18$ and $Ub-^{FLAG}RAD18$) detected with a CCD camera were plotted with those of RAD6A and RAD18 purified from *E. coli* as references. The theoretical 1:1 ratio of RAD6 and RAD18 is shown as a broken line. (B) Untagged RAD6–RAD18 complexes isolated from human cells were analyzed by SDS-PAGE as (A).

Analysis of UBZ roles in complex formation and ligase activity

Our system to purify RAD6–RAD18 complex with two different tags is a useful tool for analysis of structure–function relationships in RAD18. First, we applied this system to test whether UBZ is required for dimerization of RAD18 using two loss-of-function mutants, $RAD18^{C207F}$ and $RAD18^{D221A}$ (34,37) (Figure 3B and D). The results demonstrated that these mutants were successfully reconstituted into the complexes, $RAD6A-(RAD18^{C207F})_2$ and $RAD6A-(RAD18^{D221A})_2$ (Figure 5A) and their ligase activities were similar to that of wild-type complex (Figure 5B). These results indicate UBZ to be dispensable for complex formation and ligase activity, in line with recent reports (15,39), demonstrating reliability of our system for analysis of the structure and function of RAD6–RAD18 complexes.

Functional interaction between RAD6 and the RING domains of the two subunits of RAD18 in the complex

Next, we addressed the question whether the N-terminal part containing a RING domain mediates dimerization. We generated another truncated RAD18 mutant consisting of the 1–115 amino acid residues of RAD18 (hereafter designated as $RAD18^{\Delta C3}$, Figure 3D). When $^{His}RAD18^{\Delta C3}$ and $^{FLAG}RAD18^{\Delta C3}$ were co-produced with RAD6A in the same *E. coli* cells, we found the $^{His}RAD18^{\Delta C3}$ – $^{FLAG}RAD18^{\Delta C3}$ dimer to be reconstituted, but that RAD6A did not co-purify with the dimer. The observed elution profile of the dimer from a gel filtration column is shown in Figure 6A. $^{His}RAD18^{\Delta C3}$ and $^{FLAG}RAD18^{\Delta C3}$, confirmed by western blotting (Figure

6B), co-eluted with an apparent molecular mass of ~37 kDa, slightly larger than the calculated molecular mass of 29 kDa as a dimer (Figure 6A and B). As a complementary experiment, an N-terminal deletion mutant of RAD18 consisting of 113–495 amino acid residues (hereafter designated as $RAD18^{\Delta N1}$, Figure 3D), was generated. When $^{His}RAD18^{\Delta N1}$ was coproduced with $^{FLAG}RAD18$ and RAD6A, $^{His}RAD18^{\Delta N1}$ co-purified with RAD6A but not with $^{FLAG}RAD18$ (Figure 6D). As mentioned above, the $RAD6A-(^{His}RAD18-^{FLAG}RAD18)$ ternary complex eluted at a position corresponding to 490 kDa in gel filtration (Figure 6C). In contrast, the complex of $RAD6A-^{His}RAD18^{\Delta N1}$ eluted at a position corresponding to 220 kDa, which is much smaller than the ternary complex (Figure 6C). Furthermore the molecular ratio of RAD6A and $^{His}RAD18^{\Delta N1}$ determined by western blotting, compared with the ternary complex as a reference, was close to 1:1 (Figure 6D). These results suggest that the $RAD6A-^{His}RAD18^{\Delta N1}$ complex is a dimer composed of one RAD6A and one $^{His}RAD18^{\Delta N1}$ molecule and imply that $^{His}RAD18^{\Delta N1}$ neither self-associates nor forms a heterodimer with $^{FLAG}RAD18$. From these data shown in Figure 6, we conclude that the N-terminal region (1–115) is necessary and sufficient for dimerization, while UBZ and SAP domains are dispensable.

In general, the RING domains of E3s have an essential function in ligase activity by mediating interactions with E2s (1,2). It is of great interest to clarify how the two RING domains interact with one RAD6 subunit in the $RAD6A-(RAD18)_2$ complex. To address this point, we made several mutants in which one or two conserved amino acid residues in the RING domain were replaced