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ORIGINAL ARTICLE

# *Brca1* heterozygous mice have shortened life span and are prone to ovarian tumorigenesis with haploinsufficiency upon ionizing irradiation

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**_BRCA1_ mutation carriers have an 85% lifetime risk of breast cancer and 60% for ovarian cancer. BRCA1 facilitates DNA double-strand break repair, and dysfunction of BRCA1 leads to hypersensitivity to DNA damaging agents and consequently genomic instability of cells. In this communication, we have examined the tumor incidence and survival of _Brca1_ heterozygous female mice. _Brca1_ heterozygotes appear to have a shortened life span with 70% tumor incidence. Lymphoma, but not ovarian and mammary gland tumors, occurs commonly in these mice. After a whole-body exposure to ionizing radiation, _Brca1_ heterozygous mice have a 3–5-fold higher incidence specific to ovarian tumors, but not lymphoma, when compared with the _Brca1_+/+ mice. All the tumors from heterozygous mice examined retain the wild-type allele and the cancer cells express Brca1 protein, precluding the chromosomal mechanism for loss of heterozygosity of _Brca1_ locus. Although the manifestation of BRCA1 haploinsufficiency may be different between human and mouse, this study suggests that women carrying _Brca1_ mutations may be more prone to ovarian tumor formation after IR exposure than nonmutation carriers.**
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**Keywords:** BRCA1; heterozygous mutant; ionizing radiation; ovarian tumor; haploinsufficiency

## Introduction

Women in Western countries have about a 10% lifetime risk of breast cancer, 5–10% of which are familial. Mutations in the breast cancer susceptibility gene *BRCA1* account for up to half the hereditary breast cancer cases and almost all the hereditary breast and ovarian cancer cases (Miki *et al*., 1994; Couch *et al*., 1997). *BRCA1* mutation carriers have a lifetime risk of breast cancer up to 85% and ovarian cancer as high as 60% (Easton *et al*., 1995). Moreover, a reduced BRCA1 expression is frequently observed in sporadic breast cancer and correlated with the accelerated progression and growth (Thompson *et al*., 1995).

BRCA1 mediates DNA damage repair, cell-cycle checkpoint control and transcriptional regulation, serving as a tumor suppressor to maintain the global genomic stability. The *BRCA1* gene is located on chromosome 17q21 and 100 kb in length, encoding a 220 kDa nuclear phosphoprotein of 1863 amino acids (Miki *et al*., 1994) characterized by distinctive protein–protein interaction surfaces. The N-terminal RING finger domain dimerizes with BARD1 for ubiquitin ligase activity (Hashizume *et al*., 2001), while the C-terminus possesses two tandem copies of the BRCT motif that interact with RNA polymerase II holoenzyme (Scully *et al*., 1997), histone deacetylases (Yarden and Brody, 1999), CBP/p300 (Pao *et al*., 2000), and CtIP (Li *et al*., 1999), serving as a phosphopeptide binding module for protein targeting (Yu *et al*., 2003). Finally, the central region, mainly encoded by exon 11, possesses two nuclear localization signals and interacts with a DNA damage repair complex RAD50/MRE11/NBS1 (Zhong *et al*., 1999) and transcription repressor ZBRK1 (Zheng *et al*., 2000b). In response to DNA damage, BRCA1 becomes phosphorylated by ATM (S1387, S1423 and S1524), ATR (S1423) and Chk2 (S988) and translocates to the site of lesion (Zhang *et al*., 2004). BRCA1 facilitates DNA double-strand break (DSB) repair by homologous recombination via RAD51 and nonhomologous end-joining via RAD50/MRE11/NBS1 complex (Ting and Lee, 2004). In addition, BRCA1 controls cell-cycle checkpoint by regulating the transcription of *p21* and *GADD45* genes through interaction with CtIP at the C-terminal BRCT domain (Li *et al*., 1999, 2001).

BRCA1 deficiency renders cells sensitive to ionizing radiation (IR) and DNA cross-linkers and perturbs the genomic stability manifested as aberrant chromosomal integrity and increased mutation rate (Shen *et al*., 1998). Human tumor cells lacking BRCA1 and mouse embryonic *Brca1*−/− fibroblasts are highly sensitive to IR (Shen *et al*., 1998; Foray *et al*., 1999). However, the effect of BRCA1 haploinsufficiency on the sensitivity to genotoxic agents in peripheral lymphocytes appears controversial. It was reported that the *BRCA1*+/− peripheral lymphocytes are sensitive to irradiation and have an increased frequency of micronuclei, which can

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serve as a screening tool for *Brca1* mutation carriers (Rothfuss *et al*., 2000). Subsequent studies failed to confirm such a finding (Baria *et al*., 2001; Baeyens *et al*., 2004; Kotsopoulos *et al*., 2007). Using chromosomal rearrangements to evaluate the sensitivity to genotoxic stress, a significantly higher level of chromosomal damage in the *BRCA1* +/− lymphocytes compared with normal controls was found after irradiation (Kote-Jarai *et al*., 2006). On the other hand, Baeyens *et al*. (2004) found that there is no significant difference in the frequency of chromosomal breakages between breast cancer patients with and without a *BRCA1* mutation. Thus, whether BRCA1 heterozygosity has any effect on IR sensitivity remains uncertain. Although the reason causing this discrepancy is unclear, one possibility is that the lymphocytes may not be of a specific cell type exhibiting BRCA1 haploinsufficiency. Therefore, whether haploinsufficiency of BRCA1 manifests in animals is of interest.

In this communication, we examined tumor incidence and survival of *Brca1* heterozygous female mice and found that *Brca1* heterozygotes had a shortened life span with 70% tumor incidence. These mice commonly succumbed to lymphoma, but neither ovarian nor mammary gland tumors. Upon ionizing irradiation, *Brca1* heterozygous mice had a 3–5-fold higher incidence of ovarian tumor, while the incidence of lymphoma did not change. The tumors produced in heterozygous mice retained the wild-type allele and expressed Brca1 protein. This study implicates that women carrying *BRCA1* mutations may shorten their life spans and be prone to ovarian tumor upon IR.

## Results and discussion

Up to 5% of breast cancer patients have inherited a germline mutation of *Brca1* gene, which confers a 50–87% risk of breast cancer. In a general practice, earlier screening for breast cancer by routine mammography is recommended for *Brca1* mutation carriers (Brekelmans *et al*., 2001). In a general population, regular mammographic screening combined with appropriate and prompt treatment can reduce mortality from breast cancer by 30% in women aged 50–59 years and by 14–18% in women aged 40–49 (Fletcher *et al*., 1993). Besides, therapeutic irradiation is frequently used in treatment of breast and ovarian cancers. As cumulative studies substantiate that BRCA1 is required for radiation-induced DNA damage repair (Ting and Lee, 2004), women with defective *BRCA1* genes should have an increased sensitivity to radiation, raising a question about the prudence of medical irradiation for these subjects (Friedenson, 2000).

To approach this question, we compared the effect of irradiation on the survival and tumorigenesis in *Brca1*-proficient and *Brca1* heterozygous female virgin mice. Ninety *Brca1* +/− female mice were divided into three groups. Each group received a single dose of whole-body irradiation, 0, 2.5 or 5 Gy, at the age of 5–6 weeks. Two groups of 30 *Brca1* +/+ (wild-type) female mice that received 0 and 5 Gy of whole-body irradiation respectively served as control. The mice were monitored until a moribund state. Without irradiation, *Brca1* +/− mice have a shorter life span than *Brca1* +/+ mice with a borderline statistical significance (medium survival days: $713 \pm 146$ vs $772 \pm 102$, $P = 0.037$; log-rank test: $P = 0.07$) (Table 1) (Figure 1). The survivals of both groups were reduced significantly by ionizing irradiation in a dose-dependent manner (Figure 1) (Table 1). However, the irradiated *Brca1* heterozygous mice did not have a shorter life span than the IR-treated wild-type mice. This may be due to the high dose of gamma irradiation (5 Gy) used in the experiment that masked the underlying genetic difference.

Systemic pathological examinations were performed after sacrifice. Without irradiation, *Brca1* +/− mice had a 70% tumor incidence; 50% of them developed malignant lymphoma. Only two mice harbored fibroadenoma in mammary gland and two mice developed ovarian tumors. This tumor incidence in *Brca1* +/− mice was even higher than that of the irradiated *Brca1* +/+ mice (Table 1). Consistently, only 10% tumor incidence was observed in wild-type mice with a similar but not completely identical genetic background during their life span (Salim *et al*., 2003). This result suggests that *Brca1* +/− mice are prone to spontaneous tumor formation. In irradiated *Brca1* +/− mice, the tumor incidence increased up to 96.4%. Various types of tumors with different cellular origins were found, including malignant lymphomas, ovary tumors (granulosa cell tumors, cystadenomas and adenocarcinomas), pulmonary adenocarcinomas and hepatocellular carcinomas (Figure 2A) (Table 2). The incidence of malignant lymphoma did not vary among groups of *Brca1* +/− mice with and without irradiation. After 5.0 Gy irradiation, 57.6% of *Brca1* +/+ mice developed mainly lymphomas. Most importantly, after a whole-body exposure to IR, *Brca1* heterozygous mice had a

**Table 1** Mean survival of *Brca1* +/− and +/+ mice after γ-irradiation

| *Genotype (γ-irradiation)* | Brca1 +/− *(0 Gy)* (n = 26) | Brca1 +/− *(2.5 Gy)* (n = 27) | Brca1 +/− *(5.0 Gy)* (n = 28) | Brca1 +/+ *(0 Gy)* (n = 32) | Brca1 +/+ *(5.0 Gy)* (n = 26) |
|---|---|---|---|---|---|
| Mean survival days (±s.d.)[1] | 713 (±146)[a] | 630 (±141)[b] | 531 (±146)[c] | 772 (±102)[d] | 527 (±182)[e] |
| Mean survival days with tumor (± s.d.)[2] | 709 (±214) | 622 (±134) | 531 (±148) | NA | 526 (±167) |
| *P*-value[1,2] | 0.38 | 0.39 | 0.50 | NA | 0.49 |
| Tumor incidence (%) | 70.0 | 81.5 | 96.4 | NA | 57.6 |

Abbreviations: NA, not applicable. [a,b]$P = 0.049$, [a,c]$P = 0.00023$, [a,d]$P = 0.037$, [d,e] $P = 0.0003$, [c,e] $P = 0.48$. The *P*-value was calculated by Student's *t*-test.

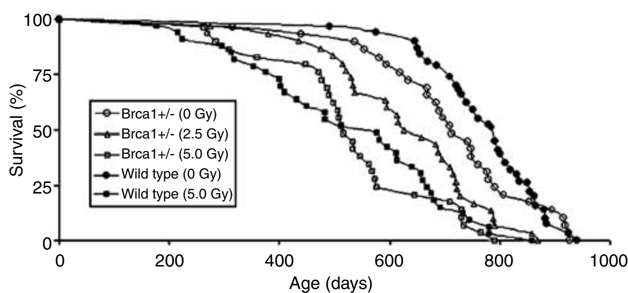


**Figure 1** Survival curves of *Brca1*+/− and +/+ (wild-type) mice that received a single dose of whole-body gamma radiation (0, 2.5 or 5.0 Gy) at the age of 5–6 weeks. Both *Brca1*+/− and *Brca1*+/+ mice showed dose-dependent shortening of life span after IR at 5–6 weeks (*Brca1*+/−: 0 vs 2.5 Gy, $P=0.01$; 0 vs 5 Gy, $P<0.0001$; *Brca1*+/+: 0 vs 5 Gy, $P<0.0001$.) Without irradiation, *Brca1*+/− mice had shorter survival than *Brca1*+/+ mice ($P=0.07$). In groups receiving 5 Gy irradiation, the survival was not different between *Brca1*+/− and *Brca1*+/+ mice ($P=0.43$). Survival analysis was performed by the Kaplan–Meier method.

3–5 fold higher incidence of ovarian tumors, but not lymphomas, when compared with the *Brca1*+/+ mice (Table 2). One possible explanation for this observation is that deficiency of *Brca1* under IR exposure affects ovary more severely than other cell types. Ovarian epithelial cells may harbor certain properties that help accumulate a higher level of DNA damage and genomic instability upon IR irradiation. For example, ovulatory cycle requires cellular proliferation to repair the ovarian surface after ovulation, allowing proliferation-associated DNA damage to occur (Schildkraut *et al.*, 1997). In general population, ovary is not a common site of IR-induced cancer. The highly increased risk of ovarian tumor formation in *Brca1*+/− mice suggests that haploinsufficiency of *Brca1* has a specific effect on ovarian cells, instead of lymphocytes, consistent with the clinical observation that Brca1 is an organ-specific tumor suppressor.

To determine whether there was a loss of heterozygosity (LOH) of *Brca1*, 10 tumors from *Brca1* heterozygous mice were microdissected and the genotypes were determined by polymerase chain reaction (PCR). As shown in Figure 2B, all *Brca1*+/− tumors retained the wild-type alleles. The expression of Brca1 was confirmed in all ovarian tumors and 10 other tumors from each group by immunohistochemistry (Figure 2Ca and b), indicating that haploinsufficiency, rather than biallelic inactivation, is the mechanism for the development of tumors in *Brca1* heterozygous mice. However, breast tumors developed in women carrying a *BRCA1* mutation frequently lose the wild-type allele (Neuhausen and Marshall, 1994). The reason of this discrepancy is not known. Nevertheless, haploinsufficiency of BRCA1 was observed in *BRCA1*+/− fibroblats in that they are impaired in DNA end-joining activity (Baldeyron *et al.*, 2002) and sensitive to IR (Rothfuss *et al.*, 2000). Thus, it is likely that the impaired DNA repair activity in *Brca1* heterozygous cells may in part account for the tumorigenesis in mice.

Breast tumors derived from *BRCA1* mutation carriers are frequently associated with p53 mutations (Greenblatt *et al.*, 2001). To test whether abnormality of p53 is associated with tumors derived from IR-irradiated *Brca1*+/− mice, we examined the expression of p53 in all ovarian tumors and 10 other tumors. These tumors were all negative for p53 staining (Figure 2Cc and d), indicating that p53 stabilization caused by point mutation or other mechanisms is not present in these tumors.

A low incidence of breast cancer, in contrast with a high incidence of ovarian cancer, in our mouse model is intriguing because *BRCA1* mutation carriers in human have up to 85% lifetime risk of breast cancer (Easton *et al.*, 1995), and homozygous *Brca1* knockout in mouse mammary epithelia frequently produces breast tumor (Deng and Xu, 2004). It is possible that inactivation of a given tumor suppressor results in different types of tumors between human and mice. The best example was revealed in the retinoblastoma gene case in that inactivation of RB causes retinoblastoma in human, but pituitary melanotroph tumor in mice (Nikitin and Lee, 1996).

Without irradiation, *Brca1* heterozygous mice have a shorter life span than the wild-type mice. This shortening of life span cannot be explained by the high incidence of lymphoma because the life span of mice carrying tumors is similar to those without tumors (Table 1). Recent studies demonstrate that DNA damage repair proteins play an essential role in life-span determination, as a number of mouse mutants carrying targeted disruption of genes involving in DNA damage repair exhibit premature aging (Vogel *et al.*, 1999; de Boer *et al.*, 2002). *Brca1*−/− *p53*+/− mice were also reported to have a decreased life span and showed a phenotype of premature aging (Cao *et al.*, 2003) even in the absence of tumor formation.

Cumulative evidence points out that BRCA1 is essential for several cellular activities including DNA DSB repair (Zheng *et al.*, 2000a; Ting and Lee, 2004). Therefore, women with *BRCA1* mutations will be likely to be defective in repairing irradiation-induced DNA damage (Foray *et al.*, 1999), allowing DNA mutations to accumulate more rapidly than nonmutation carriers. Such a pathogenic effect of BRCA1 deficiency is demonstrated by the development of breast cancer at a younger age. The median age for diagnosis of *BRCA1*-associated breast tumors is 39.5 in contrast with over the age 50 in 80% of sporadic cases (Wagner *et al.*, 1998). In a general practice, women carrying *BRCA1* mutations are advised to take annual or semiannual mammography beginning by age 25 (Burke *et al.*, 1997). Since IR is a risk factor of breast cancer (Goss and Sierra, 1998), and our data implies that *BRCA1* carriers may be more susceptible to IR-induced tumor, a safety concern on repeated screening mammography for *Brca1* carriers is raised. Recently, several large-scale studies concluded that screening mammography is not associated with a significantly increased risk of cancer formation in *Brca1* carriers (Goldfrank *et al.*, 2006; Narod *et al.*, 2006). Hence, a small dose of radiation used for diagnostic

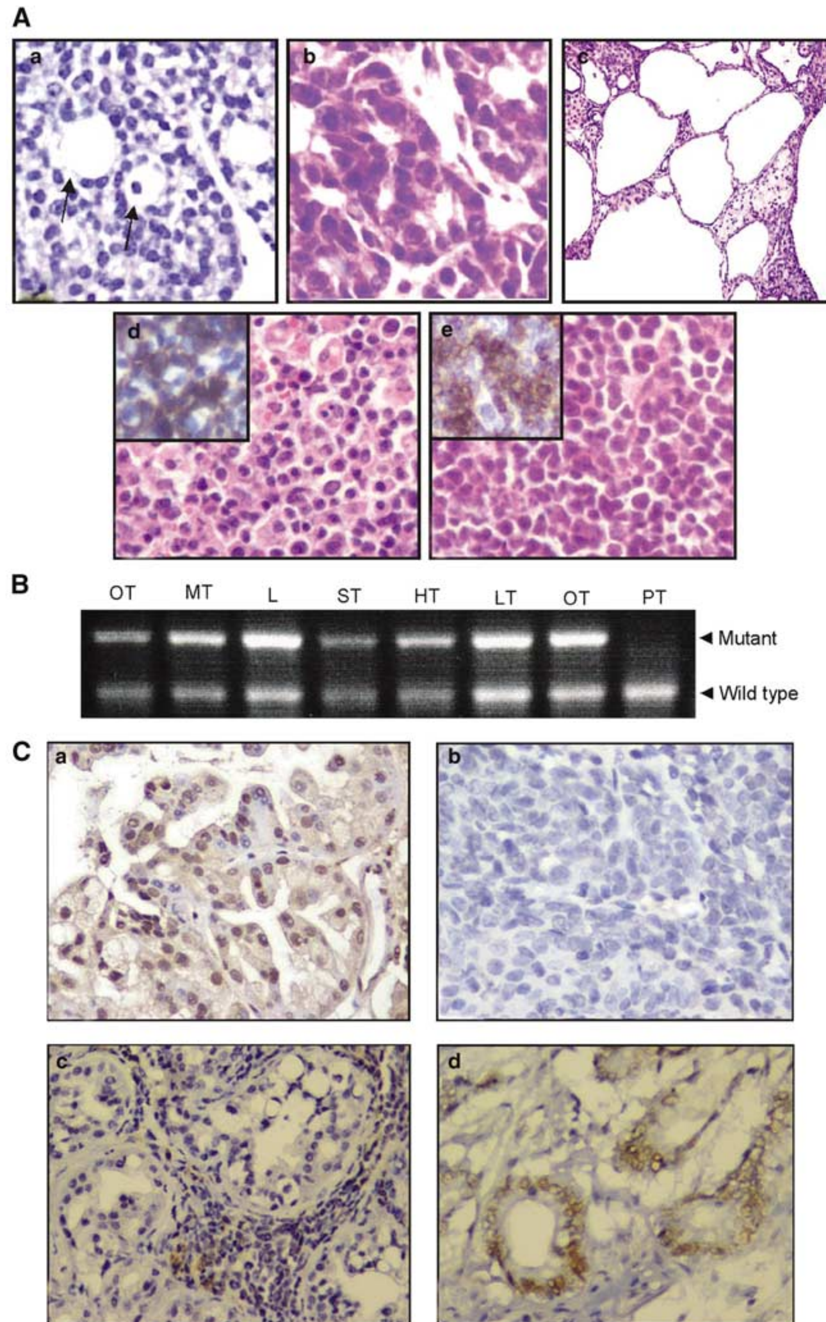


**Figure 2** (A) Histopathology of tumors developed in *Brca1* +/− mice after gamma irradiation. (a) Ovary granulosa cell tumor with Call-Exner body formation (arrow) in *Brca1* +/− mice with 2.5 Gy irradiation. (b) Ovary adenocarcinoma in Brca1 +/− mice with 5.0 Gy irradiation. (c) Ovarian cystadenoma in *Brca1* +/− mice with 5.0 Gy irradiation. (d) B-cell lymphoma. (e) T-cell lymphoma. The insets in (d) and (e) are immunohistochemical staining for CD45R for detecting B-cell origin and CD3 for detecting T-cell origin, respectively. (**B**) Test for the LOH of *Brca1* locus in *Brca1* +/− and *Brca1* +/+ mice tumor. LOH was detected by PCR using tumor DNA obtained by laser capture microdissection. All the tumors are from *Brca1* +/− with exception of pituitary tumor that is from *Brca1* +/+ mouse. OT, ovarian tumor; MT, mammary tumor; L, lymphoma; ST, stomach tumor; HT, Harderian gland tumor; LT, lung tumor; PT, pituitary tumor. (**C**) Expression of Brca1 and p53 protein in tumors. (a) An ovarian adenocarcinoma from a *Brca1* +/− mouse showed expression of Brca1 in the nuclei. (b) A breast adenocarcinoma from a *Brca1*−/−*p53*−/− mouse showed negative staining of Brca1. (c) An ovarian adenocarcinoma from a *Brca1* +/− mouse showed negative staining of p53. (d) A *p53* mutated human breast cancer showed positive staining of p53 in the nuclei of cancer cells.

purpose may not contribute substantially to the burden of breast cancer in *BRCA1* mutation carriers.

The risk of therapeutic irradiation for the secondary tumor formation in *Brca1* carriers is more obscure. Most radiotherapy protocols use a higher radiation dose than those used in our study. Germline *BRCA1* mutation carriers indeed exhibit a higher recurrence rate of another primary tumor in the contralateral breast (Verhoog *et al*., 1998; Haffty *et al*., 2002) and ovary (Metcalfe *et al*., 2005); however, this is more likely

**Table 2** Tumor spectra and incidences in *Brca1*+/− and +/+ mice with/without γ-irradiation

| *Tumor type (Gray)(No. of mice)* | Brca1+/− *(0 Gy)* (n = *20*) | Brca1+/− *(2.5 Gy)* (n = *26*) | Brca1+/− *(5.0 Gy)* (n = *28*) | Brca1+/+ *(5.0 Gy)* (n = *27*) |
|---|---|---|---|---|
| Ovary tumor | | | | |
| Solid tumor | 1 (5%)[a] | 10 (38.5%)[b] | 13 (46.4%)[c] | 3 (11.1%)[d] |
| Cystadenoma | 1 (5%) | 2 (7.7%) | 2 (7%) | 1 (3.7%) |
| Pituitary adenoma | 0 | 2 | 1 | 4 |
| Harderian gland adenocarcinoma | 1 | 3 | 5 | 2 |
| Mammary tumor | | | | |
| Fibroadenoma | 2 | 0 | 0 | 0 |
| Adenocarcinoma | 0 | 1 | 1 | 0 |
| Lung adenocarcinoma | 0 | 1 | 3 | 2 |
| Stomach adenoma | 0 | 3 | 1 | 1 |
| Liver hepatocellular carcinoma | 1 | 2 | 3 | 2 |
| Lymphoma | 10 (50%) | 13 (50%) | 14 (50%) | 10 (37.0%) |
| Sarcoma | 0 | 1 | 2 | 3 |
| Hemangioma | 0 | 2 | 2 | 1 |
| Total | 16 | 40 | 47 | 29 |

[a,b]$P = 0.008$, [a,c]$P = 0.002$, [b,d] $P = 0.021$, [c,d] $P = 0.004$. The *P*-value was calculated by $\chi^2$ test.

due to the underlying genetic defect than the DNA damage by previous regimens. A recent report demonstrated that late toxicity is not increased in 37 *Brca1* mutation carriers undergoing breast radiotherapy after a medium follow-up of 6.75 years (Shanley *et al*., 2006). Since the case number is small and radiation-induced cancers often have a long latency (Kirova *et al*., 2005), a large-scale epidemiological study should be done to clarify this issue.

Given the differences of the phenotypes of *Brca1* heterozygous mutation in mice and human, the results derived from this study may not be directly applicable to human. However, this study demonstrates a possible increase in cancer risk following radiation; therefore, a close follow-up of the development of secondary cancer should be taken into account during management of *BRCA1* mutation carriers.

## Materials and methods

### *Knockout mouse*

Animal experiments were performed under federal guidelines and approved by the Institutional Animal Care and Use Committee at UCI. To generate *Brca1* heterozygous mice, a partial deletion (amino acids 300–361) of mouse *Brca1* gene exon 11 was introduced into the genome of embryonic stem cells by homologous recombination as previously described (Liu *et al*., 1996). The knockout mice were generated in the 129O1a strain, and backcrossed extensively into the C57BL/6J genetic background. The experimental group consists of 90 *Brca1*+/− female mice divided into three groups. Each group received a single dose of whole-body irradiation, 0, 2.5 or 5 Gy, at the age of 5–6 weeks. Two groups of 30 *Brca1*+/+ (wild-type) female mice that received 0 and 5 Gy of whole-body irradiation, respectively, served as control.

### *Histology and immunohistochemistry*

Mice were killed at moribund state, and collected tissues were fixed in 4% paraformaldehyde and processed through paraffin embedding following standard procedures. Sections were stained with Mayer's hematoxylin and eosin (H&E) for histopathological examination. Immunohistochemical staining was performed following the protocol described in a Vectastain Elite ABC kit (Vector Laboratories, Burlingame, CA, USA). For antigen retrieval, slides were heated for 10 min in 10 mM citric buffer (pH 6.0) in a microwave oven. The antibodies used were CD45R/B220 (1:100) for B-cell lineage (BD Biosciences, San Diego, CA, USA), CD3 (1:50) for T-cell lineage (rabbit polyclonal, DakoCytomation, Carpinteria, CA, USA), p53 (1:100) (DO1; Santa Cruz Biotechnology, Santa Cruz, CA, USA) and a mouse polyclonal antiserum raised against amino acid residues 1400–1812 of mouse Brca1 (1:100). Two human breast cancers with p53 mutations served as positive controls for p53. A breast cancer derived from a *Brca1−/−p53−/−* mouse served as negative control for BRCA1.

### *PCR analysis on LOH*

Paraformaldehyde-fixed, paraffin-embedded tumors were sectioned and stained with H&E. The tumor cells were retrieved from slide by laser capture microdissection for analysis of the allelic change of *Brca1*. PCR was performed for 40 cycles with a pTC-100 thermal controller (MJ Research Inc., Miami, FL, USA) under the following reaction condition: denaturing temperature at 94°C for 30 s, annealing temperature at 65°C for 1 min and elongation temperature at 72°C for 1 min. To detect the mutant allele with targeted deletion of exon 11, a 236 bp product was obtained using a sense oligonucleotide, 5′-TGATATTGCTGAAGAGCTTGGCGGC-3′ and an antisense oligonucleotide, 5′-TGGGAGTGGCACCTTCCAGGG TCAA-3′, within the *pgkneopA* cassette. To detect the wild-type allele, a 150-bp product was obtained using a sense oligonucleotide, 5′-AACAGCCTGGCATAGCAGTGAGCCA-3′, and antisense oligonucleotide, 5′-TTGCGGGTGAGTCCACTT CTCTCTA-3′ within exon 11 of *Brca1*.

### *Statistical analysis*

The analyses were performed using StatCalc for Windows software (EpiInfo Version 3.3.2, Centers for Disease Control and Prevention, Atlanta, GA, USA). Tumor incidence according to pathological diagnosis was compared among the four groups by $\chi^2$ test. Survival analysis was performed by the Kaplan–Meier method. Statistical significance was assessed using Student's *t*-test and log-rank sum test. A value of $P < 0.05$ was considered statistically significant.

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