Development of thymic lymphomas in mice disrupted of *Brca2* allele in the thymus

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Abstract

Germ-line mutations in BRCA2 predispose to early-onset cancer. Homozygous mutant mouse, which has *Brca2* truncated in exon 11 exhibit paradoxic occurrence of growth retardation and development of thymic lymphomas. However, due to its large embryonic lethality, cohort studies on the thymic lymphomas were not feasible. With the aid of *Cre-loxP* system, we demonstrate here that thymus-specific disruption of *Brca2* allele without crossing it to p53-mutant background leads to the development of thymic lymphomas. Varying from 16 weeks to 66 weeks after birth, 25% of mice disrupted of *Brca2* in the thymus died of thymic lymphomas, whereas previous report did not observe lymphomagenesis using similar *Cre-loxP* system. Future analysis of thymic lymphomas from these mice presented here will provide information on the cooperative mutations that are required for the *BRCA2*-associated pathogenesis of cancer.

Keywords: BRCA2; mice; knockout; Cre recombinase; lymphoma; T cells

Introduction

Individuals who inherit one mutant allele of *BRCA2* are predisposed to early-onset breast cancer (Wooster *et al.*, 1994; Gaythery *et al.*, 1997). Studies on the functions of BRCA2 revealed that it was involved in double-strand DNA break (DSB) repair via binding to Rad51 recombinase (Patel *et al.*, 1998; Venkitaraman, 2003, 2004). Involvement of BRCA2 in DSB repair has been first evidently revealed by metaphase chromosome spreads: Brca2-deficient mouse embryonic fibroblasts (MEFs) manifest chromosome structure instability such as aberrant chromosome breaks, exchanges, and random translocations because they lack the ability to repair spontaneous DNA lesions (Patel *et al.*, 1998). Following studies succeeded in demonstrating the mechanism by which BRCA2 regulates Rad51 and DNA repair using *in vitro* biochemical studies (Davies and Pellegrini, 2007) and structural studies as well (Pellegrini *et al.*, 2002; Yang *et al.*, 2002; Huang *et al.*, 2006).

Notably, chromosome instability manifested by the dysfunctional BRCA2 is not restricted to chromosome structure instability but involves chromosome number instability, aneuploidy, as well, suggesting that it possess multiple functions including cell cycle regulation in order to keep the genome intact. Indeed studies *in vivo* suggest that BRCA2 participates in embryonic development (Suzuki *et al.*, 1997), regulation of T cell apoptosis (Cheung *et al.*, 2002), mitotic cell cycle regulation (Lee *et al.*, 1999; Lee, 2003), and cytokinesis (Daniels *et al.*, 2004). Given the multiple roles *in vivo*, understanding the pathogenesis of BRCA2-mutated tumorigenesis inevitably requires detailed studies at the animal level.

In order to understand BRCA2-associated cancer development *in vivo*, several kinds of mouse models were designed. First, targeted disruption of BRCA2 allele resulted in embryonic lethality at embryonic day 6.5 (Ludwig *et al.*, 1997; Sharan *et al.*, 1997; Suzuki *et al.*, 1997). The large exon11 is conserved between mouse and human. Importantly, this region is frequently mutated in ovarian cancers. When exon11 is targeted, embryonic survival was prolonged and some mice survived to birth. These mice exhibited paradoxic occurrence of growth retardation and thymic lymphoma; they were smaller in size compared to wild-type and succumbed to death at 12 weeks after weaning due to development of thymic lymphoma (Connor *et al.*, 1997; Friedman *et al.*, 1998). Notably, exon 11 contains 8 BRC repeats that are capable of binding to Rad51 and maintains monomeric Rad51 (Davies *et al.*, 2001; Pellegrini *et al.*, 2002). Mice which survived to birth retained at least three BRC repeats, which are considered to have a partial activity in Rad51-binding, indicating that the mutation in these

mice is hypomorphic. Although the embryonic survival was prolonged and some mice survived to birth, the numbers of surviving Brca2[Tr] mice were small and therefore analyses on the lymphomas were limited.

In order to overcome these limitations, Bern's group succeeded in generating conditional knockout mice using *Cre-loxP* system (Jonkers *et al.*, 2001). In this mouse, exon 11 of Brca2 is flanked by loxP sites, which was also the targeting site for Brca2[Tr] mice that developed tumor (Friedman *et al.*, 1998). With this conditional knockout mouse model, they recapitulated and confirmed the previous study (Lee *et al.*, 1999) that p53 inactivation is required for BRCA2-mutant cells to develop epithelial cancer. Using similar loxP-floxed *Brca2* allele, another group studied on the role of *BRCA2* in the thymus. The result showed that BRCA2 regulated T cell apoptosis but they failed to detect T cell lymphoma unless they were crossed to p53-mutant background (Cheung *et al.*, 2002).

Despite the low cohort, all the Brca2[Tr] mice that survived to birth developed thymic lymphoma (Friedman *et al.*, 1998). Because bigger cohort study on Brca2-disrupted T cell lymphomas and analysis on T cell lymphomas at the molecular level was required, we generated mice that were conditionally disrupted of *Brca2* allele in the thymus. For this aim, we crossed Brca2[F11] (Jonkers *et al.*, 2001) mice with *Lck-Cre* mice. Mouse positive for both *Brca2[F11]* allele and *Cre* (*BRCA2[F11/F11];Lck-Cre*) will lose *Brca2* in the thymus. Here, we report the tumor formation, their incidence and analysis on the cultured thymic lymphoma. The T cell lymphomas derived from Brca2- deficient thymus will be of valuable source in understanding the molecular mechanism of tumorigenesis in Brca2-deficient background.

Materials and Methods

Animal experiments

To generate mice that were disrupted of Brca2 allele specifically in the thymus, Brca2[F11] mice (Jonkers *et al.*, 2001) were crossed with *Lck-Cre* mice. Through continuous mating and screening using PCR, statistically meaningful number of each line were collected and observed for their phenotype. Each mice were observed everyday, and when they died, they were examined immediately and performed series of experiments. All experiments performed were approved by Institutional Animal Care and Use Committees (IACUC) of Seoul National University. We strictly followed the guidelines of Policy and Regulation for the Care and Use of Laboratory Animals.

PCR genotyping

Mouse tail for genotyping was cut and incubated with tail-lysis Buffer [50mM Tris, 100 mM EDTA, 1% SDS, 200 μg/ml of Proteinase K (Sigma)] for 16 h in 55°C. Supernatants were separated by centrifugation and phenol-chloroform purified. Four different primers were used to screen *loxP*-flanked alleles. Primer 10F (GGCTGTCTTAGAACTTAGGCTG) and primer 10R (CTCCACACATACATCATGTGTC) are at the 10th intron of *BRCA2*. Primer 11F (CTCATCATTTGTTGCCTCACTTC) and 11R (TGTTGGATACAAGGCATGTACAC) locate at intron 11 of *BRCA2*. For screening *Lck-Cre* mice, Cre-5 primer (CGATGCAACGAGTGATGAGGTTC) and Cre-3 primer (GCACGTTCACCGGCATCAAC) were used (Jonkers *et al.*, 2001).

Isolation and culture of T cells from thymus

In order to analyze T cells from thymus, thymuses of control mice and Brca2[F11/F11] mice were surgically isolated. Thymus was flushed several times with RPMI supplemented with 10% FBS and 50 μM of β-mercaptoethanol using 2 ml syringe without needle. Then the cells flushed from the thymus were cultured in the media (RPMI, supplemented with 10% FBS, 50 μM of β-mercaptoethanol) provided with recombinant IL-2 (BioLegend, San Diego) and IL-7 (BioLegend, San Diego) before analysis for flow cytometry.

Flow cytometric analysis

To analyze CD4 and CD8 populations, T cells adducts from dead mice were mashed. 10^6 cells of mashed cellular adducts were stained with FITC-conjugated mouse monoclonal CD4 antibody (BioLegend, San Diego) and PE-conjugated mouse monoclonal CD8b antibody (BioLegend, San Diego) for 45 min at 4°C. Stained cells were processed by FACSCalibur (BD Biosciences, NJ) and analyzed with CellQuest Program (BD Biosciences, NJ).

Results

T cell-specific disruption of *Brca2* allele

BRCA2[F11] allele has two *loxP* sites flanking exon 11. Only when Cre recombinase is expressed, it would induce the excision of exon 11 in this allele. Otherwise, the inserted *loxP* sites would not harm the allele (Figure 1A). Primers were designed to distinguish the excised exon 11 from wild-type allele

or the unexcised loxP-flanked allele (Jonkers *et al.*, 2001). Using the PCR screening method with the primers indicated, we were able to genotype for Brca2 allele. *Brca2^{F11/+}* mice were crossed with *Lck-Cre* transgenic mice to specifically excise *Brca2* allele in thymic T cells. PCR screening showed that *Brca2^{F11}* allele could be disrupted and analyzed (Figure 1B). Since we were specifically interested in tumor formation by disrupting Brca2, we chose to backcross the mice for over 10 generations to FvB strain, which is comparatively tumor-prone (Woodworth *et al.*, 2004). *Lck-Cre* mice, which express Cre recombinase that recognizes *loxP* sites only in thymus, were also backcrossed to FvB for more than 10 generations. Through continuous mating and screening by PCR (Figure 1B), we collected statistically meaningful number of each mouse line and observed for their phenotype.

Reduced survival rate in *BRCA2^{F11/F11}*; *Lck-Cre* mice compared to control mice

In humans, tumors develop with the loss-of-heterozygosity (LOH). In mouse models for cancer, LOH is rarely observed. Because LOH of tumor su-

ppressors is an important issue in cancer development, we observed the heterozygous mouse carefully. The result indicated that there was no sign of LOH in heterozygotes or did they have distinguishable phenotype compared to wild-type (data not shown). Heterozygous *BRCA2^{wt/F11}*; *Lck-Cre* mice behaved normally just like the wild-type. Previous studies on several Brca2-mutant mice also indicate that the heterozygous do not have obvious phenotype compared to wild-type mice (Connor *et al.*, 1997; Ludwig *et al.*, 1997; Sharan *et al.*, 1997; Suzuki *et al.*, 1997; Friedman *et al.*, 1998; Cheung *et al.*, 2002).

In comparison, although *BRCA2^{F11/F11}*; *Lck-Cre* mice survived to birth like the wild-type or the heterozygote, the survival rate was reduced in 70-week observation after birth (Figure 2). Survival rate of *BRCA2^{F11/F11}*; *Lck-Cre* mice were compared with those of wild-type, *BRCA2^{wt/F11}*; *Lck-Cre*, *Lck-Cre*, and *BRCA2^{F11/F11}*. Unless homozygous *BRCA2^{F11/F11}* mice are crossed with *Lck-Cre* mice, *BRCA2* allele is not disrupted, therefore all the others could serve as controls. When the survival rate was monitored, we found that only 2 mice out of 40 control mice died when monitored for 70 weeks from birth. Death in control mice were observed at 32 weeks and 58 weeks, respectively, therefore we concluded that they lived up to their normal life span. In comparison, the death rate observed was 25% for 70-week survival in *BRCA2^{F11/F11}*; *Lck-Cre* mice. The time points of death were widespread from 16 weeks to 66 weeks after birth (Figure 2).
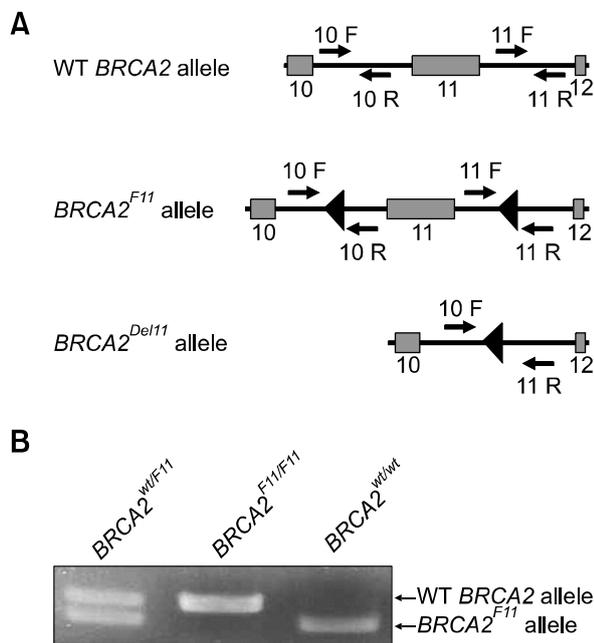

Figure 1. T-cell specific disruption of *BRCA2* using *Cre-loxP* recombination system. (A) Schematic representation of wild type and mutated *BRCA2* alleles. Two *loxP* sites as triangle flanks exon 11 of *BRCA2* (*BRCA2^{F11}*). Recombination by *Cre* recombinase can induce excision of exon 11 (*BRCA2^{Del11}*). Small arrows indicate PCR primer sites for mouse genotyping. (B) General genotyping pattern using PCR screening. Using primer 10F and 10R in Figure 1A, wild-type allele is represented as 298 bp band, but band of *BRCA2^{F11}* is shifted to 376bp, since additional *loxP* sequences add up.
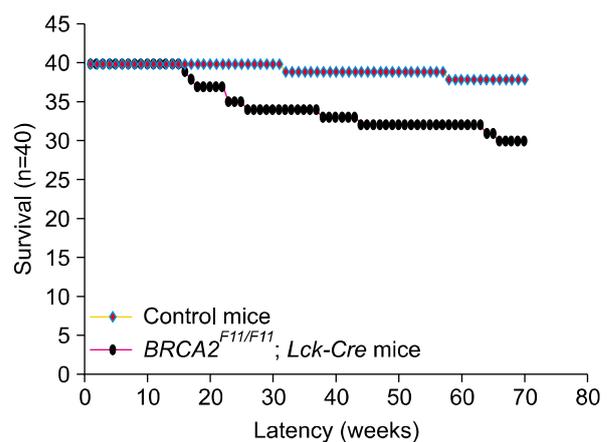

Figure 2. Survival rate of *BRCA2F^{11/F11}*; *Lck-Cre* mice and control mice. T-cell specific disruption of *BRCA2* leads to low viability in comparison with that of control mice. While only 2 control mice were died naturaly (red square) during 70 weeks, 10 mice of *BRCA2^{F11/F11}*; *Lck-Cre* mice died of thymic lymphoma (black circle). The timing of death is widespread (from 16 weeks to 66 weeks). Control mice include wild type mice, *Lck-Cre* mice and *BRCA2^{wt/F11}*; *Lck-Cre* mice. Forty mice were analyzed each.

Development of thymic lymphomas in *Brca2^F11/F11* mice

Next we analyzed for the cause of death in *BRCA2^F11/F11*; *Lck-Cre* mice. Because we engineered to disrupt *Brca2* allele in the thymus in order to analyze cohort of thymic lymphomas, we first examined for any sign of abnormality in the thymus. Thymus from 26-week old wild-type mice was white, normal sized, and well attached to the heart (Figure 3A). In contrast, thymus of dead *BRCA2^F11/F11*; *Lck-Cre* mice of similar age were severely swollen and formed tough cellular adducts, which is the evident sign of lymphoma formation. This enlarged thymus was big enough to cover all around the heart (Figure 3B and C). Using primer 10F and 11R,

we confirmed that *Brca2* allele was effectively disrupted from the thymus of *BRCA2^F11/F11*; *Lck-Cre* mice (Figure 3D). This result indicates that *BRCA2^F11/F11*; *Lck-Cre* mice died of thymic lymphoma. The causes for the death of two mice in control group were unrelated and did not show any sign of thymic lymphoma formation.

In order to analyze the T cell populations in the thymic lymphomas, we cultured the suspension cells from the swollen thymus and analyzed for their CD4 and CD8 profiles (Figure 4). If they were thymic lymphomas, the CD4 and CD8 profiles will differ from that of normal thymus; it is likely that they form monoclonal populations. Thymus is the place for early T-cell development and maturation.
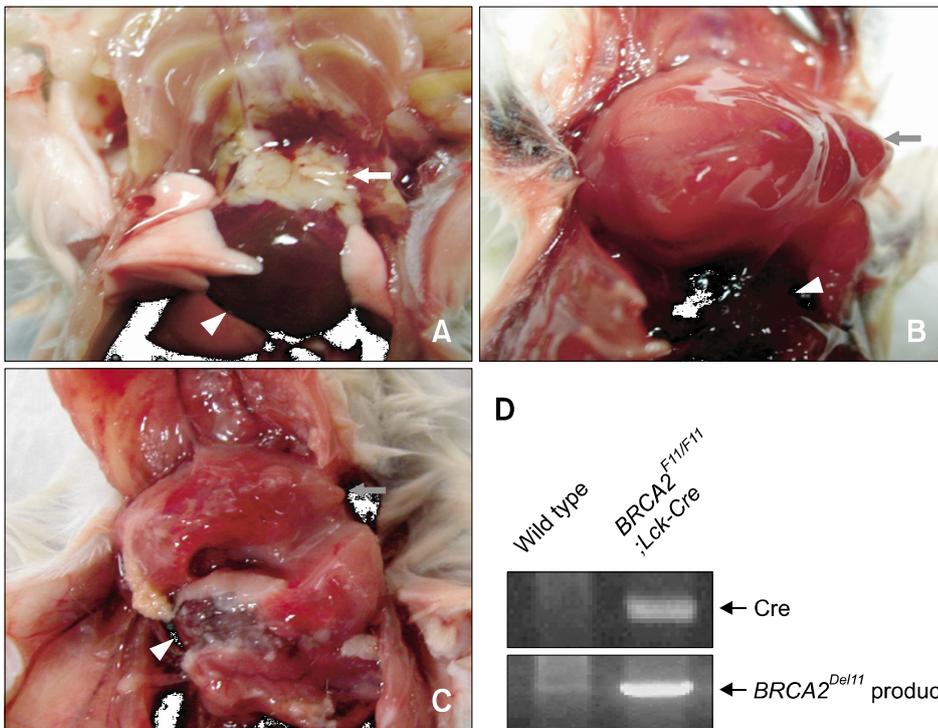

Figure 3. The thymuses of dead *BRCA2^F11/F11*; *Lck-cre* mice were swollen and formed cellular adducts. All of 10 dead *BRCA2^F11/F11*; *Lck-cre* mice developed thymic lymphomas. (A) Wild type thymuses are white and small. (B, C) Thymuses of dead *BRCA2^F11/F11*; *Lck-cre* mice are swollen and large that they could cover all the way to surround the heart. These thymuses formed tough cellular adducts. (D) Using PCR screening with primer 10F and 11R (Figure 1A), we confirmed that *BRCA2* was disrupted along with the expression of Cre in thymic adducts of dead *BRCA2^F11/F11*; *Lck-cre* mice. Triangle indicates heart, white arrow indicates normal thymus, and gray arrow indicates thymic adduct.
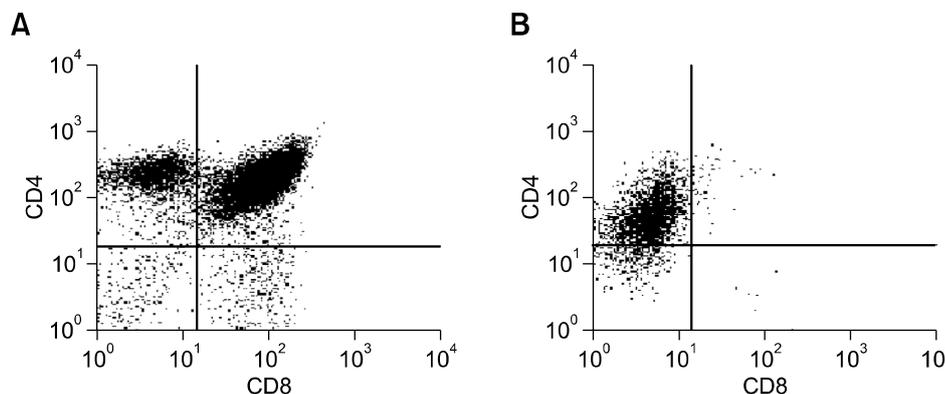

Figure 4. CD4 and CD8 property in thymus of dead *BRCA2^F11/F11*; *Lck-cre* mouse. T cells in thymus from one of the dead *BRCA2^F11/F11*; *Lck-cre* mice were stained with CD4-FITC and CD8-PE for flow cytometric analysis. (A) Wild-type mice show normal profile of T cell populations in the thymus; CD4 single positive, CD8 single positive, double positive and double negative cells. (B) T cells from dead *BRCA2^F11/F11*; *Lck-Cre* only contain CD4 single positive cells, suggesting that it is a monoclonal thymic lymphoma.

Therefore, normal thymus contains all four types of T cell repertoire; double positive, double negative, CD4 single popsitive or CD8 single positive cells. As anticipated, T cells from wild-type mice showed all four populations (Figure 4A). In contrast, thymus from the dead $BRCA2^{F11/F11}$; *Lck-Cre* mouse contained only CD4 single positive population, indicating that they were monoclonal tumor cells (Figure 4B). This result is in agreement with the previous report that thymoma from mice with truncated *Brca2* allele contains monoclonal populations (Lee, 2003). Taken together, disruption of *Brca2* allele in the thymus promotes T cell lymphoma.

Discussion

We have shown here that thymic T cell-specific disruption of *BRCA2* using *Cre-loxP* system leads to thymic lymphoma. Our results correlate with previous study in Brca2$^{Tr/Tr}$ mice where *Brca2* allele is disrupted from the germ-line (Connor *et al.*, 1997; Friedman *et al.*, 1998; Patel *et al.*, 1998; Lee *et al.*, 1999). However, similar study by Cheung and colleagues has drawn a different conclusion (Cheung *et al.*, 2002). The report suggested that T cell-specific disruption of *BRCA2* itself was not sufficient to induce T cell lymphoma but exhibited aberrant chromosomes and increased apoptosis in response to DNA damage (Cheung *et al.*, 2002). The difference may be due to different targeting site between two mice or different genetic background. The mice that were used in our experiments had targeted exon 11 of Brca2 (Jonkers *et al.*, 2001), whereas the mice that were used by Cheng and colleagues had targeted exon 9 and 10 of Brca2 allele (Cheung *et al.*, 2002). It should be emphasized that the very large exon 11 of Brca2 contains 6~8 BRC repeats, which are implicated in Rad51 binding and modulating DNA repair. Therefore, we speculate that targeting exon 11 but not the other regions provided the basis of better survival and genetic instability. Also, we used FvB strain which has increased incidence for tumor formation (Woodworth *et al.*, 2004), whereas Cheung and colleagues used 129/C57BL/6 or C57BL/6 (Cheung *et al.*, 2002). Nevertheless, our results indicate that mutation in *Brca2* in T cells predispose the cells to acquire higher rate mutations, leading to cancer. Therefore, these results indicate that the first hit in *BRCA2* mutation is necessary for the initiation of genetic instability. Using this mouse model, we will be able to identify genes that cooperate with *BRCA2* on the road to cancer.

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