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Impaired phosphorylation and mis-localization of Bub1 and BubR1 are responsible for the defective mitotic checkpoint function in Brca2-mutant thymic lymphomas

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Abbreviations: BRCA2, human BRCA2; Brca2, murine BRCA2

Abstract

Breast cancer susceptibility gene, *BRCA2*, is a tumor suppressor and individuals who inherit one defected copy of *BRCA2* allele experience early onset breast cancer or ovarian cancer accompanied by the loss of the wild type allele. Mouse model for Brca2 mutation shows growth retardation and paradoxical occurrence of thymic lymphomas. Thymic lymphomas from Brca2-mutant mice harbor mutations in *p53*, *Bub1*, and *BubR1*, which function as mitotic checkpoint proteins. Therefore, interplay between Brca2 and mitotic checkpoint has been suggested in the maintenance of genetic fidelity, although it has not been assessed whether the unique mutations in *Bub1* and *BubR1* found in Brca2-mutant mice are responsible for the abolishment of mitotic checkpoint function. This report demonstrates that Bub1 and BubR1 mutant proteins from Brca2$^{-/-}$ thymic lymphomas have defects in the phosphorylation and kinetochore localization after spindle damage. Thus, the mutations of Bub1 and BubR1 found in Brca2-mutant mice indeed are responsible for the chromosome instability in Brca2-mutated tumors.

Keywords: breast neoplasms; BRCA2 protein; mitosis; lymphoma; tumor suppressor proteins; T-cell; tumor

Introduction

Individuals who inherit one defective copy of BRCA2 gene are predisposed to early onset breast cancer and ovarian cancer (Wooster *et al*., 1995; Tavtigian *et al*., 1996). Tumor cells from predisposed individuals usually contain mutations in both alleles (Collins *et al*., 1995; Gudmundsson *et al*., 1995), thus BRCA2 works as a tumor suppressor. The function of BRCA2 has been largely ascribed to its role in DNA repair. BRCA2 binds to Rad51, a RecA homologue involved in homologous recombination and double-strand break repair (Venkitaraman, 2001; 2002). Murine cells homozygous for Brca2 truncations in exon 10 (Ludwig *et al*., 1997; Sharan *et al*., 1997; Suzuki *et al*., 1997) or exon 11 (Connor *et al*., 1997; Friedman *et al*., 1998) exhibit hypersensitivity to γ-irradiation, although checkpoint response to DNA damage is largely intact (Patel *et al*., 1998).

Mice homozygous for *Brca2* truncations exhibit growth retardation due to activation of checkpoint response (Patel *et al*., 1998). Paradoxically, they develop thymic lymphomas (Connor *et al*., 1997; Friedman *et al*., 1998), and it has been shown previously, that the tumorigenesis in Brca2$^{Tr/Tr}$ mice involves inactivation of mitotic checkpoint function and exhibit chromosomal instability (Lee *et al*., 1999). Thus, it is likely that inactivating mutations in mitotic checkpoint genes cooperate with BRCA2 deficiency in the pathogenesis of inherited breast cancer.

Mitotic checkpoint, also called spindle assembly checkpoint, delays the onset of anaphase until all chromosomes are attached with spindle, and ensures proper chromosome segregation to the daughter cells (Rudner and Murray, 1996; Taylor, 1999). Mitotic checkpoint is activated by the presence of unattached kinetochores with spindle (Rudner and Murray, 1996). Lack of microtubule attachment or lack of tension at the kinetochore activates phosphorylation of kinases that activate components of mitotic checkpoint, Bub1, Bub3, Mad 1-3, and Mps1 (Rudner and Murray, 1996), at the kinetochore. Chromosome loss or gain due to defects in mitotic checkpoint can kill cells, cause birth defects or contribute to tumorigenesis.

Progression to cancer required gross changes in genetic information. Calculations using human non-germline cells show the mutation rate of 10^{-7} per gene per cell generation (Loeb, 1991). However, the appearance of cancer cells required mutations of half-dozen or more mutations in cellular genes, which directly or indirectly affect proliferation. Hence, with the normal mutation rate, tumor formation is impos-

sible during a human life time. Dysfunctional BRCA2 enforces mutagenic repair (Venkitaraman, 2001) that activates checkpoint response and apoptosis. Surviving cells that acquired mutations in mitotic checkpoint function are exposed to another level of change in genetic information, aneuplody, resulting from uncontrolled chromosome segregation. Therefore, cooperation of truncation in *Brca2* and mutation in mitotic checkpoint results in gross change in genetic information that in effect achieves high mutability and progress to early onset breast and ovarian cancer.

Previous report showed that the tumors from Brca2-deficient mouse had defects in mitotic checkpoint and concurrently found mutations in *p53*, *Bub1*, and *BubR1*. However, whether the unique *Bub1* and *BubR1* mutations found in Brca2-deficient mouse contribute to neoplastic transformation has not been formally assessed in the previous report. In this report, a Brca2-deficeint T cell lymphoma showing defects in the phosphorylation of mitotic checkpoint components Bub1 and BubR1 is described. Furthermore, another Brca2-deficeint thymic lymphoma showed mis-localization of Bub1 in response to microtubule disruption, demonstrating that mitotic checkpoint is indeed not functioning in Brca2-mutant tumors. These results strongly support the notion that mitotic checkpoint inactivation accelerates the chromosome instability in *BRCA2*-mutant cells.

Materials and Methods

Culture of thymic lymphomas in Brca2$^{Tr/Tr}$ mice

The *Brca2*$^{Tr/Tr}$ homozygous mice that survived weaning developed thymic lymphomas between 12 to 14 weeks of age. Single cell suspensions were made from each thymus by passing through the syringe without a needle. Cells were cultured with RPMI medium containing 50 nM of β-mercaptoethanol, 10% FBS, IL-2 (Roche Biochemicals, Germany) according to the recommended concentration. The next day, cells were washed by centrifugation in 1,000 rpm for 5 min. The pellet was once again resuspended in RPMI medium described above. After several weeks of culture in the cytokine-containing medium, cells were tested of their viability in culture in the presence or absence of cytokines by the MTT assay (Roche Biochemicals).

Western blot analysis of Bub1 and BubR1

2×10^4 cells were either treated or untreated with 200 ng/ml nocodazole for 24 h and subjected to Western blotting. Human monoclonal antibody to BubR1 or Bub1 were kind gifts of Dr. Frank MaKeon and Dr. Stephen Taylor (Harvard Medical School). For loading control, the blot was reprobed with anti-β-actin (Ab-2, Calbiochem).

Immunohistochemistry

Suspension cell cultures were treated with 250 ng/ml of nocodazole for 24 h before fixation. At the indicated time point, suspension cells were put onto the poly-L-lysine (Sigma) coated cover slips. Nocodazole treated cells were rinsed twice in room temperature with phem (60 mM Pipes, 25 mM Hepes, pH 7.0, 10 mM EGTA, 4 mM $MgSO_4$) (phem). Cells were extracted for 5 min in 0.5% triton X-100 (Sigma) in phem. Then cells were fixed with 1% paraformaldehyde, freshly prepared in phem for 15 minutes followed by rinsing twice in mbst (10 mM Mops, pH 7.3, 150 mM NaCl, 0.05% Tween-20 (mbst)). Blocking was processed in 20% boiled goat serum in mbst for 1 h followed by 1:100 dilution of Bub1 antibody incubation. After antibody staining, cells were counter-stained with propidium iodide for visualization of DNA. Localization of Bub1 was visualized by the observation under the confocal microscopy using Bio-Rad MRC 1,000.

Results and Discussion

Previously, using Brca2$^{Tr/Tr}$ mice (Friedman *et al*., 1998), we have shown that mitotic checkpoint inactivation overcomes growth retardation in Brca2$^{Tr/Tr}$ MEFs and fosters transformation. Furthermore, mutations in *p53*, *Bub1*, or *BubR1* are found in Brca2$^{Tr/Tr}$ thymic lymphomas, indicating that mitotic checkpoint function is lost (Lee *et al*., 1999). However, it has not been assessed in which way mutations in *Bub1* and *BubR1* affect mitotic checkpoint response, and whether they are dominant negative mutants.

To address this issue, analysis on the status and function of mitotic checkpoint proteins Bub1 and BubR1 were performed in Brca2$^{Tr/Tr}$ thymic lymphomas. First, cells isolated from three independent Brca2$^{Tr/Tr}$ thymus were cultured for 5 days and analyzed for their surface markers. The cells display markers characteristic of an early stage of thymic T cell development (Figure 1). They are positive for TCRβ and Thy1, indicating that they are T-cell originated. In addition, when these cells were stained with CD4 and CD8 antibodies, they showed negative staining for CD4, but stained weakly with CD8 (CD4$^-$ CD8^{low}; lymphoma A) (Figure 1). Second and third thymic lymphomas isolated from different Brca2$^{Tr/Tr}$ mice revealed that one was CD4 single positive (CD4$^+$ CD8$^-$; lymphoma B), and the other was CD8 single positive (CD4$^-$ CD8$^+$; lymphoma C) (data not shown). These data suggest that *Brca2* disruption in mice did not interfered with early T cell differentiation since the thymic lymphomas were monoclonal and showed dif-

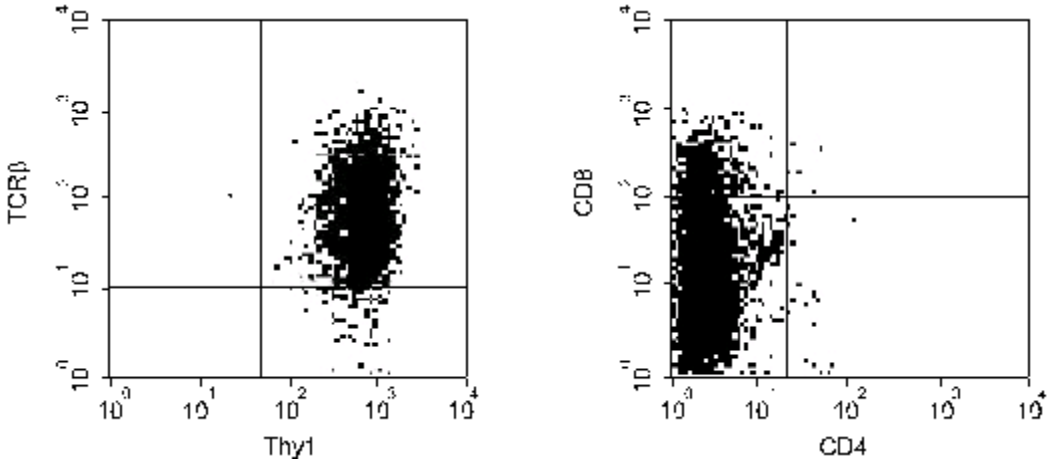

Figure 1. Cells isolated from Brca2$^{Tr/Tr}$ thymus show markers characteristic of early T cell differentiation.

ferent stages of T cell differentiation. Unlike normal thymocytes, they divide continuously in culture independent of cytokine growth factors (data not shown).

As discussed in the previous report (Lee *et al*., 1999), all three of Brca2$^{Tr/Tr}$ thymic lymphomas used in this study, exhibit defects in mitotic checkpoint function after spindle disruption. Karyotyping of these thymic lymphomas revealed that Brca2$^{Tr/Tr}$ lymphoma cells exhibit aberrant chromosome number and structure, characterized as chromosome instability (Lee *et al*., 1999). Interestingly, sequencing result revealed that one mutated *Bub1* allele and wild-type *BubR1* are found in lymphoma A and lymphoma B. Lymphoma C has wild type *Bub1* and one mutated allele of *BubR1* (Lee *et al*., 1999). In order to test if *Bub1* and *BubR1* mutations in Brca2$^{Tr/Tr}$ thymic lymphomas are responsible for the inactivation of mitotic checkpoint function, phosphorylation status was investigated after nocodazole treatment. Bub1 kinase is autophosphorylated upon spindle disruption, or lack of tension at the kinetochore, in yeast (Roberts *et al*., 1994; Farr and Hoyt, 1998), and the autophoshprylation is required for its kinase activity (Roberts *et al*., 1994; Taylor *et al*., 1998). Therefore, the phosphorylation status of Bub1 in Brca2$^{Tr/Tr}$ thymic lymphomas was tested to ask if it is functional. Upon nocodazole treatment, in control Pim-1 T cell lymphomas (Jonkers *et al*., 1997), up-shifted phosphorylated Bub1 band was detected by Western blotting with a monoclonal antibody to murine Bub1 (Taylor and McKeon, 1997) (Figure 2). Phosphatase treatment revealed that the shifted band is indeed a product of phosphorylation (data not shown). In lymphoma A and C, phosphorylated band was also detected upon nocodazole treatment, suggesting that Bub1 phosphorylation after spindle disruption is intact in these cells (Figure 2). However, in lymphoma B, the level of Bub1 was

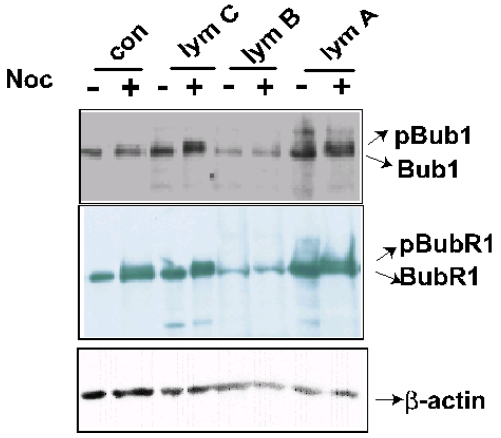

Figure 2. Mitotic checkpoint proteins Bub1 and BubR1 in Brca2$^{Tr/Tr}$ cells show altered response to microtubule disruption. Bub1 and BubR1 in control cells (lymphomas from Pim-1 transgenic), lymphoma C, and lymphoma A show phosphorylated forms (shifted bands) upon nocodazole treatment. The level of β-actin in the lower panel indicates that the protein amounts loaded on each lane are approximately equal.

significantly low and phosphorylation was lost upon nocodazole treatment. Reprobing the blot with antibody to β-actin revealed that similar amounts of protein were loaded (Figure 2). Therefore, it is likely that the machinery that controls Bub1 level and phosphorylation is altered in lymphoma B. Since lymphoma A and B both have one mutated allele of *Bub1*, and yet Bub1 phosphorylation in lymphoma A is intact, it is speculated that an unknown mechanism in lymphoma B is deregulated that in effect resulted in a low level of Bub1 and inability of phosphorylation (Figure 2).

BubR1 has been cloned by its ability to bind to Bub3 (Taylor *et al*., 1998). Simultaneously, its muta-

tion has been found in certain colon cancer cell lines that exhibited chromosome instability (Cahill *et al*., 1998). Of note, mutation in *BubR1* is also found in Brca2$^{Tr/Tr}$ lymphomas. To ask if similar defect in phophorylation is present in Brca2$^{Tr/Tr}$ lymphomas, the blot used to detect Bub1 was reprobed with antibody to BubR1. Since the antibody to BubR1 does not cross-react with Bub1 (personal communications with Dr. S. Taylor), the blot shown in Figure 2 reveals the level of murine BubR1. Comparing with control Pim-1 lymphoma (Jonkers *et al*., 1997), phosphorylation of BubR1 upon spindle disruption is more or less intact in lymphoma C (Figure 2, middle panel). High level of BubR1 was detected in lymphoma A, and the phosphorylation of BubR1 upon nocodazole treatment was intact. Interestingly, BubR1 level was 10th the level in lymphoma B and the phosphorylation after microtubule disruption was absent, as Bub1 was (Figure 2). Note that sequencing result showed that *BubR1* in lymphoma B was wild type (Lee *et al*., 1999). Taken together, it is very likely that an unidentified mechanism which regulates Bub1 level and phosphorylation is impaired in lymphoma B. And as a result, BubR1 level and phosphorylation is impaired, since BubR1 is likely to act downstream of Bub1 (Rudner and Murray, 1996; Taylor, 1999; Taylor *et al*., 1998). It is speculative that at least in lymphoma B, defect in phosphorylation of Bub1 and BubR1 that governs the activation of mitotic checkpoint kinases, is responsible for inactivation of mitotic checkpoint function in Brca2$^{Tr/Tr}$ thymic lymphoma.

Mitotic checkpoint proteins function at the kinetochore since they sense and check if all the chromosomes are aligned at the metaphase plane and attached with spindles, before the onset of anaphase (Rudner and Murray, 1996). One way to detect defective mitotic checkpoint function is to test whether the protein correctly localizes to the kinetochores when microtubule spindles are disrupted. It has been shown that 3F3/2 staining is absent in Brca2$^{Tr/Tr}$ lymphomas after spindle disruption (Lee *et al*., 1999). 3F3/2 anti-phosphoepitope antibody recognizes kinetochores that are not attached to the spindle (Campbell and Gorbsky, 1995; Nicklas *et al*., 1995; Daum *et al*., 2000). Thus, lack of 3F3/2 staining at the kinetochore after nocodazole treatment supports the notion that mitotic checkpoint is not functioning in Brca2$^{Tr/Tr}$ lymphomas. However, 3F3/2 staining does not directly speak to the functionality of Bub1 and BubR1, since the epitope detected by 3F3/2 is not Bub1 nor BubR1 (Daum and Gorbsky, 1998; Daum *et al*., 2000).

To directly test if mutant Bub1 in Brca2$^{Tr/Tr}$ lymphoma cells were functional, lymphoma A were treated with nocodazole and stained with anti-Bub1. As seen in Figure 3, Bub1 colocalized with propidium iodide stained DNA when treated with nocodazole in

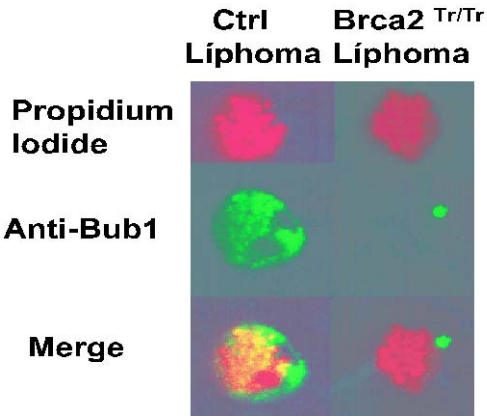

Figure 3. Mis-localization of Bub1 in Brca2$^{Tr/Tr}$ lymphoma A after nocodazole treatment. Nocodazole treatment in control cells causes co-staining of Bub1 (green in color) with DNA stained by propidium iodide (colored in red). In contrast, in Brca2Tr/Tr lymphoma A, Bub1 fails to localize to the kinetochore.

control Pim-1 transgenic lymphoma cells. In contrast, propidium iodide and Bub1 staining is not merged into one picture in Brca2$^{Tr/Tr}$ thymic lymphoma. This result shows that the genetic mutation in Bub1, found in Brca2$^{Tr/Tr}$ thymic lymphoma A, encodes a dominant negative mutant protein, since one mutated allele is sufficient for mis-localization of Bub1.

The data presented here show that mitotic checkpoint kinase Bub1 and BubR1 are functioning as dominant negative mutants in Brca2$^{Tr/Tr}$ lymphomas, either by defect in phosphorylation or localization to the kinetochore. In lymphoma C, heterozygous mutation in BubR1 is found (Lee *et al*., 1999). In Western blot, a molecule of 90 kDa, in addition to the wild type 119 kDa, is detected unlike controls. Although the consequences of the mutations need to be investigated further, mitotic checkpoint function assayed by 3F3/2 staining showed absence of phosphorylation at the kinetochore in lymphoma C (Lee *et al*., 1999). In summary, all three thymic lymphomas showed defect in mitotic checkpoint function after spindle damage. However, attempts to detect Bub1 staining in lymphoma B was not successful, probably due to low level of Bub1. Therefore, whether phosphorylation of Bub1 is required for proper kinetochore localization or vice versa remains to be investigated further. The mechanism underlying the difference between lymphoma A and B with same *Bub1* mutation is not yet solved. Since phosphorylation of Bub1 in lymphoma A was intact yet showed mis-localization of Bub1, it is possible that phosphorylation and localization of Bub1 is a separate event. It is also possible that in addition to *Bub1* and *BubR1* mutations, other unknown regulatory molecules have been mutated that resulted in the difference between lymphoma A and

lymphoma B.

Most tumor cells exhibit aneuploidy early or late in their progression to cancer. In this regard, it is important to note that accumulating reports suggest the involvement of mitotic checkpoint dysfunction in tumorigenesis. Certain types of cancer that show aneuploidy earlier in their tumorigenesis step are thought to involve mutations in checkpoints that monitor chromosome segregation (Cahill *et al*., 1998; Lee *et al*., 1999; Ru *et al*., 2002).

The data presented in this report support the notion that mutations in mitotic checkpoint components contribute to genetic instability by deregulated chromosome segregation, which results in gross genetic information change by chromosome gain or loss. *Bub1* and *BubR1* mutation resulted in defected phosphorylation and mis-localization. The consequences of these events would result in massive alteration in chromosome number that normal cells cannot endure. It should be stressed that the mutations in *Bub1* and *BubR1* have been found in Brca2-deficient background. In *Brca2* mutated cells, mutation rate can increase, that in a subset of cells critical mutations in mitotic checkpoint can take place that overcome growth retardation and apoptosis. Thus, this report suggest that Brca2-involved repair machinery and Bub1/BubR1-contained mitotic checkpoint machinery crosstalk in keeping the genetic integrity and cell survival.

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