Histological Findings of Mammary Gland Development and Risk of Breast Cancer in BRCA1 Mutant Mouse Models

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ABSTRACT

Purpose: The breast cancer susceptibility gene, BRCA1, is involved in normal development and carcinogenesis of mammary glands. Here, we aimed to evaluate the relationship between histological findings of mammary gland development and breast cancer risk in BRCA1 mutant mice.

Methods: Five BRCA1 mutant mice and five non-mutant FVB/NJ mice were used for each group of 1-month-old (pubertal), 3-month-old (fertile), and 8-month-old (menopausal) mice. In another experiment, 15 BRCA1 mutant mice were followed up to 8 months after birth and classified into tumor-bearing (11 mice) and tumor-free (4 mice) groups. Excised mammary gland tissues were stained with Carmine Alum, and the number of terminal end buds (or alveolar buds), branching density, and duct elongation were measured using image analysis programs. Differences between the two groups were assessed using paired t-test.

Results: One-month-old BRCA1 mutant mice showed a higher number of terminal end buds (23.8 ± 1.0 vs. 15.6 ± 0.8, p = 0.0002), branching density (11.7 ± 0.4 vs. 9.6 ± 0.5%, p = 0.0002), and duct elongation (9.7 ± 0.7 vs. 7.3 ± 0.4 mm, p = 0.0186) than controls. However, there was no difference between the 3- and 8-month-old groups. In BRCA1 mutant mice, the tumor-bearing group showed a significantly higher number of alveolar buds (142.7 ± 5.5 vs. 105.5 ± 5.4, p = 0.0008) and branching density (30.0 ± 1.0 vs. 24.1 ± 1.1%, p = 0.008) than the tumor-free group; however, duct elongation was not different (23.9 ± 0.6 vs. 23.6 ± 0.6 mm, p = 0.008) between the groups.

Conclusion: BRCA1 mutant mice exhibited early pubertal mammary gland development and delayed age-related mammary gland involution was associated with breast cancer. Our results may have clinical implications for predicting breast cancer risk and developing prevention strategies for BRCA1 mutation carriers.

Keywords: Genes, BRCA1; Mammary glands, animal; Mammary neoplasms, experimental; Mice, transgenic; Risk factors

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Conflict of Interest
The authors declare that they have no competing interests.
**INTRODUCTION**

Germline BRCA1/2 mutations are a common cause of hereditary breast cancer, accounting for approximately 5%-10% of all breast cancer patients [1]. Especially, mutations in the BRCA1 gene, which is responsible for repairing DNA and controlling the cell cycle, increase the risk of breast and ovarian cancers. The cumulative risk estimate for developing breast cancer by the age of 70 years is approximately 60%-70%, which is 10 times higher than that for an average woman without BRCA1/2 mutations [1]. The level of risk, however, varies from individual to individual and appears to have increased in recent generations [2]. Women with a BRCA1 mutation typically develop breast cancer at an early age compared to those diagnosed with sporadic breast cancers and often have basal-like or triple-negative breast cancer [3].

More than 20 BRCA1 transgenic mice have been developed, including those having null, hypomorphic, isoform, point, or conditional mutations, to study the functions of BRCA1 gene in the formation of breast tumors [4-6]. Previous studies on mammary gland development in conditional BRCA1 mutant mouse models yielded important information necessary to understand tumor suppressive functions of BRCA1 and molecular mechanisms of breast cancer susceptibility caused by the BRCA1 mutation [5,6]. In normal mice, the BRCA1 gene is required for proliferation and differentiation of cells in mammary glands as a negative regulator of mammary epithelial cell growth in response to ovarian hormones. However, a mutation in the BRCA1 gene results in marked mammary development or abnormal ductal morphogenesis [7-10]. BRCA1 as well as BRCA2 genes are expressed at high levels in terminal end buds, which are puberty-specific structures that contain rapidly proliferating cells undergoing differentiation [8]. Several studies have shown that susceptibility of mammary glands to carcinogenesis is related to the state of the mammary gland development at the time of exposure to mutagenic factors; immature breasts are particularly susceptible to early events related to carcinogenesis [11,12].

In humans, histological findings of breast biopsy tissues, including those showing atypical hyperplasia, have been used to predict the risk of breast cancer development [13]. In addition, levels of lobular involution, lobule type, and the number of epithelial cells in normal breast tissue were also associated with increased breast cancer risk, and risk prediction based on tissue-based features was superior to that based on the Gail model [14-16]. However, only a few studies have investigated histological findings of normal breast tissue and breast cancer risk in BRCA1 mutation carriers. Despite our knowledge of the many determinants of breast cancer risk, our ability to predict the risk for individual women is limited [14]. Thus, the aim of this study is to evaluate the relationship between histological findings of mammary gland development and risk of breast cancer in BRCA1 mutant mice. Histological findings associated with increased or decreased risk of breast cancer in BRCA1 mutant mice could be translated into a more precise measure of breast cancer risk and to develop strategies for preventing cancer in BRCA1 mutation carriers.

**METHODS**

Animal care and experimental procedures were performed in accordance with the Guidelines on Ethical Use of Animals approved by the Institutional Animal Care and Use Committee of Seoul National University Hospital (authorization No. 16-0044-S1A0). BRCA1 mutant mice were imported from Jos Jonkers’ laboratory in Netherlands Cancer Institute (Amsterdam,
Netherlands) and non-mutant FVB/NJ mice from the Jackson Laboratory (Bar Harbor, USA). 

BRCA1 mutant mice were developed by inducing tissue-specific loss of BRCA1 and p53 genes using the K14-Cre system, thereby leading to the transformation of mammary epithelial cells of wild type FVB/NJ mice [6].

In this study, 45 nulliparous female mice, which included 30 BRCA1 mutant and 15 non-mutant FVB/NJ mice, were used for two separate experiments. Five BRCA1 mutant mice and five non-mutant FVB/NJ mice were used for each group of 1-month-old (pubertal), 3-month-old (fertile), and 8-month-old (menopausal) mice. In addition, 15 BRCA1 mutant mice were subjected to palpation to detect the formation of tumors until 8 months after birth.

For observation of mammary gland tissue, the fourth inguinal mammary gland was removed from the left side of each female mouse; in the case of tumor-bearing mice, mammary glands located on the opposite side were used. Following anesthesia, the obtained mammary fat pads containing whole mammary glands were placed on whole-mount slides [17]. The mammary gland tissues were stained with Carmine Alum to distinguish mammary epithelial cells using the method described by Munoz-de-Toro et al. [18] (Figure 1).

The number of terminal end buds at pubertal stages (or alveolar buds at fertile or menopausal stages), branching density, and duct elongation were measured on whole-mount mammary gland images using ImageJ software (Wayne Rasband; National Institutes of Health, Bethesda, USA). In high-magnification scanned images, mammary duct extremities in mice are darker than the rest of the ductal tree. With the application of a size criterion of 0.02 mm², the number of terminal end buds (or alveolar buds) was assessed using the thresholding function of the ImageJ software [19]. The branching density, defined as the number of branching intersection points by unity of the length of the ductal tree, was assessed using Sholl analysis [20]. Using ImageJ software, duct elongation was measured taking into account the lateral growth, which is the distance in mm from the inguinal lymph node of the epithelial tree to the outermost point of ductal growth [20]. Terminal end buds are bulb-shaped structures, unique to the mammary gland in peripubertal stages, which direct the growth of ducts throughout the rest of the fat pad [21]. At a fertile stage, the terminal end buds get transformed into terminal ducts and alveolar structures. Therefore, the term “alveolar buds” is used for such structures during fertile or menopausal stages.

Statistical analysis

Data, including the number of terminal end buds (or alveolar buds), branching density, and duct elongation were obtained from at least four independent samples and are expressed as the mean ± standard deviation for each group, i.e., BRCA1 mutant mice, non-mutant FVB/
NJ mice, tumor-bearing mice, or tumor-free mice found in BRCA1 mutant mice. Statistical analyses, including the paired t-test were conducted using GraphPad Prism software (version 5.0; GraphPad, Inc., La Jolla, USA). \( p < 0.05 \) was considered statistically significant.

RESULTS

Comparison between BRCA1 mutant and non-mutant mice

One-month-old BRCA1 mutant mice at the pubertal stage of mammary gland development showed a significantly higher number of terminal end buds (23.8 ± 1.0 vs. 15.6 ± 0.8, \( p = 0.0002 \)), branching density (11.7 ± 0.4 vs. 9.6 ± 0.5\%, \( p = 0.0082 \)), and duct elongation (9.7 ± 0.7 vs. 7.3 ± 0.4 mm, \( p = 0.0186 \)), compared to FVB/NJ mice (controls) (Figure 2). All the 3-month-old mice at fertile stage from both the groups showed no difference in the number of alveolar buds (80.6 ± 1.1 vs. 80.2 ± 1.2, \( p = 0.8171 \)), branching density (19.0 ± 1.2 vs. 19.7 ± 3.4\%, \( p = 0.6411 \)), and duct elongation (16.5 ± 0.3 vs. 16.3 ± 0.4 mm, \( p = 0.6386 \)) compared to control mice. Similarly, 8-month-old mice at menopausal stage showed no significant difference in the number of alveolar buds (112.4 ± 6.7 vs. 104.6 ± 3.9, \( p = 0.3416 \)), branching density (28.1 ± 1.3 vs. 25.2 ± 0.4\%, \( p = 0.06 \)), and duct elongation (22.8 ± 0.7 vs. 22.5 ± 0.4 mm, \( p = 0.714 \)) compared to control mice (Table 1).

Comparison between tumor-bearing and tumor-free BRCA1 mutant mice

In the group of BRCA1 mutant mice, which was followed until 8 months, 11 out of 15 (73\%) mice developed breast cancer showing histopathological features of high-grade, triple-

![Figure 2](https://ejbc.kr)
negative breast cancer. Moreover, the tumor-bearing group (11 mice) showed a significantly higher number of alveolar end buds (142.7 ± 5.5 vs. 105.5 ± 5.4, \( p = 0.0008 \)) and branching density (30.0 ± 1.0 % vs. 24.1 ± 1.1 %, \( p = 0.008 \)) compared to the tumor-free group (4 mice) (Figure 3; Table 2). However, no difference was observed in duct elongation between the two groups (23.9 ± 0.6 mm vs. 23.6 ± 0.6 mm, \( p = 0.8099 \)).

**DISCUSSION**

In this study, we first quantified histological indicators of mammary gland development in both normal and *BRCA1* mutant mice throughout each developmental stage to evaluate the relationship between mammary gland development and risk of breast cancer. The results showed that *BRCA1* mutant mice tended to have increased terminal end buds, branching density, and ductal elongation compared with controls at one month of age, indicating precocious development of mammary glands in the *BRCA1* mutant mice. However, no difference in these characteristics was observed in the 3-month (fertile) and 8-month (menopausal)-old groups. Our results are concordant with those of previous studies regarding mammary gland development in *BRCA1* mutant mice and suggest that the normal function of the *BRCA1* gene is temporally and developmentally restricted [7,8].

Table 1. Comparison of mammary development parameters between *BRCA1* mutant and FVB/NJ mice

| Mice                              | Number of terminal end buds or alveolar buds | Branching density (%) | Duct elongation (mm) |
|-----------------------------------|---------------------------------------------|-----------------------|----------------------|
| FVB/NJ, 1-month-old (n = 5)       | 15.6 ± 0.8                                  | 9.6 ± 0.5             | 7.3 ± 0.4            |
| *BRCA1* mutant, 1-month-old (n = 5)| 23.8 ± 1.0                                  | 11.7 ± 0.4            | 9.7 ± 0.7            |
| \( \rho \)-value                  | 0.002                                       | 0.0082                | 0.0186               |
| FVB/NJ, 3-month-old (n = 5)       | 80.2 ± 1.2                                  | 19.7 ± 3.4            | 16.3 ± 0.4           |
| *BRCA1* mutant, 3-month-old (n = 5)| 80.6 ± 1.1                                  | 19.0 ± 1.2            | 16.5 ± 0.3           |
| \( \rho \)-value                  | 0.8171                                      | 0.6411                | 0.6386               |
| FVB/NJ, 8-month-old (n = 5)       | 104.6 ± 3.9                                 | 25.2 ± 0.4            | 22.5 ± 0.4           |
| *BRCA1* mutant, 8-month-old (n = 5)| 112.4 ± 6.7                                 | 28.1 ± 1.3            | 22.8 ± 0.7           |
| \( \rho \)-value                  | 0.3416                                      | 0.06                  | 0.714                |

Values are means ± standard deviation.

Table 2. Comparison of mammary development parameters between tumor-free and tumor-bearing *BRCA1* mutant mice

| Mice                                      | Number of alveolar buds | Branching density (%) | Duct elongation (mm) |
|-------------------------------------------|-------------------------|-----------------------|----------------------|
| *BRCA1* mutant, tumor-free, 8-month-old (n = 4)| 105.5 ± 5.4             | 24.1 ± 1.3            | 23.6 ± 0.6           |
| *BRCA1* mutant, tumor-bearing, 8-month-old (n = 11)| 142.7 ± 5.5             | 30.0 ± 1.0            | 23.9 ± 0.6           |
| \( \rho \)-value                           | 0.0008                  | 0.008                 | 0.8099               |

Values are means ± standard deviation.

**Figure 3.** Histologic images of a mammary gland in 8-month-old, tumor-free (A) and tumor-bearing (B) *BRCA1* mutant mice. The quantification results are shown as bar graphs (C). Values are reported as the mean ± standard deviation.

NS = not significant.

\( ^* p < 0.01, ^{†} p < 0.001. \)
The *BRCA1* gene is upregulated in the breast during puberty than during menopause, and a mutation in the *BRCA1* gene results in marked lobuloalveolar development, particularly in the proliferation of terminal end buds [7,10]. We followed the *BRCA1* mutant mice up to the menopausal stage and compared the histological findings of normal mammary glands between tumor-bearing and tumor-free *BRCA1* mutant mice. The results showed that *BRCA1* mutant mice with more alveolar buds and higher branching density were more likely to develop breast cancer. This finding is concordant with the Mayo Benign Breast Disease Cohort study, which showed that reduced levels of age-related lobular involution in normal breast tissue were associated with an increased risk of breast cancer [14]. In our study, there was no difference in mammary duct elongation between tumor-bearing and tumor-free *BRCA1* mutant mice. This could be explained by the fact that in both mice and humans, age-related mammary gland involution begins in the lobules and is pronounced, whereas duct elongation occurs at a later stage; therefore, it is affected to a lesser extent [22,23].

Early menarche, late menopause, nulliparity, obesity after menopause, taking hormones, family history of breast cancer, *BRCA1*-gene mutation, and chest radiation therapy in childhood are known breast cancer risk factors that interact with each other and determine the onset of breast cancer in a woman [2]. Our experiments support the importance of two new risk factors found in *BRCA1*-related breast cancer: early mammary gland development and reduced levels of age-related lobular involution. These features can be measured quantitatively using breast biopsy tissues and can be incorporated along with the estimation of mammographic breast density into risk assessment models to improve prediction of breast cancer risk [14-16,23]. Accurately identifying individuals at an increased risk for breast cancer is important, because more intense screening and specific interventions, such as risk-reducing surgery or medications, can be used more effectively [24]. In epidemiological studies, an important risk factor to be considered is an early age at thelarche, which shows a continuous trend towards early development [25]. Additional studies on the interaction of genes, hormones, and environment at puberty are needed, because our study suggests that mutations in predisposing genes related to breast cancer, such as *BRCA1*, could be a cause of early mammary gland development [26,27].

There are some limitations of our study. First, molecular mechanisms linking altered mammary gland development and high rates of breast cancer in *BRCA1* mutant mice were not investigated. Recently, receptor activator of NF-κB (RANK) signaling by *BRCA1* mutation was suggested to be a cellular mechanism required for hormone-independent abnormal proliferation of mammary luminal progenitors and an increased risk of breast cancer [28,29]. The RANK ligand is a potential target for breast cancer prevention in *BRCA1* mutation carriers [30]. Second, only nulliparous mice were used in our study. Thus, the effects of pregnancy and lactation on delayed age-related mammary gland involution and breast cancer risk could not be determined. High levels of *BRCA1* expression have been reported in rapidly growing alveolar buds during pregnancy [7]. Third, the effect of *BRCA2* mutation that is known to be more closely related to hormone receptor-positive breast cancers was not compared in our study. In a previous study, however, no differences were observed in the expression of *BRCA1* and *BRCA2* genes between normal and mutant mice at pubertal and fertile stages [8].

In conclusion, pubertal mammary gland development occurred at an early stage in *BRCA1* mutant mice, and delayed age-related mammary gland involution was associated with breast cancers. If these findings are confirmed in human studies, measurement of early mammary gland development or age-related lobular involution can be incorporated into risk assessment models, to improve breast cancer risk prediction for individual women. Further research on the molecular
mechanisms linking mammary gland development and breast cancer risk is needed to develop a physiological approach for prevention of breast cancer in carriers of the BRCA1 mutation.

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