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Microsatellite Instability in Breast Cancers with Special Reference to Patients' Age and Bilaterality

Satoshi FUJII, Yukio TAKESHIMA, Koji ARIHIRO, Mayumi KANEKO and Kouki INAI

2nd Department of Pathology, Hiroshima University School of Medicine, 1-2-3 Kasumi, Minami-ku, Hiroshima 734-8551, JAPAN

ABSTRACT

We examined breast cancers from 67 female patients to ascertain the possible correlation between RER or LOH status, age and bilaterality using eight microsatellite markers on chromosomes 2p, 3p, 16q, 17p and 17q. The frequencies of RER in young patients (25–35 years old), patients with double primary disease (43–77 years old) and patients with contralateral metastases (46–72 years old) were 35%, 63%, and 80%, respectively, while that in elderly patients (60–81 years old) was 0%. In contrast, there were no statistically significant differences in the frequency of LOH between these groups. Our results suggest that RER might play an important role in the occurrence of breast cancer at a younger age and in bilateral breast cancer.

Key words: *Replication error (RER), Loss of heterozygosity (LOH), Young, Bilateral breast cancer*

Epidemiological studies have indicated that a positive family history, obesity and hormonal imbalance are etiological risk factors for breast cancer, and it has recently been suggested that somatic or inherited genetic alterations play an important role in the occurrence, development and progression of this disease [5-7,13,17,21,28,37,38,41,43,45,46,51,52,57,59,62,64]. There have been now many reports of genetic alterations in breast cancer. However, it remains unclear whether these are critical events in the early stages of carcinogenesis. The correlation between risk factors and genetic alterations is also unclear.

In contrast, a distinctive type of genetic alteration based on misalignment mutagenesis has been reported in colorectal and gastric cancer. This type of mutagenesis involves microsatellites which are defined as tandem arrays of short, simple nucleotide sequences dispersed within the human genome. It is now believed that such repeat sequences indicate a predisposition to mutation and that alterations in microsatellites reflect genuine genetic instability, although it is still unknown whether this genetic instability has a general effect on activating oncogenes or inactivating tumor suppressor genes. The phenotype associated with genetic instability has been termed "microsatellite instability" or "replication error (RER)".

Colorectal cancer was the first type of human malignancy in which RER was detected. RER has been very frequently demonstrated in patients with hereditary non-polyposis colorectal cancer (HNPCC). Moreover, it has been found that these patients have germline mutations in the known mismatch repair genes[8,42]. Also, non-HNPCC colorectal cancers in patients aged 35 years or younger exhibit a higher prevalence of RER than those in elderly patients[32]. RER has subsequently been found in many tumor types including breast cancer[11,18,48]. If similar abnormalities of the mismatch repair gene system affect the genesis of breast cancer, RER might occur in the early stages of breast cancer in young patients. Moreover, bilateral breast cancer may have a background of RER because patients with multiple primary cancers have frequently been reported to show RER[20,39].

Loss of heterozygosity (LOH) at microsatellite loci on various chromosomes is a feature of microsatellite instability, and various rates of LOH have been reported in breast cancer. However, there are few reports concerning the correlation between LOH status and the age of the patients or bilaterality.

Therefore, we examined the prevalence of RER and LOH in breast cancer, in relation to the age of the patients and bilaterality, to elucidate the role of microsatellite instability in the tumorigenesis of breast cancer.

MATERIALS AND METHODS

Patient selection

The correlation between RER and LOH status, age and bilaterality was evaluated in 67 female patients with breast cancer, identified from our surgical files over the period 1986 to 1996. We classified the patients into five groups: young (25–35 years old, 20 patients, Group I; 36–39

TEL: +81-82-257-5152 FAX: +81-82-257-5154

Table 1. Clinicopathological Profiles of Patients with Breast Cancer

Group	No. of patient	Age[a]	Size[b] (range)	Histological type[c]				LN[d]	
				NIDC	IDC	ILC	Special type[e]	(+)	(–)
Young patients									
Group I	20	31	2.4 (0.7–6.0)	0	19	0	1	9	11
Group II	18	37	3.0 (0.9–7.0)	3	14	0	1	9	9
Elderly patients									
Group III	16	69	2.4 (1.0–6.0)	1	14	1	0	2	14
Bilateral cancer									
Group IV	8	60	2.8 (0.3–10.5)	1	13	2	0	3	5
Group V	5	61	4.4 (0.8–15.0)	0	5	0	0	5	0

NIDC: nonivasive ductal carcinoma; IDC: invasive ductal carcinoma; ILC: invasive lobular carcinoma.
[a]Age, average age of patients; [b]Size, average maximum tumor size (cm); [c]Pathological classification of breast cancer was confirmed according to the general rules for clinicopathological records of breast cancer of the Japan Breast Cancer Society; [d]LN, No. of patients with lymph node metastasis; [e]Special type, other histological types.

years old, 18 patients, GroupII), elderly (60–81 years old, 16 patients, Group III) and those with bilateral disease (43–77 years old, 13 patients). The patients with bilateral breast cancer included 8 with independent primary carcinomas of both breasts (double primary disease, 43–77 years old, GroupIV) and 5 with primary carcinoma of one breast which had metastasized to the contralateral breast (46–72 years old, GroupV). Of the 16 elderly patients, 14 had no lymph node metastasis. Of the 20 patients in Group I, 9 had lymph node metastases and of the 18 patients in Group II, 9 had lymph node metastases. The clinicopathological profiles of the patients are summarized in Table 1. Patients with family history of breast cancers were not included in the present study.

Sections of paraffin-embedded cancerous tissues were stained with hematoxylin and eosin and examined routinely under the microscope according to the guidelines of the Japan Breast Cancer Society.

DNA extraction

From tissues fixed in 10% formalin and embedded in paraffin, 10μm-thick unstained sections were cut. After dewaxation with xylene and 100% ethanol, microscope slides of the cancerous area were prepared by scraping off only cancerous cells with sterile needles. Slides from areas with a high proportion (>50%) of cancerous cells were prepared for microscopic DNA analysis. Using the same method, we extracted matched normal DNA from lymph node tissue free of metastases. Genomic DNA was prepared by the standard method[53].

Polymerase chain reaction-based microsatellite analysis

Sixty-seven matched DNA pairs obtained from normal and cancerous tissues were examined for RER and LOH at eight microsatellite loci by polymerase chain reaction (PCR) amplification. Eight primers were used to target and examine two microsatellite loci on chromosome 16 *(D16S301 and D16S303)*[10,55], four on chromosome 17 *(D17S796, TP53, D17S855 and D17S579)*[3,23], one locus on chromosome 2 *(D2S136)*[58] and one on chromosome 3 *(D3S1067)*[24]. Each PCR was performed in 25μl reaction mixture containing 200–500ng DNA, 2.5μl 10×buffer (500mM KCl, 100mM Tris-HCl (pH8.8), 15mM $MgCl_2$, 1%Triton), 0.05mM dNTP, 1.25 units *Taq* DNA polymerase, 0.05nM of the counterpart of each primer and 2μCi [α-^{32}P-dCTP]. After heating at 95°C for 8 min, 35 PCR cycles comprising 1 min at each annealing temperature, 2 min at 72°C for strand elongation and 40s at 95°C for denaturing were performed. A final elongation was performed over 7 min at 72°C. The reaction product (4μl) was then denatured and electrophoresed in 6% denaturing polyacrylamide gel containing urea. After electrophoresis, the gel was fixed on paper, dried and exposed to X-ray film for 24–48h. If a shift and/or gain of electrophoretic bands was observed with the DNA allele from the cancer, that cancer was categorized as showing RER. We defined a patient whose cancer showed RER using at least one marker, as a patient with RER. If the signal intensity of one allele in the cancer was reduced to less than 50% of the normal intensity observed among informative cases, that patient was categorized as showing LOH.

Statistical analysis

Correlations between the RER or LOH status and clinicopathological parameters (histological type, tumor size, lymph node metastasis and stage) were analyzed using chi-square method or Fisher's exact test.

RESULTS

A summary of the RER and LOH status of the young patients are shown in Table 2 and 3, and the RER and LOH status of the patients with bilateral disease are summarized in Table 4. Typical electrophoretic photographs are shown in

Table 2. The Frequency of RER in Young Patients

Group	n	Microsatellite markers							Total
		D16S301	*D16S303*	*D17S796*	*TP53*	*D17S855*	*D17S579*	*D2S136*	*D3S1067*	
I	20	1/20(5)[a]	0/20(0)	2/20(10)	1/20(5)	1/20(5)	4/20(20)	0/20(0)	0/20(0)	7/20(35) [b]
II	18	0/18(0)	0/18(0)	0/18(0)	0/18(0)	1/18(6)	2/18(11)	0/18(0)	0/18(0)	2/18(11)
III	16	0/16(0)	0/16(0)	0/16(0)	0/16(0)	0/16(0)	0/16(0)	0/16(0)	0/16(0)	0/16(0)

[a]Number of cases with RER/total cases (%).
[b]$p<0.05$.

Table 3. The Frequency of LOH in Young Patients

Group	n	Microsatellite markers							Total
		D16S301	*D16S303*	*D17S796*	*TP53*	*D17S855*	*D17S579*	*D2S136*	*D3S1067*	
I	20	1/20(5)[a]	0/20(0)	2/17(12)	1/20(5)	1/20(5)	1/18(6)	0/20(0)	1/18(6)	7/20(35) [b]
II	18	2/17(12)	0/18(0)	1/13(8)	2/18(11)	1/18(6)	0/17(0)	0/18(0)	2/17(12)	5/18(28) [b]
III	16	0/16(0)	0/16(0)	0/16(0)	2/14(14)	2/16(13)	1/16(6)	2/16(13)	0/16(0)	5/16(31)

[a]Number of cases with LOH/number of infomative cases (%).
[b]not significant.

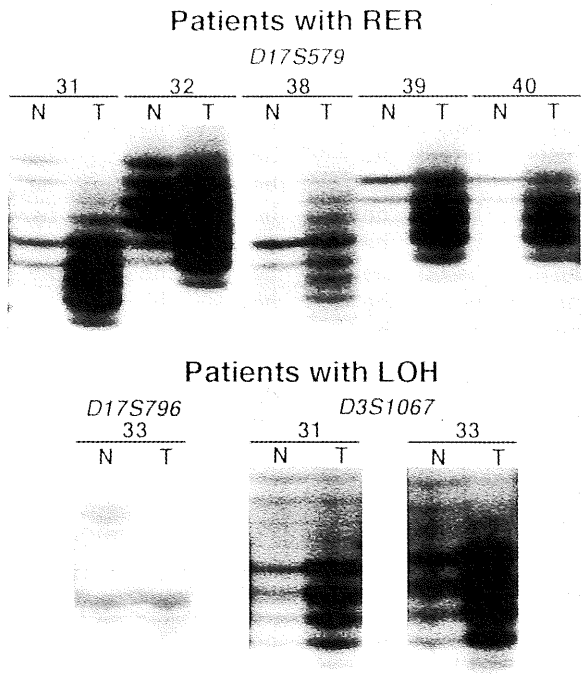

Fig. 1. Replication Error and Loss of Heterozygosity in Breast Cancer Specimens from Young Patients
For each patient, lane N shows DNA from non-cancerous tissue and lane T shows DNA from the breast cancer. The tumors from patients 31, 32, 38, 39 and 40 (upper row) exhibit RER, while those from patients 31 and 33 (lower row) exhibit LOH. The shift and loss of band in electrophoretic mobility was reproducible in replicate experiments.

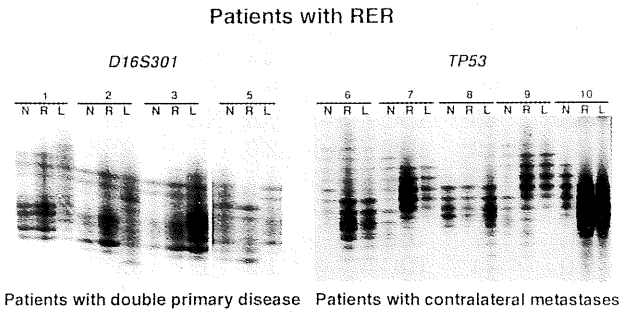

Fig. 2. Replication Error in Breast Cancer Specimens from Patients with Bilateral Disease
For each patient, lane N shows DNA from non-cancerous tissue, and lanes R and L DNA from the tumors in the right and left breasts. Patients 1, 2, 3 and 5 had double primary disease. In these patients, the tumor in one breast exhibits RER at *D16S301*, whereas both tumors in patients with metastasis to the contralateral breast (patients 6, 7, 9 and 10) exhibit RER at *TP53*.

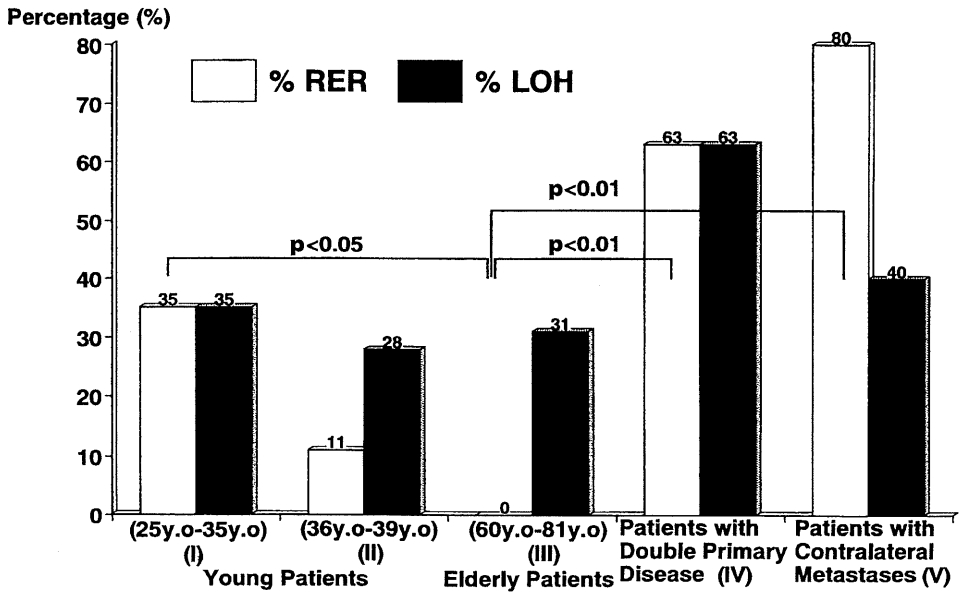

Fig. 3. Percentage of RER and LOH in Breast Cancer Specimens from Different Groups

Table 4. Result of Microsatellite Assay in the Patients with Bilateral Breast Cancer

Patients with Double Primary Desease

Patients' No.	Age	Histolgy	LN	Size	Stage	Microsatellite marker							
						D16S301	*D16S303*	*D17S796*	*TP53*	*D17S855*	*D17S579*	*D2S136*	*D3S1067*
1R	63	IDC	(+)	10.5	III	he	he	he	he	he	he	he	he
1L	63	IDC	(+)	1.7	III	RER	he	he	he	he	he	he	he
2R	57	IDC	(–)	4.5	I	he	he	he	he	he	he	he	he
2L	57	IDC	(–)	1.7	I	RER	he	LOH	LOH	he	he	he	he
3R	69	IDC	(+)	NA	NA	he	he	LOH	he	he	he	ho	he
3L	69	IDC	(–)	NA	NA	RER	he	he	he	he	he	ho	he
4R	43	IDC	(–)	NA	NA	he	he	he	he	he	he	he	he
4L	43	IDC	(–)	2.6	I	RER	he	he	he	he	he	he	he
5R	74	IDC	(–)	1.5	I	RER	he	he	he	he	he	LOH	he
5L	74	ILC	(–)	2.3	I	he	he	he	he	he	he	he	he
11R	47	ILC	(–)	1.2	I	he	LOH	ho	he	he	he	he	he
11L	44	IDC	(–)	1.2	I	LOH	LOH	ho	he	he	he	he	he
12R	54	IDC	(–)	2.4	I	he	he	he	he	he	he	he	he
12L	54	IDC	(–)	0.3	I	he	he	he	he	he	he	he	he
13R	77	NIDC	(–)	5	I	he	he	LOH	LOH	he	he	he	he
13L	77	IDC	(+)	0.9	I	he	he	he	he	he	he	he	he

Patients with Contralateral Metastases

Patients' No.	Age	Histolgy	LN	Size	Stage	Microsatellite marker							
						D16S301	*D16S303*	*D17S796*	*TP53*	*D17S855*	*D17S579*	*D2S136*	*D3S1067*
6R	48	IDC	NA	3	NA	he	he	he	RER	he	he	he	he
6L	46	IDC	(+)	NA	NA	he	he	he	RER	he	he	he	he
7R	54	IDC	NA	3	NA	he	he	he	RER	he	he	he	he
7L	53	IDC	(+)	15	II	he	he	he	RER	he	he	he	he
8R	64	IDC	(+)	5	III	he	he	he	he	he	LOH	he	he
8L	67	IDC	NA	1.2	NA	he	he	LOH	he	he	LOH	LOH	he
9R	72	IDC	NA	0.8	NA	he	he	he	RER	he	he	ho	he
9L	72	IDC	(–)	4.5	I	he	he	LOH	RER	he	he	ho	he
10R	64	IDC	(+)	2.5	III	he	he	he	RER	he	he	ho	he
10L	66	IDC	(+)	NA	NA	he	he	he	RER	he	he	ho	he

LN: lymph node metastasis; he: non-LOH; RER: replication error; LOH: loss of heterozygosity; ho: not informative; NA: not available; IDC: invasive ductal carcinoma; ILC: invasive lobular carcinoma.

Fig. 1 and 2. The percentages of RER and LOH observed in each group are shown in Fig. 3. The percentage of RER in young patients was considerably higher than that in elderly patients (Fig. 3). The difference in RER between 25 to 35 year-old and elderly patients (Group I and III) was statistically significant ($p<0.05$). RER was observed more frequently in both patients with double primary disease and those with contralateral metastases (Group IV and V) than in elderly patients (Group III) ($p<0.01$).

In contrast, the percentage of LOH in young patients was similar to that in elderly ones (Fig. 3). A tendency towards a higher percentage of LOH was observed in patients with double primary disease, but there were no significant differences in the percentage of LOH between the groups.

The microsatellite markers *D2S136* and *D3S1067* were used to identify changes in the *MSH2* and *MLH1* genes in this study. There were few patients with alterations at these microsatellite loci (Table 3 and 4) (1 in Group I at *D3S1067*, 2 in Group II at *D3S1067*, 2 in Group III at *D2S136*, 1 in Group IV at *D2S136* and 1 in Group V at *D2S136*), that is, there was no striking association between the presence of RER and alterations at microsatellite loci on the *MSH2* and *MLH1* genes.

Patients with double primary disease showed a predisposition towards alterations at *D16S301* and *D16S303* on chromosome 16q more frequently. In 5 of these 8 the tumor on one side exhibited RER at *D16S301* (Table 4). On the other hand, patients with metastatic tumors exhibited a predisposition towards more frequent alterations at *D17S796* and *TP53* on chromosome 17p (Table 4). In 4 of 5 patients with contralateral metastases, both tumors showed RER at *TP53*.

There was a few patients with genetic alterations at microsatellite locus *(D17S855)* on the *BRCA1* gene (Table 2, 3 and 4) (2 in Group I, 2 in Group II, 2 in Group III and none in Groups IV or V).

No significant correlation between RER or LOH and any clinicopathological parameter was observed in this study.

DISCUSSION

Cancers with RER can be classified into three types. The first is a subset of sporadic colorectal cancers. The great majority of these patients have no family history of cancer, are over 60 years of age, and have no detectable germline mutations in their mismatch repair genes[26,34,54]. The second type is a subset of colorectal, endometrial and other cancers arising in related patients fulfilling the criteria for HNPCC[1,44,48]. These patients have a strong family history of cancer, are often young, and most have germline mutations in their mismatch repair genes. The third type is a subset of many tumor types including cancers of the breast, as well as pancreas and stomach[9,11,18,36,60].

It has been reported that RER in sporadic human breast cancers was low[2,14,30,44,50,56,63]. However, in the present study, RER was more frequently observed in younger patients than in elderly patients. This discrepancy may reflect the difference of criteria of RER in the previous study[2,14,30,50,56,63]. The significant difference of the RER rate, however, may have some significant meanings. It has been reported that RER correlates with lobular carcinoma, lymph node involvement and poor prognosis[2,14,30]. Despite this, the present study found no evidence that RER correlates with lobular carcinoma, lymph node involvement or noninvasive ductal carcinoma. It has also been suggested that RER is an early event in the genesis of occasional breast cancers[50,56,63]. Moreover, another study in our laboratory indicated the presence of RER in preneoplastic breast cancer lesions[25], and in the present study, RER was not seen in any of the 16 elderly patients, 14 of whom had no lymph node metastasis. This may reflect a difference in etiology between young and elderly patients in the early carcinogenic process.

It is well known that individuals with mutations in one of the known mismatch repair genes (including *MSH2*, *MLH1* and *PMS1*) have an increased likelihood of accumulating genetic alterations that lead to tumorigenesis[8,19,33,40]. It has been reported that a subset of breast cancer occurs due to the inheritance of a mutant mismatch repair gene[47]. However, mutations in mismatch repair genes have not been reported in sporadic breast cancer. In the present study, RER or LOH were infrequently found at microsatellite loci on the *MSH2* and *MLH1* genes in all groups. However, these mismatch repair genes, including unknown genes, are likely to be involved in RER in young patients and those with bilateral breast cancer. Endometrial carcinoma which is the second commonest malignancy of the HNPCC syndrome is reported to show RER more frequently[48]. However, it is also reported that primary endometrial carcinoma has low frequency of mutations in *MLH1* and *MSH2*[29]. These findings suggest that other mismatch repair genes are responsible for the RER phenotype in endometrial carcinoma as well as breast cancer. It will therefore be necessary to examine mutations in other mismatch repair genes. Also, it has been reported that there were frequent replication errors in post hepatitis B viral cirrhosis, even though hepatocellular carcinoma showed high frequency of LOH using microsatellite markers[49]. These findings suggest RER was generated from another system different from germline and/or somatic mutation in mismatch repair genes.

In general, breast cancer in younger women (40 years of age or younger) has a poorer prognosis

than that in older women[12]. It is probable that breast cancers in younger women proliferate more aggressively than those in postmenopausal women. Therefore, it is supposed that markers of poor prognosis exist at the molecular level without significant histopathologic differences. However, the prognosis of cancers with RER remains undetermined. For example, occasional colorectal cancers with RER and a right-sided anatomical distribution have a favorable prognosis[26,34,54], and endometrial carcinomas with RER have a diploid or near diploid DNA content and a low recurrence rate[48]. In contrast, RER has been reported to be more frequently observed in the poorly differentiated type of gastric cancer and in patients with multiple primary cancers[18,20]. In our study, there was no significant correlation between RER and any clinicopathological factor (histological grade, tumor size and lymph node metastasis). Consequently, it is still unknown whether RER in breast cancer is a biological marker of poor prognosis.

RER has been frequently found in the tumors of patients with multiple primary cancers. Genetic instability is therefore believed to play an important role in the development of multiple primary cancers[20,39]. Our results showed that RER occured more frequently in patients with double primary disease than in those with single cancers. Moreover, it was interesting that RER was found more frequently in the patients with contralateral metastases, and that RER was frequently detected at microsatellite loci on the *p53* gene in these patients. Since abnormalities of the *p53* gene have been reported to be more common in bilateral breast cancer than in unilateral breast cancer[27], it is suggested that RER in bilateral breast cancer and breast cancer with contralateral metastases might correlate with an abnormality of the *p53* gene.

Inherited mutations in the *BRCA1* and *BRCA2* genes are known to confer a predisposition to breast cancer[16,61]. *BRCA1* mutation has been associated with estimated life-time risks of approximately 85 percent for breast cancer and 50 percent for ovarian cancer[15,35]. Recently, alterations in the *BRCA1* gene were identified in approximately 10 percent of a cohort of young women who were diagnosed before the age of 35 and were not selected on the basis of family history[31]. Thus, mutations of the *BRCA1* gene are thought to play an important role in breast-ovarian cancer families. Although the prevalence of *BRCA1* gene mutations in young Japanese women with breast cancer has not been reported, the proportion of Japanese breast cancer and breast-ovarian cancer families who inherit the mutated *BRCA1* allele appears to be small[22]. Loss of heterozygosity at microsatellite loci on the *BRCA1* and *BRCA2* genes might correlate with larger tumor size and higher histological grade in some breast cancers[4]. In the present study, we found only a few patients with alteration at one microsatellite locus *(D17S855)* on the *BRCA1* gene in every groups, but it will be necessary to examine alterations at many microsatellite loci on the *BRCA1* gene as well as investigating mutations using direct sequencing and on the basis of family history.

In summary, our preliminary data indicated that the percentage of RER in young patients (25–35 years old) and those with bilateral breast cancer was significantly higher than in elderly patients, while there was no significant correlation between the incidence of LOH and the ages of the patients or bilaterality. These findings suggest that RER may play an important role in early onset (occurrence at a young age) and bilateral breast cancer. Genetic analysis including RER or LOH in middle age patients (40–59 years old) is warranted. It is possible that genetic instability has general effects on activating oncogenes or inactivating tumor suppressor genes. However, further studies will be required to determine the true role of RER in the natural history of breast cancer.

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