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ORIGINAL ARTICLE

Harms of screening mammography for breast cancer in Japanese women

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Abstract
Background The US Preventative Services Task Force assesses the efficacy of breast cancer screening by the sum of its benefits and harms, and recommends against routine screening mammography because of its relatively great harms for women aged 40–49 years. Assessment of the efficacy of screening mammography should take into consideration not only its benefits but also its harms, but data regarding those harms are lacking for Japanese women.
Methods In 2008 we collected screening mammography data from 144,848 participants from five Japanese prefectures by age bracket to assess the harms [false-positive results, performance of unnecessary additional imaging, fine-needle aspiration cytology (FNA), and biopsy and its procedures].
Results The rate of cancer detected in women aged 40–49 years was 0.28%. The false-positive rate (9.6%) and rates of additional imaging by mammography (5.8%) and ultrasound (7.3%) were higher in women aged 40–49 years than in the other age brackets. The rates of FNA (1.6%) and biopsy (0.7%) were also highest in women aged 40–49 years. However, they seemed to be lower than the rates reported by the Breast Cancer Surveillance Consortium (BCSC) and other studies in the US.
Conclusions The results, although preliminary, indicate the possibility that the harms of screening mammography for Japanese women are less than those for American women.

Keywords Breast cancer screening · Harm · Mammography

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Introduction

Recent years have seen increased awareness of the need for assessment of the efficacy of cancer screening on the basis of both its benefits and harms [1–5]. The US Preventative Services Task Force (USPSTF) [3, 4] reported their updated guidelines for screening mammography. They comprehensively assessed the efficacy of breast cancer screening in terms of the net benefit, which is the sum of benefits (mortality reduction) and harms (radiation exposure, pain, anxiety, over-diagnosis, and false-negative and false-positive mammography results). For women in their 40s and 50s, screening mammography had a 15 and 14% mortality reduction effect as the benefit in eight and six meta-analysis studies [3]. On the other hand, the harms (especially false-positive mammography, unnecessary additional imaging tests and histological examinations) were relatively greater in women aged 40–49 years when comparing the analyzed data [6–12] with the data of the Breast Cancer Surveillance Consortium (BCSC) [3]. The USPSTF thus recommended against routine screening mammography in women aged 40–49 years (grade C recommendation) [4]. That recommendation, however, has not escaped criticism [5]. That paper reevaluated the data that served as the basis for preparation of the USPSTF guidelines and argued that, for maximization of the survival results, it would be necessary to start screening once annually beginning from the age of 40 years [5].

On the other hand, in Japan, screening mammography, which was endorsed in 2000 for women aged 50 years and over, was expanded to cover women aged 40–49 years in 2004. However, at the time of that endorsement, data regarding the improvement in survival and the harms of screening mammography were not yet available. It will take considerable time to elucidate the improvement in survival, and a conclusion cannot be drawn at this time. Conversely, the harms of the technique can be investigated. Accordingly, the present study was designed to evaluate the harms of breast cancer screening by mammography in Japanese women. We studied the harms of screening mammography using the initial test data collected from five prefectures. The analyzed harms consisted of false-positive results, unnecessary additional imaging tests, and the need for biopsies and their procedures, which were compared with the US data.

Materials and methods

We collected community-based screening mammography data for 144,848 participants that had been recorded in fiscal 2008 in five prefectures—Gunma, Ibaraki, Fukui, Miyagi and Tokushima—with the support of the Japan Association of Breast Cancer Screening. Participants undergo—in principle—biennial screening mammography, based on the guidelines of the Japanese Ministry of Health, Labour and Welfare. Using a questionnaire, we inquired about the following items by age bracket (40–49, 50–59, 60–69, and 70 years or over): the number of participants, number recalled, number of responders and number of detected breast cancer cases. Recalls were defined as women who required further examinations after the initial screening. Responders were defined as women who were recalled after the initial screening because of a positive finding and actually presented for further examination. False-positive screenings were defined as the proportion of recalls except cases whose further examinations proved to be breast cancer.

We ascertained the methods used for additional imaging [mammography, ultrasound (US)] and interventions [cytological examination by fine-needle aspiration cytology (FNA) and histological examination] for women with or without breast cancer by age bracket. We used data from only four of those Japanese prefectures (Gunma, Ibaraki, Fukui and Miyagi) for analysis of the details of the further examinations [additional mammography, additional US, cytological examination by FNA and biopsy (any method)], because the data from Tokushima lacked adequate details.

Furthermore, we ascertained the details of biopsy, such as core-needle biopsy (CNB), vacuum-assisted biopsy

Table 1 Total data of this analysis of all five prefectures

Prefecture	Participants	Recalled		Responders		Cancer		PPV	False positive	
	(*n*)	(*n*)	(%)	(*n*)	(%)	(*n*)	(%)		(*n*)	(%)
Gunma	22,893	1,172	5.1	1,124	95.9	75	0.33	6.4%	1,097	4.8
Ibaraki	63,451	3,451	5.4	3,055	88.5	121	0.19	3.5%	3,330	5.2
Fukui	13,796	1,534	11.1	1,418	92.4	43	0.31	2.8%	1,491	10.8
Miyagi	32,847	3,066	9.3	3,036	99.0	115	0.35	3.8%	2,951	9.0
Tokushima	11,861	1,134	9.6	1,061	93.6	51	0.43	4.5%	1,083	9.1
Total	144,848	10,357	7.2	9,694	93.6	405	0.28	3.9%	9,952	6.9

PPV positive predictive value

(VAB) and open surgical biopsy (OSB), for each age bracket. CNB was defined as percutaneous histological examination using an 11, 14- or 16-gauge needle without aspiration. VAB was defined as percutaneous histological examination using a needle with aspiration by Mammotome® (Johnson & Johnson Ethicon Endo-Surgery, Inc., Cincinnati, OH) and Vacora® (BARD, Murray Hill, NJ, USA). Data from only three prefectures (Gunma, Ibaraki and Fukui) were used for analysis of the details of the histological examination methodology (CNB, VAB or OSB), because the data from Miyagi and Tokushima lacked sufficient detail.

Differences in the recall rate, response rate, cancer yields, positive predictive values, false positives, additional imaging (screening mammography, US), FNA and biopsy between ages 40–49 years and the other age brackets were statistically evaluated using the chi-square test. The Japanese data and BCSC data on the harms were also comparatively analyzed. Differences were regarded as significant if the two-sided P value was <0.05.

Results

Table 1 shows the region-specific data. The data obtained from the five prefectures and the age-specific data are summarized in Table 2. In women aged 40–49 years, the recall rate (9.9%) and false-positive rate (9.6%) were higher than in the other age brackets, with statistical significance ($p < 0.001$). The response rate for detailed examinations (92.2%) and the positive predictive value (2.8%) were slightly lower in the women in their 40s than in women in the other age brackets. Cancer detection rates in the 40s, 50s, 60s and above 70 were 0.28, 0.25, 0.24 and 0.43%, respectively.

Table 3 shows the data for the additional imaging and interventions performed in the four analyzed prefectures. The respective rates of performance of mammography, US, cytological examination (FNA) and biopsy (histological examination) as the detailed investigations were 4.0, 4.8, 0.9 and 0.4% among the total participants, and 5.8%, 7.3%, 1.6% and 0.7% in women aged 40–49 years. The rates of additional imaging, FNA and biopsy were significantly higher in the 40s than in the other age brackets ($p < 0.001$).

Table 4 presents the details of the information obtained by histological examinations (CNB, VAB and OSB) performed in Gunma, Ibaraki, Fukui and Miyagi prefectures. CNB, VAB and OSB were performed in 0.26, 0.08 and 0.04% of the total participants, respectively. Each of those rates was highest for women in their 40s: 0.38, 0.16 and 0.07%. Next, the Japanese and BCSC data on the harms were comparatively analyzed (Table 5) [3]. The harms in terms of false positivity and unnecessary additional

Table 2 Analysis by age bracket (data from all five prefectures)

Age Bracket	Participants	Recalled			Responders			Breast cancer cases (cancer yield)			PPV		False positives		
(years)	(*n*)	(*n*)	(%)	(*p*)	(*n*)	(%)	(*p*)	(*n*)	(%)	(*p*)	(%)	(*p*)	(*n*)	(%)	(*p*)
40–49	33,924	3,357	9.9		3,096	92.2		95	0.28		2.8		3,262	9.6	
50–59	43,144	3,051	7.1	$p < 0.001$	2,836	93.0	$p = 0.267$	107	0.25	$p = 0.388$	3.5	$p = 0.121$	2,944	6.8	$p < 0.001$
60–69	46,650	2,591	5.6	$p < 0.001$	2,461	95.0	$p < 0.001$	113	0.24	$p = 0.296$	4.4	$p < 0.01$	2,478	5.3	$p < 0.001$
70 -	21,130	1,358	6.4	$p < 0.001$	1,301	95.8	$p < 0.001$	90	0.43	$p < 0.05$	6.6	$p < 0.001$	1,268	6.0	$p < 0.001$
Total	144,848	10,357	7.2		9,694	93.6		405	0.28		3.9		9,952	6.9	

PPV positive predictive value

Table 3 Rates of additional imaging and interventions (data from four prefectures)

Age (years)	Participants	Further evaluation of responders											
		Additional MMG			Additional US			FNA			Biopsy[a]		
	(*n*)	(*n*)	(%)[b]	(*p*)	(*n*)	(%)[b]	(*p*)	(*n*)	(%)[b]	(*p*)	(*n*)	(%)[b]	(*p*)
40–49	31,323	1,813	5.8		2,298	7.3		503	1.6		215	0.7	
50–59	40,199	1,728	4.3	$p < 0.001$	2,003	5.0	$p < 0.001$	359	0.9	$p < 0.001$	160	0.4	$p < 0.001$
60–69	42,789	1,304	3.0	$p < 0.001$	1,521	3.6	$p < 0.001$	204	0.5	$p < 0.001$	105	0.2	$p < 0.001$
70–	18,676	522	2.8	$p < 0.001$	586	3.1	$p < 0.001$	83	0.4	$p < 0.001$	78	0.4	$p < 0.001$
Total	132,987	5,367	4.0		6,408	4.8		1,149	0.9		558	0.4	

MMG Mammography, *US* ultrasound, *FNA* fine-needle aspiration cytology

[a] Number of cases undergoing histological examination

[b] % of participants

Table 4 Rates of each type of biopsy (data from three prefectures)

Age (years)	Participants	Type of biopsy								
		CNB			VAB			OSB		
	(*n*)	(*n*)	(%)[a]	(*p*)	(*n*)	(%)[a]	(*p*)	(*n*)	(%)[a]	(*p*)
40–49	25,159	95	0.38		39	0.16		17	0.07	
50–59	30,526	80	0.26	$p < 0.05$	26	0.09	$p < 0.05$	13	0.04	$p = 0.206$
60–69	32,491	49	0.15	$p < 0.001$	12	0.04	$p < 0.001$	9	0.03	$p < 0.05$
70–	11,964	34	0.28	$p = 0.153$	4	0.03	$p < 0.001$	4	0.03	$p = 0.196$
Total	100,140	258	0.26		81	0.08		43	0.04	

CNB Core-needle biopsy, *VAB* vacuum-assisted biopsy, *OSB* open surgical biopsy

[a] % of participants

imaging and biopsy were greatest for women in their 40s in Japan, but less than in the BCSC in all age brackets. In addition, the cancer detection rate per 1,000 screened in Japanese women aged 40–49 years was 2.8, which was slightly higher than the 2.6 recorded in the BCSC data.

Discussion

The USPSTF recommended against routine screening mammography in women aged 40–49 years [4]. As background to that recommendation, in terms of the benefit, screening mammography in the 40s results in 15% mortality reduction, and it was acknowledged to have a benefit in eight RCT meta-analyses. However, in terms of the harms, the BCSC data indicated that they (especially false positivity, unnecessary additional imaging and biopsy) were relatively greater for women in their 40s [4].

In this study, as well, the harms in terms of false positivity and performance of unnecessary additional imaging and biopsy were greatest for Japanese women in their 40s, but less than in the BCSC in all age brackets. Thus, screening mammography appears to be less harmful in Japan than in the US. In a report from the US [13], the relative proportions of biopsy performed using CNB, VAB and OSB were 23.2, 40.0 and 36.8% for women as a whole, and 25.3, 40.4 and 34.2% for women aged 40–49 years, respectively. That study found that the proportion of OSB has declined by the year, but it remains at approximately 30%. Figure 1 illustrates, per 1,000 screened women in their 40s, the estimated numbers of additional imaging, FNA, biopsy and its procedures, false positives and detected cancers. The number of biopsy procedures was calculated from the data for three prefectures in Japan and from the data of the US report [13]. As biopsy procedures, the respective numbers of CNB, VAB and OSB are approximately 3.8, 1.6 and 0.7 per 1,000 screened women in Japan, and 2.4, 3.8 and 3.2 in the US. These data suggest that once US women in their 40s go for screening, they undergo more biopsies and OSBs than in Japan. Based on these results, in addition to the lower rates of false positives, additional imaging and biopsy, the invasiveness of biopsy is lower in Japan. Accordingly, we speculate that the harms of breast cancer screening in women in their 40s are less in Japan than in the US. The costs associated with CNB, VAB and OSB in Japan are 19,300, 55,800 and

Table 5 Comparison of the data from the BCSC and this study

	Source	Age bracket (years)			
		40–49	50–59	60–69	70–
Outcomes per screening round (per 1,000 screened)					
False-positive mammogram	BCSC[a]	97.8	86.6	79.0	68.8
	This study[b]	96.2	68.2	53.1	60.1
Additional imaging	BCSC[a]	84.3	75.9	70.2	64.0
	This study[c]	73.4	49.8	35.5	31.4
Biopsy	BCSC[a]	9.3	10.8	11.6	12.2
	This study[c]	6.9	4.0	2.5	4.2
Screening-detected breast cancer[d]	BCSC[a]	2.6	4.7	6.5	7.9
	This study[b]	2.8	2.5	2.4	4.3

[a] Data from BCSC (Breast Cancer Surveillance Consortium) were cited from Ref. [3]

[b] Calculated from Table 2

[c] Calculated from Table 3

[d] Including invasive cancer and DCIS

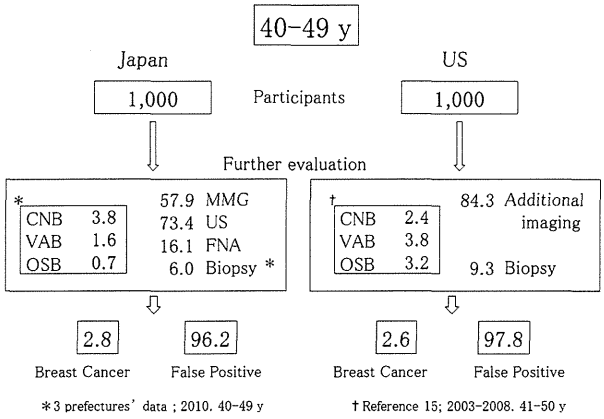

Fig. 1 The estimated numbers of additional imaging, FNA, biopsy and its procedures, false positives and detected cancers per 1,000 screened women in their 40s

65,600 Yen (including the pathological diagnosis fee), respectively, which are only 1/8 to 1/18 of the costs in the US [13]. We need to evaluate the harms of screening in consideration of the different economic circumstances between the countries. The benefit of screening can be assessed by a single measure of the decrease in mortality. With regard to its harms, however, the weight of each criterion differs by country, region, economic status and personal values. An overall net benefit should be decided upon, accounting for all the above factors.

One of the limitations of this study was that our data were taken from only 5 of the 47 prefectures in Japan. The participating prefectures had been conducting breast cancer screening for a long period, and their data can be assumed to be relatively accurate. However, analysis of larger data sets for the whole nation will be necessary before any firm conclusions can be drawn about the net benefit of breast cancer screening for Japanese women. A second limitation is that we did not focus on the other harms of breast cancer screening, such as psychological harm, over-diagnosis, radiation exposure and false-negative results. Psychological harm is said to be transient [14]. Over-diagnosis tends to occur mainly in older women, and methods for calculating it are not well established. Radiation exposure resulting from screening mammography might itself cause breast cancer, but the risk appears negligible [3].

We conclude that the major harms, consisting of false-positive results, unnecessary additional imaging, and the need for biopsy and its invasiveness, are greatest in women in their 40s undergoing breast cancer screening mammography in Japan, but they seemed to be less than those reported by the BCSC and other studies in the US. In the future, it will be necessary to compile more data regarding the mortality reduction and the accompanying harms in order to prove the efficacy of screening mammography in Japanese women age 40–49 years.

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ORIGINAL ARTICLE

Analysis of clinically relevant values of Ki-67 labeling index in Japanese breast cancer patients

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Abstract
Background It has become important to standardize the methods of Ki-67 evaluation in breast cancer patients, especially those used in the interpretation and scoring of immunoreactivity. Therefore, in this study, we examined the Ki-67 immunoreactivity of breast cancer surgical specimens processed and stained in the same manner in one single Japanese institution by counting nuclear immunoreactivity in the same fashion.
Methods We examined 408 Japanese breast cancers with invasive ductal carcinoma and studied the correlation between Ki-67 labeling index and ER/HER2 status and histological grade of breast cancer. We also analyzed overall survival (OS) and disease-free survival (DFS) of these patients according to individual Ki-67 labeling index.
Results There were statistically significant differences of Ki-67 labeling index between ER positive/HER2 negative and ER positive/HER2 positive, ER negative/HER2 positive or ER negative/HER2 negative, and ER positive/HER2 positive and ER negative/HER2 negative groups (all $P < 0.001$). There were also statistically significant differences of Ki-67 labeling index among each histological grade ($P < 0.001$, respectively). As for multivariate analyses, Ki-67 labeling index was strongly associated with OS (HR 39.12, $P = 0.031$) and DFS (HR 10.85, $P = 0.011$) in ER positive and HER2 negative breast cancer patients. In addition, a statistically significant difference was noted between classical luminal A group and "20 % luminal A" in DFS ($P = 0.039$) but not between classical luminal A group and "25 % luminal A" ($P = 0.105$).
Conclusions A significant positive correlation was detected between Ki-67 labeling index and ER/HER2 status and histological grades of the cases examined in our study. The suggested optimal cutoff point of Ki-67 labeling index is between 20 and 25 % in ER positive and HER2 negative breast cancer patients.

Keywords Ki-67 · Breast cancer · Cutoff point · Estrogen receptor · HER2 · Histological grade

Introduction

Tumor proliferation fraction has become an established predictive marker for clinical outcome of breast cancer patients [1–3]. Uncontrolled cell proliferation has also been considered a hallmark of malignancy and can be assessed by various laboratory methods, including counting mitotic figures under light microscopy, flow or image cytometric evaluation of the fraction of the cells in S phase, and immunohistochemistry of various nuclear antigens associated with cell proliferation [3–5]. The proliferation antigen Ki-67 is localized in nuclei of the cells at all phase of the cell cycle except for those at G0 phase and, in particular, the Ki-67 labeling index (percentage of cells with Ki-67

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positive nuclear immunoreactivity) is considered to represent the status of tumor proliferation [1–3, 6, 7].

The statistically significant correlation between the Ki-67 labeling index of carcinoma cells and clinical outcome has been reported in human breast cancer patients [8–10]. Trihia et al. reported that a relatively higher Ki-67 labeling index within the carcinoma was significantly associated with adverse clinical outcome regardless of the subtypes of breast cancer [9, 10]. These results indicate that the Ki-67 labeling index in breast carcinoma cells may confer a higher risk of relapse and subsequently a worse overall survival in those with early breast cancer [8–10].

While results obtained using the Ki-67 labeling index of carcinoma cells resemble those obtained by the Oncotype Dx assay in ER positive and lymph node negative breast cancer patients (largely because the results of the Oncotype Dx assay are based on the status of cell proliferation genes) [11], additional information can be gained from assessing the Ki-67 labeling index within the carcinoma cells. The information obtained from such an assessment is not limited to predictions of prognosis or clinical outcome but also includes prediction of relative responsiveness or resistance to chemotherapy or endocrine therapy in adjuvant settings and the treatment efficacy in tissue specimens obtained before, during, and after neoadjuvant therapy, particularly neoadjuvant endocrine therapy [3]. Because of this additional predictive value, results of the Ki-67 labeling index in carcinoma cells have been incorporated into surgical pathology reports of breast cancer patients in an increasing number of diagnostic pathology laboratories in many countries [3].

However, as in any study utilizing immunohistochemical staining to evaluate clinical samples, it is cardinal and pivotal to standardize the method of Ki-67 measurement, including pre-analytical, analytical, interpretation, and scoring assessment [3], because otherwise results are far from reproducible and applicable in routine clinical settings. This may be particularly true of the methodology used in the stratification of early breast cancer patients into high and low proliferation groups. This stratification is markedly important in clinical settings and many attempts have been made to define the optimal cutoff value [12–14]; however, the reported value suggested to optimally distinguish these two groups of patients has been strikingly variable, from 1 to 28.6 %, thereby markedly limiting its clinical utility [3]. The 12th St. Gallen International Breast Cancer Conference 2011 recommended that patients with ER positive and HER2 negative breast cancer with a Ki-67 labeling index of 14 % or more may be recommended to receive adjuvant chemotherapy in addition to endocrine therapy [12]. The use of this cutoff point must, however, be approached with some caution as Nishimura et al. [13] recently demonstrated that the optimal cutoff of Ki-67 was 25 % in Japanese early breast cancer patients. In addition, the International Ki-67 in Breast Cancer Working Group also proposed that the direct application of specific cutoffs for decision making must be considered unreliable unless analyses were conducted in a highly experienced laboratory with its own reference data [3].

Careful and critical review of the previously reported studies of Ki-67 in human breast cancer revealed that the great majority of Ki-67 labeling index studies have not necessarily been performed under stringent conditions as described above, especially under those recommended by the International Ki-67 in Breast Cancer Working Group. Therefore, in this study, we evaluated the Ki-67 labeling index in breast cancer surgical pathology specimens processed in the same manner in a single institute, Tohoku University Hospital, Sendai, Japan and by the same observers using the same evaluation criteria. We then evaluated the correlation between the Ki-67 labeling index and ER/HER2 status and histological grade in Japanese cases of invasive ductal carcinoma. We then attempted to determine the clinical relevant cutoff value or the percentage of Ki-67 positive invasive breast carcinoma cells that could differentiate eventual clinical outcome of ER positive breast cancer cases.

Materials and methods

Carcinomas

We examined 408 Japanese patients with invasive ductal carcinomas of the breast, all of whom had undergone surgery at Tohoku University Hospital, Sendai and Nahanishi Clinic Okinawa. The study protocol was approved by the Ethics Committee at Tohoku University Graduate School of Medicine. The median age of the patients was 56 years (range 25–89 years). Estrogen receptor (ER) and human epidermal growth factor receptor 2 (HER2) status were reevaluated and summarized as follows: ER positive and HER2 negative, ER positive and HER2 positive, ER negative and HER2 positive, and ER negative and HER2 negative. These specimens had been first cut into 5-mm slices after carefully inking the margins, fixed in 10 % formalin for 46–48 h at room temperature, and embedded in paraffin wax.

Immunohistochemistry

Immunohistochemical analyses were all performed by a single experienced histotechnician at the Department of Pathology, Tohoku University Hospital using the same protocol. All the blocks were freshly cut into 4-μm sections, placed on glue-coated glass slides (Matsunami Glass

Ind., Ltd, Osaka, Japan), and left at room temperature for 3–5 days. Sections were then deparaffinized in xylene, and hydrated with graded alcohols and distilled water at room temperature. Endogenous peroxidase activity was blocked with freshly prepared 3 % hydrogen peroxidase for 10 min at room temperature. Antigen retrieval was performed in an autoclave (Tomy SX-500 high pressure steam sterilizer, Tomy Seiko Co., Ltd., Tokyo, Japan) using citrate buffer for Ki-67 heated at 121 °C for 5 min. Sections were subsequently incubated for 30 min at room temperature in a blocking solution of 10 % rabbit serum (Nichirei Biosciences, Tokyo, Japan) for Ki-67, and then immunostained for 16 h at 4 °C with the primary antibody. The primary antibody of Ki-67 was MIB-1 mouse monoclonal antibody (code M7240; Dako, Copenhagen, Denmark) diluted at 1:300. Secondary antibody reaction for Ki-67 immunohistochemistry was performed using biotinylated rabbit anti-mouse antibody (Nichirei Bioscience) at a dilution of 1:100 for 30 min at room temperature and peroxidase-conjugated avidin (Nichirei Bioscience) was used according to the manufacture's instruction. Reacted sections were visualized using 3,3′-diaminobenzidine-tetrachloride (DAB)/30 % H_2O_2 in 0.05 mol/l Tris buffer (pH 7.6) and counterstained with hematoxylin for nuclear staining. We used the avidin–streptavidin immunoperoxidase method using the clone 6F11 antibody (Ventana, Tucson, AZ, USA) in an automated immunostainer (Benchmark System; Ventana) for immunohistochemistry of ER. A standardized immunohistochemistry kit (Hercep-Test for Immunoenzymatic Staining; Dako) was used for HER2 staining as previously reported [15, 16].

Histopathological analysis

Histopathological evaluations were based on the World Health Organization (WHO) histological classification of tumors of breast and *Rosen's Breast Pathology* [17, 18]. Histological grades were assessed according to the criteria of Elston and Ellis [17, 18]. The Ki-67 immunoreactivity was evaluated independently by two of the authors by first identifying the areas of the most densely stained areas in the whole tissue sections by scanning at low power fields and then counting 1000 carcinoma cells in these areas [3]. We used an Olympus BX50 (Olympus, Tokyo, Japan) and ×20 objectives for the analysis. Figure 1 represents characteristic immunohistochemical findings of Ki-67 positive and negative carcinoma cells (Fig. 1). The presence of ER was determined by distinctive nuclear immunoreactivity and was graded from 0 to 8 using the Allred score, with positivity of the cases defined as a score of 3 [19]. With regard to HER2 evaluation, membranous staining was graded as 0–1+, 2+, and 3+ [20]. The cases scored as 2+ were subjected to FISH to calculate the gene copy ratio of

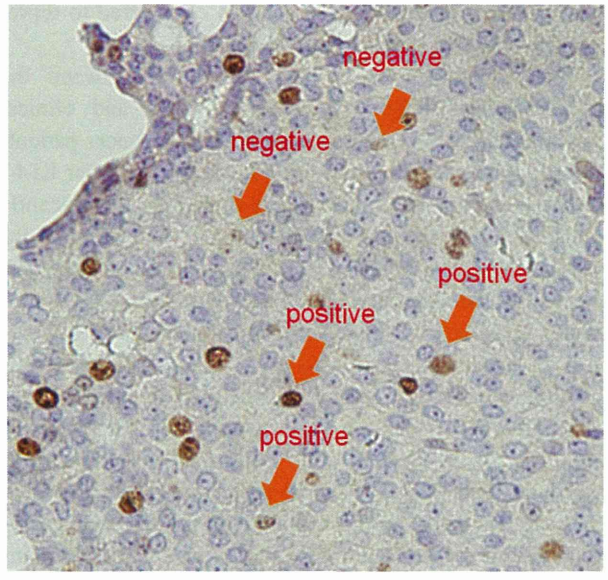

Fig. 1 Representative immunohistochemical findings of Ki-67 positive and negative carcinomas. The specimens were fixed in neutral buffered 10 % formalin and sections stained for Ki-67 with MIB1 antibody (*brown stain*) and counterstained with Mayer's hematoxylin (*blue stain*) (color figure online)

HER2 to CEP17 (PathVysion HER2 DNA Probe kit; Abbott, Chicago, IL, USA), as previously reported [15, 21]. HER2 positive cases were defined as a HER2/CEP17 signal ratio (FISH score) greater than 2.2 [20].

On the basis of the values obtained in the manner above, we examined the correlation between the Ki-67 labeling index and ER/HER2 status and histological grade. We also analyzed overall survival (OS) and disease-free survival (DFS) stratified according to the Ki-67 labeling index, in order to examine the utility of various cutoff points of Ki-67 in predicting clinical outcome within various ER+ breast cancer subgroups (luminal A, luminal B). In order to do this we tentatively assigned luminal A cases as follows: "classical luminal A" as the ER positive and HER2 negative group [22]; "14 % luminal A", based upon the proposal made at the St. Gallen 2011 consensus meeting [12], with a Ki-67 labeling index of less than 14 %; "20 % cutoff luminal A" with a Ki-67 labeling index of less than 20 %; "25 % cutoff luminal A" with a Ki-67 labeling index of less than 25 %; and "30 % cutoff luminal A" with a Ki-67 labeling index of less than 30 % [14, 23]. As for luminal B, we defined "classical luminal B" as ER positive and HER2 positive [24]; "14 % luminal B", proposed at St. Gallen 2011 [12], with a Ki-67 labeling index of more than 14 %; "20 % cutoff luminal B" with a Ki-67 labeling index of more than 20 %; "25 % cutoff luminal B" with a Ki-67 labeling index of more than 25 %; and "30 % cutoff luminal B" with a Ki-67 labeling index of more than 30 % [14, 22].

Statistical analyses

Statistically analyses were performed using StatMate IV for Windows (ATMS, Tokyo, Japan). The Mann–Whitney test was used to assess the correlation between the Ki-67 labeling index and ER/HER2 status and histological grade. The Cox proportional hazards regression model was used for multivariate analyses to evaluate each factor including the Ki-67 labeling index, TNM stages, ER expression, HER2 status, and adjuvant therapy of the patients. The analyses of OS or DFS curves were performed using the Kaplan–Meier method. The results were considered significant at $P < 0.05$.

Results

Correlation between Ki-67 labeling index and ER and HER2 status

Figure 2 summarizes the Ki-67 labeling index results according to ER and HER2 status of the cases examined. The Ki-67 labeling index in carcinoma cells was 11 % (median) and 17.9 % (average) in ER positive/HER2 negative, 40 % (median) and 36.4 % (average) in ER positive/HER2 positive, 40 % (median) and 46.8 % (average) in ER negative/HER2 positive, and 60 % (median) and 56.3 % (average) in ER negative/HER2 negative groups. There were statistically significant differences of the Ki-67 labeling index between ER positive/HER2 negative and ER positive/HER2 positive, ER negative/HER2 positive or ER negative/HER2 negative, and ER positive/HER2 positive and ER negative/HER2 negative groups (all $P < 0.001$).

Correlation between Ki-67 labeling index and histological grades

Figure 3 summarizes the Ki-67 labeling results index in each histological grade of the cases examined. The Ki-67 labeling index was 6 % (median) and 8.5 % (average) in grade 1, 19 % (median) and 24.0 % (average) in grade 2, and 60 % (median) and 55.8 % (average) in grade 3. The Ki-67 labeling index was significantly different between histological grades ($P < 0.001$, respectively).

OS of luminal A and B groups according to Ki-67 labeling index

Table 1 shows the distribution of patients according to the subtypes classical luminal, 14 % luminal, 20 % luminal, 25 % luminal, and 30 % luminal. The 5-year OS rates of patients in luminal A groups were 0.949 in classical luminal A, 1.000 in "14 % luminal A", 1.000 in "20 % luminal A", 1.000 in "25 % luminal A", and 1.000 in "30 % luminal A". There were no statistically significant differences of OS rates among these groups. The 5-year OS rates of luminal B were 1.000 in classical luminal B, 0.875 in "14 % luminal B", 0.853 in "20 % luminal B", 0.822 in "25 % luminal B", and 0.812 in "30 % luminal B". No statistically significant differences were detected among these groups.

DFS of luminal A and B groups according to the Ki-67 labeling index

Figure 4 summarizes the DFS rates of the patients according to each subgroup determined by the Ki-67 labeling index of individual cases. The 5-year DFS rates of patients in luminal A groups were 0.956 in classical luminal A, 1.000 in "14 % luminal A", 0.993 in "20 % luminal A", 0.989 in "25 % luminal A", and 0.983 in "30 % luminal A". There were statistically significant differences between classical luminal A and "14 % luminal A" or "20 % luminal A" ($P = 0.010$ and $P = 0.039$, respectively). A similar tendency was also noted between classical luminal A and "25 % luminal A" or "30 % luminal A" ($P = 0.105$ and 0.159, respectively) but the difference did not reach statistical significance. The 5-year DFS rates of patients in luminal B groups were 0.885 in classical luminal B, 0.880 in "14 % luminal B", 0.871 in "20 % luminal B", 0.840 in "25 % luminal B" and 0.835 in "30 % luminal B". There were no statistically significant differences among these groups above.

Multivariate analyses of OS and DFS according to Ki-67 labeling index

Among the factors examined, including the Ki-67 labeling index, tumor size, nodal status, stage, and adjuvant chemotherapy status, the Ki-67 labeling index was markedly associated with OS (HR 39.12, $P = 0.031$) and DFS (HR 10.85, $P = 0.011$) in ER positive and HER2 negative breast cancer patients. However, the Ki-67 labeling index was not statistically associated with OS (HR 9.28, $P = 0.198$) and DFS (HR 5.76, $P = 0.420$) in all cases including ER positive/HER2 positive, ER negative/HER2 negative, and ER negative/HER2 positive breast cancer patients.

Determination of Ki-67 labeling index cutoff values of carcinoma cells according to the clinical outcome of ER positive breast cancer cases

We evaluated the statistical significance of cutoff values of the Ki-67 labeling index in carcinoma cells segregated by 5 %. There were no statistically significant differences in OS of the patients. A statistically significant difference was

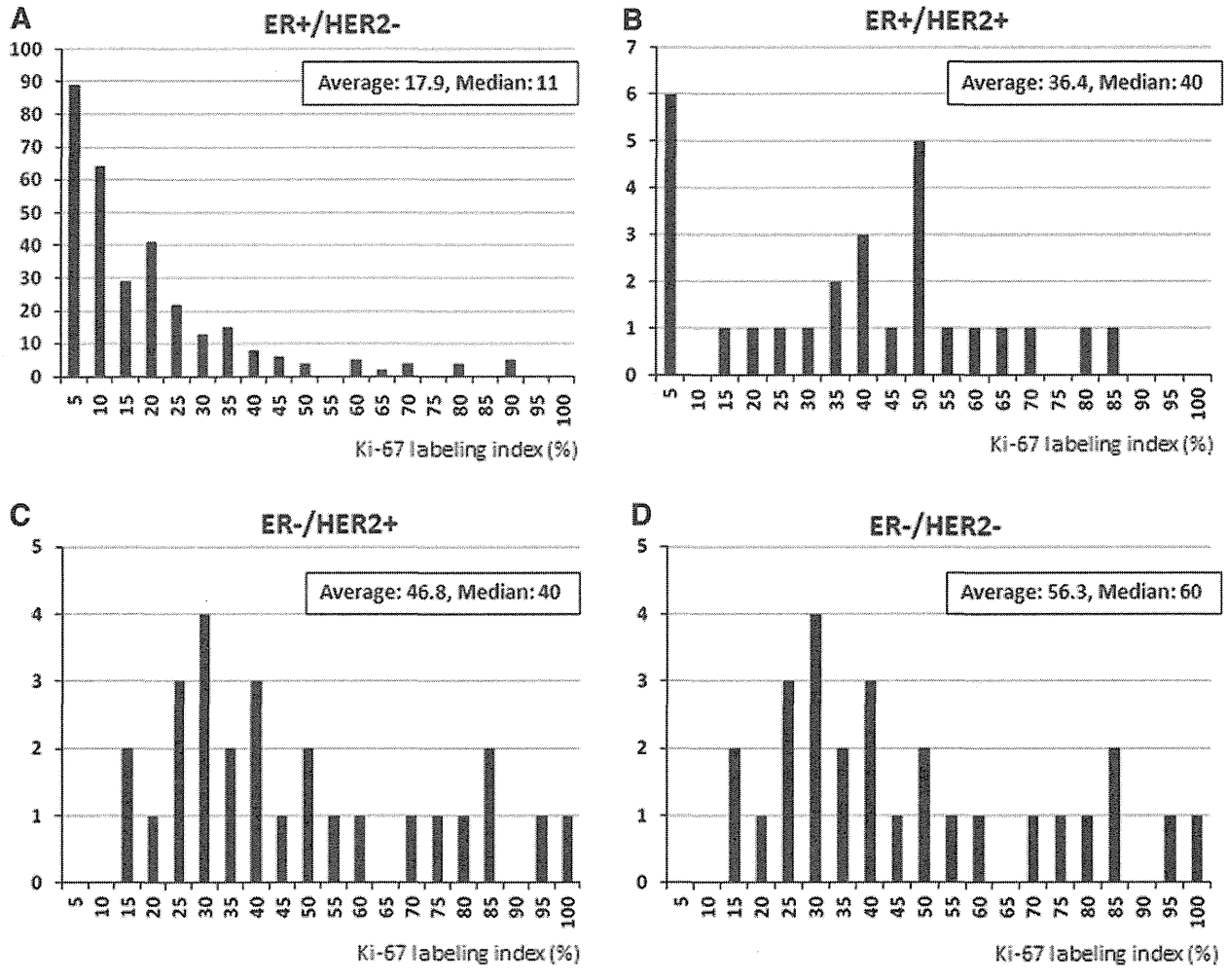

Fig. 2 Correlation between Ki-67 labeling index and ER or HER2 status. The distribution of Ki-67 labeling index in **a** ER positive and HER2 negative cases, **b** ER positive and HER2 positive cases, **c** ER negative and HER2 positive cases, **d** ER negative and HER2 negative cases

noted between classical luminal A group and "20 % luminal A" in DFS ($P = 0.039$) but not between classical luminal A group and "25 % luminal A" ($P = 0.105$). Therefore, the optimal cutoff point of the Ki-67 labeling index was suggested to be between 20 and 25 %.

Discussion

Ki-67 has been established as a well-known biomarker of cell proliferation in many human malignancies including breast cancer. The Ki-67 labeling index has been utilized to obtain both prognosis and prediction of the sensitivity to systemic therapy of breast cancer patients [2, 10, 21]. Some examples of this are the statistically significant correlation between a high Ki-67 labeling index of carcinoma cells and increased risk of cancer relapse and death in breast cancer patients [10] and the utility of mid-course evaluation of Ki-67 labeling index, even after 2 weeks of endocrine therapy, in predicting the subsequent response to endocrine therapy in ER positive breast cancer patients [23]. In addition the group of breast cancer patients associated with a high Ki-67 labeling index studied in the Breast International Group trial (BIG) 1-98 was associated with a potential clinical benefit in selecting letrozole over tamoxifen in post-menopausal patients [2]. Despite these important aspects of Ki-67 immunohistochemistry, the necessary standardized guidelines have not been developed [12, 25].

The International Ki-67 in Breast Cancer Working Group recently recommended the fixation of the specimens with neutral buffered formalin for 4–48 h or more and the counting of at least 500 invasive carcinoma cells using MIB-1 mouse monoclonal antibody [3]. In our present study, all the specimens examined had been processed in the same manner and according to the guidelines above and the Ki-67 labeling index was also evaluated accordingly.

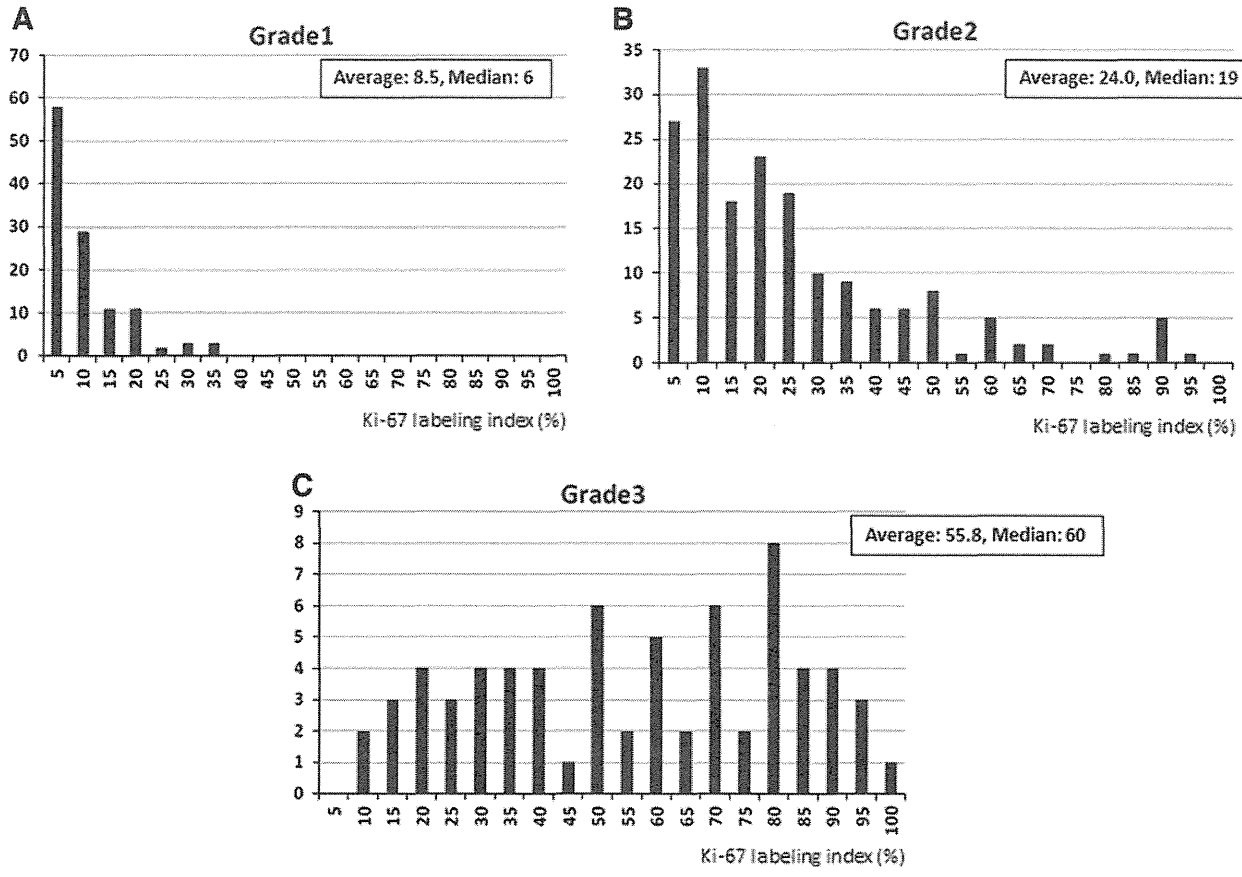

Fig. 3 Correlation between Ki-67 labeling index and histological grade of the patients. The distribution of Ki-67 labeling index in **a** grade 1, **b** grade 2, **c** grade 3 groups

Table 1 Distribution of patients according to the subtypes classical luminal, 14 % luminal, 20 % luminal, 25 % luminal, and 30 % luminal

	n	Ki-67 (median %)	Ki-67 (average %)
Classical lum A	289	11	17.9
14 % lum A	160	5	6.0
20 % lum A	186	6	7.5
25 % lum A	215	8	9.2
30 % lum A	225	9	10.1
Classical lum B	23	40	36.4
14 % lum B	152	27	33.2
20 % lum B	126	31	36.7
25 % lum B	97	35	41.1
30 % lum B	87	40	42.9

Previous studies conducted by Nishimura et al. [26–28] on Japanese breast cancer patients demonstrated that the Ki-67 value as significantly higher in triple negative cases. However, the Ki-67 labeling index was also statistically lower in ER positive/HER2 negative cases [26–28]. We therefore examined the correlation between the Ki-67 labeling index and hormone receptor, HER2 status, or histological grade using surgical pathology specimens processed in the same manner and immunostained in the same fashion by one single experienced histotechnician in one single institution.

The results of our present study demonstrated that the ER positive and HER2 negative group was associated with a significantly lower Ki-67 labeling index of carcinoma cells than in other subtypes examined. The cases with a high Ki-67 labeling index in the ER positive and HER2 negative group have been considered as potential candidates for receiving chemotherapy in addition to endocrine therapy as in the patients with a high histological grade [12–14]. In our present study, there was also a statistically significant correlation between the Ki-67 labeling index and histological grades of individual cases. Collectively our findings suggest that it may be better to review the slides when there is a significant discrepancy between the results of Ki-67 labeling index and histological grade in invasive ductal carcinoma cases. The results of our present study also demonstrated that subtyping of the tumors using immunohistochemical surrogate markers such as ER,

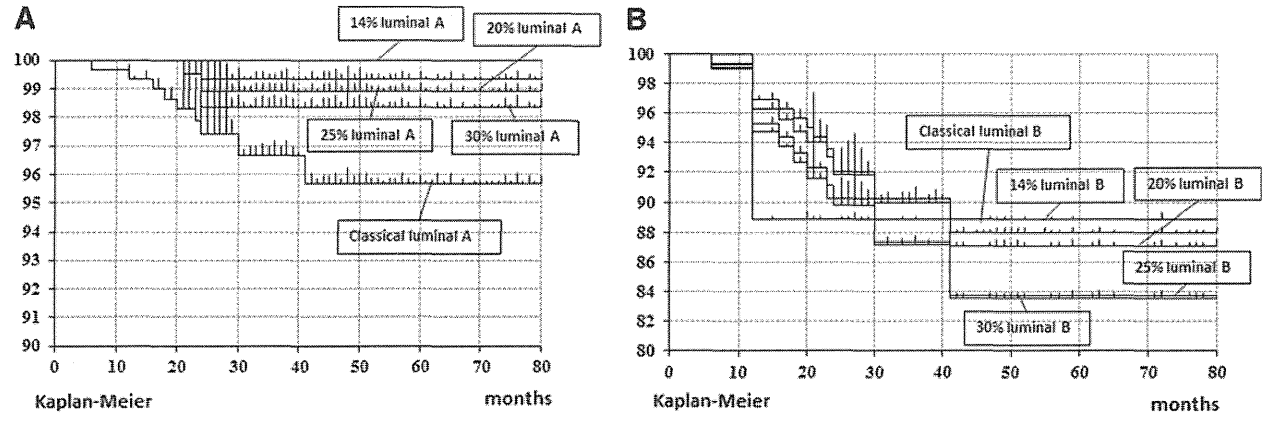

Fig. 4 DFS according to Ki-67 labeling index of the patients. **a** Luminal A: *classical luminal A* ER positive and HER2 negative; *14 % luminal A* Ki-67 labeling index less than 14 %; *20 % luminal A* Ki-67 labeling index less than 20 %; *25 % luminal A* Ki-67 labeling index less than 25 %; *30 % luminal A* Ki-67 labeling index less than 30 %. **b** Luminal B: *classical luminal B* ER positive and HER2 positive; *14 % luminal B* Ki-67 labeling index more than 14 %; *20 % luminal B* Ki-67 labeling index more than 20 %; *25 % luminal B* Ki-67 labeling index more than 25 %; *30 % luminal B* Ki-67 labeling index more than 30 %

HER2, and Ki-67, if using appropriately processed surgical pathology specimens and well-controlled immunohistochemical procedures, could at least contribute to identifying high-risk Japanese breast cancer patients within the hormone receptor positive subgroup of breast cancers. Nishimura et al. [26] also indicated that ER/PgR, HER2, and Ki-67 are all important biological markers for predicting prognosis and making effective treatment decisions in Japanese breast cancer patients by using only these biomarkers. The combination of these markers has been proposed at least in defining luminal A and B types of breast cancer without necessarily performing gene profiling studies with some exceptions [12, 29]. Luminal B type breast cancer represents a clinically important subgroup generally associated with adverse clinical outcome regardless of systemic adjuvant therapy [19]. It was recently recommended at the St. Gallens consensus meeting that chemotherapy was indicated for the majority of these patient defined as ER positive and with a Ki-67 labeling index of more than 14 % [12]. However, it is also true that the optimal cutoff points of the Ki-67 labeling index in these cases have been reported as 10–25 % [3, 12]. For instance, no pathological responders were reported in the cases with more than 25 % Ki-67 in neoadjuvant chemotherapy of Japanese breast cancer patients [13]. These discrepancies or variations of proposed values of Ki-67 labeling may be all due to differences of methodologies involved in obtaining the Ki-67 labeling index including pre-analytical factors such as fixation of the specimens and/or ethnical or racial backgrounds of the patients and further investigations are required for clarification.

The direct application of a specific cutoff for clinical decision making may be considered unreliable unless analyses are conducted in a highly experienced laboratory with its own reference data [3]. The International Ki-67 in Breast Cancer Working Group demonstrated that no consensus has been reached regarding the ideal cutoff point of the Ki-67 labeling index. The results of our present study demonstrated that there were statistically significant differences of DFS between classical luminal A and luminal with a 14 or 20 % cutoff of Ki-67. In addition, we examined the cutoff values of the Ki-67 labeling index segregated by 5 %. A statistically significant difference was noted between classical luminal A group and "20 % luminal A" in DFS but not between classical luminal A group and "25 % luminal A". Therefore, we propose an optimal cutoff point of the Ki-67 labeling index of between 20 and 25 %. These results were similar to that of a previous study from Japan mentioned above [13]. Therefore, ER positive and HER2 negative Japanese breast cancer patients with a Ki-67 labeling index of 20–25 % are associated with more aggressive biological course than those not and additional chemotherapy may be of further help or benefit to these patients.

It was recently proposed that the prognostic information provided by ER, PgR, HER2, and Ki-67 immunostaining performed in a rigorously controlled fashion was considered at least equivalent to that provided by 21 gene signature analysis and highlights the relevance of these readily available routine histopathological parameters in the clinical management of early ER positive breast cancer [30]. In addition, we demonstrated using multivariate analysis that the Ki-67 labeling index was one of the most important prognostic factors for the ER positive and HER2 negative group in this study. Therefore, it has become important to standardize the type of fixation, time to fixation, appropriate primary antibody, and methods of immunostaining and interpretation, especially in countries like Japan where

the expensive gene signature tests are and will be out of reach for the great majority of breast cancer patients. We also noted the statistically significant correlation between the Ki-67 labeling index and ER/HER2 status and histological grade of individual patients performed in a single institution. It is true that our present study was retrospective, the number of the patients is relatively small, and the patients were all Japanese but the results still provided sufficient evidence to support the value of the Ki-67 labeling index in the clinical management of breast cancer patients. Further investigations employing larger numbers of patients with longer periods of clinical follow-up may be required for determining the most clinically relevant cutoff points of the Ki-67 labeling index in breast cancer patients, especially those in the early stage in order to confer the maximal clinical benefits upon individual breast cancer patients.

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Conflict of interest The authors have no conflict of interest.

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Reproductive factors and breast cancer risk in relation to hormone receptor and menopausal status in Japanese women

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The associations between menstrual and reproductive factors and breast cancer risk in relation to estrogen/progesterone receptor (ER/PgR) status have been unclear in Japanese women. This case-control study evaluated these associations, overall and separately, by menopausal status. A total of 1092 breast cancer cases and 3160 controls were selected from among female patients aged 30 years and over admitted to a single hospital in Miyagi Prefecture between 1997 and 2009. The receptor status distribution among the cases (missing: 8.4%) was 571 ER+/PgR+, 133 ER+/PgR−, 24 ER−/PgR+ and 271 ER−/PgR−. Menstrual and reproductive factors were assessed using a self-administered questionnaire. Polytomous logistic regression and tests for heterogeneity across ER+/PgR+ and ER−/PgR− were conducted. Later age at menarche was significantly associated with a decreased risk of both ER+/PgR+ and ER−/PgR− cancer among women overall (P_{trend} = 0.0016 for ER+/PgR+; P_{trend} = 0.015 for ER−/PgR−) and among postmenopausal women (P_{trend} = 0.012 for ER+/PgR+; P_{trend} = 0.0056 for ER−/PgR−). Nulliparity was associated with an increased risk of ER+/PgR+, but not ER−/PgR− cancer among women overall ($P_{heterogeneity}$ = 0.019) and among postmenopausal women (odds ratio for ER+/PgR+ = 2.56, 95% confidence interval = 1.61–4.07; $P_{heterogeneity}$ = 0.0095). A longer duration of breastfeeding tended to be associated with a decreased risk in all subtypes among women overall. Later age at menarche has a protective effect against both ER+/PgR+ and ER−/PgR− cancer. However, parity might impact differently on various subtypes of breast cancer. Further studies are needed to clarify the etiology of the rare ER+/PgR− and ER−/PgR+ cancer subtypes. (*Cancer Sci* 2012; 103: 1861–1870)

Over the past few decades, numerous epidemiologic studies of breast cancer have been conducted, based mainly on Caucasian populations. These studies show that menstrual and reproductive factors and menopausal status are associated with breast cancer risk.[1,2] In Japan, cohort studies,[3,4] case-control studies[5–7] and a meta-analysis have revealed similar associations.[8]

Breast cancers are known to express the estrogen receptor (ER) or progesterone receptor (PgR). Tumor subtypes defined by these receptors represent biologically different entities.[9] In Western countries, many studies have evaluated breast cancer risk according to hormone receptor status.[10–13] A meta-analysis shows that nulliparity is associated with a higher risk of ER+ tumors, but not ER− tumors.[13] Another meta-analysis suggests that nulliparity is associated with an increased risk of ER+/PgR+ tumors, but not ER−/PgR− tumors. The protective effects of late age at menarche and longer duration of breastfeeding do not differ across ER/PgR status.[12]

Among studies conducted in the Asian region, a large-scale case-control study from China evaluates risk factors defined according to the four types of hormone receptor status and finds an association with parity history similar to that in the abovementioned meta-analysis.[14] Although a few epidemiologic studies focus on the hormone receptor status of breast cancer in Japan,[15–17] their results are inconsistent. One study shows that parity, the number of births and age at menarche have different associations with the risk of breast cancer according to ER and PR status.[15] Another study shows that only age at menarche is differently associated with the risk of breast cancer according to ER status.[16] A third study shows no gradient in the risk associated with reproductive factors,[17] including age at menarche, age at menopause, age at first birth, parity number and duration of breastfeeding. In most of the Japanese studies, however, hormone receptor data are incomplete, and, therefore, the percentage of breast cancer cases for which the hormone receptor status is unknown is relatively large. Consequently, the sample sizes might have been too small to allow comprehensive evaluation of breast cancer risk according to hormone receptor status. The inconsistencies among the results obtained in these Japanese studies are likely attributable to such limitations.

Therefore, we conducted a hospital-based case-control study to precisely evaluate the association between reproductive factors and breast cancer risk according to hormone receptor status. Data were obtained from women aged 30 years and over who were admitted to a single hospital in Miyagi Prefecture, Japan. Analyses were performed based on joint ER and PR status; that is, ER+/PgR+, ER+/PgR−, ER−/PgR+ and ER−/PgR−. In this study, data on hormone receptor status were available for over 90% of the breast cancer cases included.

Methods

Data collection. In January 1997, we began a questionnaire survey in connection with the present study. Information on lifestyle and personal history was collected from all patients at their first admission to the Miyagi Cancer Center Hospital (MCCH) using a self-administered questionnaire. The questionnaire was distributed to patients on the day of their reservation for initial admission (i.e. 10–15 days before admission) and collected by nurses on the actual day of admission. The MCCH is located in Natori City, situated in the southern part of Miyagi Prefecture, and functions as a hospital for both

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cancer and benign disease. Details of the questionnaire survey have been described elsewhere.[18–21]

The questionnaire covered demographic characteristics, personal and family histories of cancer and other diseases, including family history of breast cancer in mother or sisters, current height and weight, general lifestyle factors before the development of current symptoms, including cigarette smoking, alcohol drinking, physical activity, occupation, menstrual and reproductive histories, and history of oral contraceptives (OC) and other exogenous female hormone uses. Items related to the referral base were also included. The items related to menstrual and reproductive histories included age at menarche, menopausal status, age at menopause, parity history, parity number, age at first birth, history of breastfeeding, duration of breastfeeding and quantity of milk secretion. A question on the duration of breastfeeding was added after 2000. Between January 1997 and December 2009, the questionnaire was distributed to 23 531 first-admitted patients, of whom 21 056 responded.

Study subjects. Cases and controls were selected from among patients who responded to the above questionnaire survey. To identify incident cases of female breast cancer, a list of the patients was linked with the hospital-based cancer registry files. The registry records all cancer cases confirmed by clinical, cytological and/or histopathological examination at the MCCH. Through linkage to the registry, 21 056 patients were classified into 1812 with a past history of cancer, 6848 male patients with cancer, 1096 female patients with breast cancer, 4171 female patients with other cancers, and 7129 non-cancer patients (3708 male and 3421 female patients). Among the 1096 female patients with breast cancer, 1092 aged 30 years and over were included as the cases for the present study.

Controls were selected from among female non-cancer patients. Patients with benign tumors were classified as non-cancer patients for the present study. Accordingly, 3160 female non-cancer patients aged 30 years and over were identified as controls. The diagnoses among the controls were as follows: benign tumor in 1824 (57.7%), cardiovascular disease in 116 (3.7%), digestive tract disease in 377 (11.9%), respiratory tract disease in 122 (3.9%), urologic-gynecologic disease in 170 (5.4%), other benign disease in 302 (9.5%) and no abnormal findings in 249 patients (7.9%). The sites of benign tumors were the digestive tract in 637 subjects, gynecologic organs in 375, urologic organs in 17, breast in 36, bone or connective tissue in 545 and other sites in 214. The final response rate in the questionnaire survey was 94.1% for the case group and 89.8% for the control group.

This study was approved by the ethical review board of the Miyagi Cancer Center and was conducted in accordance with the principles specified in the Declaration of Helsinki. We considered the return of self-administered questionnaires signed by the subjects to imply their consent to participate in the study.

Hormone receptor status. Information on hormone receptor status (i.e. expression of the ER and PgR in breast cancers) was extracted from medical records. In brief, enzyme immunoassays (EIA) were used in the early period of the study to determine hormone receptor status. After mid-2003, immunohistochemistry (IHC) assays were conducted on tumor tissue samples. The cut-off point for receptor positivity in the EIA was 14 fmol/mg for ER and 13 fmol/mg for PgR. In the IHC assay, a histology score (HSCORE) of ≥ 20 for ER and one of ≥ 6 for PgR were evaluated as positive.[22] The concordance between the two assays was 94.3% for ER and 100% for PgR in the laboratory of the MCCH.[22] Among the total of 1092 cases, data on joint ER/PgR status were available for 1000 (91.6%); 571 cases were ER+/PgR+, 133 were ER+/PgR−, 24 were ER−/PgR+ and 271 were ER−/PgR−.

Statistical analysis. We used multiple polytomous unconditional logistic regression analysis to estimate odds ratios (OR) and 95% confidence intervals (CI) for hormone receptor-defined breast cancer risk in relation to menstrual and reproductive factors, family history of breast cancer, use of OC, and use of exogenous female hormones other than OC.

The exposure variables analyzed in the present study were menstrual and reproductive factors (age at menarche, menopausal status, age at menopause, parity, parity number, age at first birth, history of breastfeeding, total duration of breastfeeding and quantity of milk secretion), family history of breast cancer in mother or sisters (yes or no), history of OC use (ever or never) and use of exogenous female hormones other than OC (ever or never). For history of breastfeeding (formula only, mixed breastfeeding and formula, or breastfeeding only), use of formula only was recognized as no history of breastfeeding, and used as a reference. Breastfeeding only and mixed breastfeeding and formula were both regarded as a positive history of breastfeeding.

We considered the following variables to be potential confounders: age, referral base (from screening or other), area of residence (southern Miyagi Prefecture or other), year of recruitment, smoking (ever or never), alcohol drinking (ever or never), occupation (housewife or other), body mass index (BMI) and physical activity (more or less than 1 h per week). BMI was calculated as weight divided by squared height (kg/m^2). In the analysis, menstrual and reproductive factors and history of breast cancer in mother or sisters were also adjusted for each other. Missing values for confounders were treated as an additional variable category, and were included in the model.

In the analysis, we stratified case subjects according to joint hormone receptor status. Stratification by menopausal status was also performed. Menopause was defined as the cessation of menstrual periods due to natural or other reasons, including surgery. With regard to menopause due to other reasons, we were unable to obtain any information about history of oophorectomy; therefore, case subjects aged 45–57 years and controls aged 43–57 years (defined as the mean age at natural menopause ±2 SD) were regarded as patients with unknown menopausal status. In the analysis stratified by menopausal status, case subjects who had ER+/PgR− or ER−/PgR+ tumors were too few to allow precise estimation of OR in comparison with subjects who had ER+/PgR+ or ER−/PgR− tumors; therefore, we excluded these subjects from the analysis according to menopausal status.

Dose-response relationships were tested by treating each exposure category as a continuous variable. We conducted Wald tests for estimating the heterogeneity of breast cancer risk across ER+/PgR+ and ER−/PgR−. Values were considered significant if the two-sided P were <0.05. All analyses were performed using SAS version 9.3 (SAS Institute, Cary, NC, USA).

Results

The background characteristics of the study subjects are presented in Table 1. Among the case subjects included in the analysis (n = 1000), 416 were premenopausal, 555 were postmenopausal and 29 were undefined. Among the premenopausal subjects, 260 (62.5%) were ER+/PgR+, 44 (10.6%) were ER+/PgR−, 12 (2.9%) were ER−/PgR+ and 100 (24.0%) were ER−/PgR−. Among the postmenopausal subjects, 300 (54.1%) were ER+/PgR+, 87 (15.7%) were ER+/PgR−, 11 (2.0%) were ER−/PgR+ and 157 (28.3%) were ER−/PgR−. Among the control subjects (n = 3160), 1081 (34.2%) were premenopausal, 1963 (62.1%) were postmenopausal and 116 (3.7%) were undefined. Cases with ER+/PgR+ tumors tended to be heavier, and were more likely to be referred from screening, to engage in physical activity and to be drinkers. Cases with

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Table 1. Background characteristics in cases and controls

	All					
	Cases					
	Hormone receptor status					Controls
	ER+/PgR+	ER+/PgR−	ER−/PgR+	ER−/PgR−	Missing	
Total (*n*)	572	133	24	271	92	3160
Menopausal status (*n*)[a]						
Premenopausal	260	44	12	100	22	1081
Postmenopausal	300	87	11	157	43	1963
Unknown menopausal status	12	2	1	14	27	116
Age group (years old) (%)						
30–39	6.1	3.8	4.2	5.9	10.9	8.4
40–49	25.0	18.8	41.7	23.2	13.0	17.6
50–59	28.5	28.6	20.8	30.6	34.8	22.1
60–69	23.4	28.6	12.5	22.9	20.7	25.3
≥70	17.0	20.3	20.8	17.3	20.7	26.5
Average	57.2	59.2	56.3	57.2	57.7	59.6
SD	12.6	11.7	14.0	12.1	12.9	13.7
BMI (%)						
<18.5	4.9	6.0	—	4.8	9.8	5.8
18.5–25	59.4	62.4	62.5	65.3	60.9	63.4
25–30	27.6	26.3	37.5	24.0	22.8	26.0
≥30	8.0	5.3	—	5.5	3.3	4.2
Missing	—	—	—	0.4	3.3	0.7
Average	24.1	23.6	23.9	23.5	23.0	23.5
SD	3.8	3.6	3.1	3.8	3.6	3.6
Year of recruitment (%)						
1997–2002	24.7	39.8	58.3	45.4	50.0	54.7
2003–2009	75.3	60.2	41.7	54.6	50.0	45.3
Area of residence (%)						
Southern Miyagi Prefecture	82.7	85.0	87.5	83.4	78.3	88.4
Other	17.3	15.0	12.5	16.6	21.7	11.6
Referral base (%)						
From screening	21.2	20.3	16.7	13.3	8.7	18.1
Other	78.8	79.7	83.3	86.7	91.3	81.9
Occupation (%)						
Housewife	20.1	21.8	25.0	20.7	31.5	21.4
Other	68.2	68.4	54.2	66.4	54.3	61.7
Missing	11.7	9.8	20.8	12.9	14.1	16.9
Physical activity (%)						
More than 1 h per week	43.9	43.6	41.7	40.2	41.3	44.9
<1 h per week	50.2	50.4	54.2	51.7	50.0	47.4
Missing	5.9	6.0	4.2	8.1	8.7	7.7
Smoking (%)						
Never	79.9	79.7	66.7	81.5	80.4	80.0
Ever	17.7	17.3	20.8	16.2	17.4	15.6
Missing	2.4	3.0	12.5	2.2	2.2	4.4
Alcohol drinking (%)						
Never	68.4	78.9	70.8	69.7	75.0	71.3
Ever	28.7	20.3	12.5	26.9	19.6	23.3
Missing	3.0	0.8	16.7	3.3	5.4	5.3

[a]Menopause was defined as the cessation of menstrual periods due to natural or other reasons including surgery. BMI, body mass index; ER, estrogen receptor; PgR, progesterone receptor.

ER−/PgR− tumors tended to be lighter, and were less likely to be referred from screening, to engage in physical activity, and to be smokers. Cases with unknown ER/PgR status were less likely to be referred from screening in comparison with the other subtypes.

Table 2 shows the OR and 95% CI for menstrual and reproductive factors, family history of breast cancer, and exogenous female hormone use according to the four hormone receptor subtypes. A later age at menarche is significantly associated with a decreased risk of ER+/PgR+ (P_{trend} = 0.0016; OR = 0.61, 95% CI 0.45–0.83 for ≥15 years) and ER−/PgR− (P_{trend} = 0.015; OR = 0.57, 95% CI 0.38–0.86 for ≥15 years) cancer. Natural menopause (OR = 0.64, 95% CI 0.49–0.84) and menopause due to other reasons (OR = 0.53, 95% CI 0.35–0.80) are associated with a lower risk of ER+/PgR+ cancer in comparison with premenopause. Nulliparity is associated with a higher risk of ER+/PgR+ cancer (OR = 1.30, 95% CI 0.96–1.78; P = 0.094), but

Table 2. OR (95% CI) of breast cancer risk by hormone receptor status associated with risk factors

	Control	ER+/PgR+ (n = 572)				ER+/PgR− (n = 133)				ER−/PgR+ (n = 24)				ER−/PgR− (n = 271)				$P_{heterogeneity}$ ER+/PgR+ *vs* ER−/PgR−
		Case	OR	95% CI	P	Case	OR	95% CI	P	Case	OR	95% CI	P	Case	OR	95% CI	P	
Age at menarche																		
≤12	691	187	1.00 (reference)[a]			27	1.00 (reference)[a]			7	1.00 (reference)[a]			81	1.00 (reference)[a]			
13	600	133	0.93	0.71–1.21		25	1.10	0.62–1.95		5	0.95	0.29–3.16		60	0.85	0.59–1.22		
14	574	105	0.83	0.62–1.11		36	1.82	1.05–3.15		4	0.83	0.22–3.13		57	0.89	0.61–1.31		
≥15	1021	128	0.61	0.45–0.83		41	1.28	0.71–2.32		7	0.73	0.20–2.74		61	0.57	0.38–0.86		
P for trend					0.0016				0.23				0.62				0.015	0.93
Menopausal status																		
Premenopause	1081	260	1.00 (reference)[b]			44	1.00 (reference)[b]			12	1.00 (reference)[b]			100	1.00 (reference)[b]			
Natural menopause	1424	241	0.64	0.49–0.84		74	1.20	0.72–2.02		11	0.59	0.19–1.89		128	1.22	0.83–1.80		
Menopause due to other reason	539	59	0.53	0.35–0.80		13	0.71	0.32–1.58		0	–	–		29	0.95	0.53–1.69		
Parity																		
Parous	2590	460	1.00 (reference)[c]			112	1.00 (reference)[c]			21	1.00 (reference)[c]			234	1.00 (reference)[c]			
Nulliparous	235	69	1.30	0.96–1.78	0.094	10	0.94	0.47–1.85	0.85	1	0.48	0.06–3.72	0.48	16	0.65	0.38–1.11	0.12	0.019
Age at first birth[i]																		
≤24	1242	179	1.00 (reference)[d]			49	1.00 (reference)[d]			2	1.00 (reference)[d]			102	1.00 (reference)[d]			
25–29	1071	213	1.26	1.00–1.59		47	1.09	0.71–1.66		15	9.04	1.92–42.68		99	0.97	0.72–1.32		
≥30	211	57	1.57	1.08–2.30		15	1.77	0.91–3.44		3	7.80	1.13–54.07		30	1.31	0.81–2.11		
P for trend					0.0086				0.17				0.009				0.48	0.26
Parity number[i]																		
1	273	58	1.00 (reference)[e]			13	1.00 (reference)[e]			4	1.00 (reference)[e]			36	1.00 (reference)[e]			
2	1243	250	1.03	0.73–1.45		57	1.07	0.55–2.05		13	0.67	0.19–2.30		120	0.77	0.51–1.17		
3	773	115	0.91	0.62–1.35		35	1.22	0.59–2.50		1	0.12	0.01–1.21		64	0.71	0.44–1.15		
4	214	29	1.02	0.60–1.73		4	0.58	0.18–1.90		1	0.43	0.04–4.71		11	0.52	0.25–1.08		
≥5	87	8	0.87	0.38–1.99		3	1.23	0.32–4.77		2	2.28	0.29–18.15		3	0.39	0.11–1.36		
P for trend					0.59				0.94				0.64				0.045	0.17
Breastfeeding[i]																		
Formula only	410	89	1.00 (reference)[f]			26	1.00 (reference)[f]			3	1.00 (reference)[f]			41	1.00 (reference)[f]			
Mixed breastfeeding and formula	1268	262	0.99	0.75–1.32		58	0.78	0.48–1.27		13	1.70	0.45–6.44		134	1.10	0.75–1.60		
Breastfeeding only	891	107	0.73	0.53–1.02		28	0.60	0.33–1.08		4	0.72	0.14–3.74		59	0.88	0.57–1.37		
Total month of breastfeeding[i]																		

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Table 2 (continued)

	Control	ER+/PgR+ (n = 572)				ER+/PgR− (n = 133)				ER−/PgR+ (n = 24)				ER−/PgR− (n = 271)				$P_{heterogeneity}$ ER+/PgR+ *vs* ER−/PgR−
		Case	OR	95% CI	P	Case	OR	95% CI	P	Case	OR	95% CI	P	Case	OR	95% CI	P	
0–3	394	143	1.00 (reference)[f]			29	1.00 (reference)[f]			7	1.00 (reference)[f]			56	1.00 (reference)[f]			
3–12	302	74	0.70	0.50–0.97		17	0.79	0.42–1.49		3	0.39	0.08–1.97		37	0.84	0.53–1.32		
12–24	396	89	0.65	0.47–0.89		18	0.61	0.32–1.13		3	0.47	0.11–2.10		31	0.57	0.35–0.93		
>24	478	94	0.68	0.48–0.97		21	0.59	0.30–1.16		1	0.07	0.004–0.99		36	0.61	0.36–1.03		
P for trend					0.013				0.082				0.04				0.023	0.58
Quantity of breast milk secretion[i]																		
Poor or no	761	168	1.00 (reference)[f]			43	1.00 (reference)[f]			11	1.00 (reference)[f]			74	1.00 (reference)[f]			
Fair	876	141	0.82	0.64–1.06		28	0.61	0.37–1.01		6	0.44	0.14–1.37		82	1.08	0.77–1.52		
Good	885	141	0.80	0.62–1.04		38	0.82	0.52–1.31		3	0.30	0.08–1.16		67	0.90	0.63–1.29		
Family history of breast cancer in mother or sisters																		
No	3037	524	1.00 (reference)[g]			116	1.00 (reference)[g]			21	1.00 (reference)[g]			238	1.00 (reference)[g]			
Yes	123	48	2.14	1.49–3.08	<.0001	17	3.52	2.03–6.09	<.0001	3	4.06	1.15–14.31	0.029	33	3.51	2.32–5.31	<.0001	0.044
Oral contraceptives use																		
Never	2604	504	1.00 (reference)[h]			115	1.00 (reference)[h]			22	1.00 (reference)[h]			241	1.00 (reference)[h]			
Ever	158	30	0.90	0.59–1.37	0.62	8	1.22	0.57–2.59	0.61	0	–	–	–	16	1.03	0.60–1.78	0.91	0.68
Use of exogenous female hormones other than oral contraceptives																		
Never	2588	498	1.00 (reference)[h]			112	1.00 (reference)[h]			21	1.00 (reference)[h]			241	1.00 (reference)[h]			
Ever	134	26	0.86	0.55–1.36	0.52	9	1.56	0.76–3.19	0.23	1	0.79	0.10–6.17	0.82	11	0.79	0.42–1.50	0.47	0.82

All models were adjusted by age, BMI (<18.5, 18.5–25, 25–30, ≥30), smoke (never, current or past), alcohol (never, current or past), occupation (housewife, other), physical activity (<1 h per week, more than 1 h per week), year of recruitment (continuous), area (southern Miyagi Prefecture, other) and reference (from screening, other). [a]Additionally adjusted by family history of breast cancer (yes, no), parity number (0, 1, 2, 3, 4, ≥5). [b]Additionally adjusted by family history of breast cancer, age at menarche (≤12, 13, 14, ≥15), parity number (0, 1, 2, 3, 4, ≥5). [c]Additionally adjusted by family history of breast cancer, age at menarche. [d]Additionally adjusted by family history of breast cancer, age at menarche, parity number (1, 2, 3, 4, ≥5). [e]Additionally adjusted by family history of breast cancer, age at menarche, age at first birth (≤24, 25–29, ≥30). [f]Additionally adjusted by family history of breast cancer, age at menarche, age at first birth, parity number (1, 2, 3, 4, ≥5). [g]Additionally adjusted by parity number (0, 1, 2, 3, 4, ≥5). [h]Additionally adjusted by family history of breast cancer, age at menarche, parity number (0, 1, 2, 3, 4, ≥5). [i]For parous women only. BMI, body mass index; CI, confidence interval; ER, estrogen receptor; OR, odds ratio; PgR, progesterone receptor.