not ER−/PgR− cancer ($P_{heterogeneity}$ = 0.019). An older age at first birth is significantly associated with an increased risk of ER+/PgR+ cancer ($P_{trend}$ = 0.0086; OR = 1.26, 95% CI 1.00–1.59 for ≥25–≤29 years; OR = 1.57, 95% CI 1.08–2.30 for ≥30 years) and ER−/PgR+ cancer ($P_{trend}$ = 0.009; OR = 9.04, 95% CI 1.92–42.68 for ≥25–≤29 years; OR = 7.80, 95% CI 1.13–54.07 for ≥30 years). Multiparity is associated with a decreased risk of ER−/PgR− cancer ($P_{trend}$ = 0.045). Breastfeeding only and a good quantity of breast milk secretion are associated with a decreased risk of cancers for all hormone receptor subtypes, but not statistically significantly. Data on duration of breastfeeding were available for 2222 subjects (52.3%). A longer period of breastfeeding is associated with a lower risk of cancers of all subtypes, although the result for ER+/PgR− is not statistically significant ($P_{trend}$ = 0.013 for ER+/PgR+, $P_{trend}$ = 0.082 for ER+/PgR−, $P_{trend}$ = 0.04 for ER−/PgR+ and $P_{trend}$ = 0.023 for ER−/PgR−). A family history of breast cancer in mother or sisters is significantly associated with an increased risk of all subtypes. The heterogeneity test for a family history of breast cancer reveals a significant difference in risk across ER+/PgR+ and ER−/PgR− tumors ($P_{heterogeneity}$ =0.044). The use of OC and exogenous female hormones other than OC is not significantly associated with breast cancer risk for any of the subtypes.

Table 3 shows the results according to ER+/PgR+ and ER−/PgR− status among premenopausal women. A later age at menarche is marginally associated with a decreased risk of ER+/PgR+ cancer ($P_{trend}$ = 0.056). An older age at first birth is significantly associated with an increased risk of ER+/PgR+ cancer ($P_{trend}$ = 0.027). However, tests of heterogeneity between the risks of ER+/PgR+ and ER−/PgR− cancer show non-significance for these factors. A family history of breast cancer is positively associated with the risk of both ER+/PgR+ and ER−/PgR− cancer.

Table 4 shows the results for postmenopausal women. A later age at menarche is associated with a decreased risk of both ER+/PgR+ and ER−/PgR− cancer ($P_{trend}$ = 0.012 and 0.0056, respectively). Nulliparity is positively associated with a risk of ER+/PgR+ cancer, but not ER−/PgR− cancer ($P_{heterogeneity}$ = 0.0095). Among parous women, no dose-response relationship with parity number is observed for either of the receptors. A longer period of breastfeeding is associated with a lower risk of both ER+/PgR+ and ER−/PgR− cancer; however, this is not statistically significant ($P_{trend}$ = 0.062 and 0.076, respectively). A family history of breast cancer is associated with an increased risk of both ER+/PgR+ and ER−/PgR− cancer; the magnitude of the risk appears to be greater for ER−/PgR− cancer ($P_{heterogeneity}$ = 0.052; OR = 3.23, 95% CI 1.86–5.62).

## Discussion

This hospital-based case-control study revealed the associations between menstrual and reproductive factors and breast cancer risk in terms of joint hormone receptor status. A few epidemiologic studies conducted in Japan have focused on tumor subtypes.[15–17] However, it has been difficult to determine whether the associations among Japanese women differ from those among Western women. It is known that the proportion of tumor subtypes differs across menopausal status[23] and the breast cancer patients' survival rates are reported to be different according to tumor subtypes.[24] Etiology and biology of subtypes might be different from each other. Therefore, the present study is important for clarifying the impact of menstrual and reproductive factors on breast cancer risk in relation to tumor subtypes and menopausal status among Japanese women.

Regarding menstrual factors, a meta-analysis showed that a late age at menarche is associated with a decreased risk of ER+/PgR+ and ER−/PgR− cancers.[12] A recent study from China demonstrates a similar association.[14] In the present study, a later age at menarche is associated with a decreased risk of ER+/PgR+ and ER−/PgR− cancers among both women overall and postmenopausal women. It has been hypothesized that a later age at menarche might result in a shorter proliferation of mammary gland cells, which might be more susceptible to carcinogenesis.[25] This hypothesis might explain the association between a later age at menarche and a lower risk for both ER+/PgR+ and ER−/PgR− cancer.

Parity, parity number and age at first birth have been recognized as factors affecting breast cancer risk in Japan and other countries.[12,14,15,17] A meta-analysis has shown that nulliparity[13] and a higher age at first birth[12,13] are associated with an increased risk of ER+/PgR+ cancer, indicating that the effects differ between ER+/PgR+ and ER−/PgR− status.[12] In the present study, nulliparity was associated with an increased risk of ER+/PgR+ cancer among both women overall and postmenopausal women, and higher age at first birth was associated with an increased risk of ER+/PgR+ cancer among women overall. A significant association with age at first birth was also observed for ER−/PgR+ cancer. However, as the confidence interval was wide, the result for ER−/PgR+ cancer is questionable. Our overall analysis also showed that multiparity was associated with a decreased risk of ER−/PgR− cancer. Nulliparity showed a decreased risk, but not statistically significant. Although the precise mechanism is unknown, it has been reported that successive multiparity induces a protective effect through sequential differentiation of mammary gland stem cells;[26] such cells are thought to be associated with ER−/PgR− cancer.[27]

A meta-analysis has shown that breastfeeding is associated with a decreased risk of ER+/PgR+ and ER−/PgR− cancer.[12] A previous study from the Asian region demonstrates an association between a longer duration of breastfeeding and a decreased risk of ER+/PgR+ cancer, but not ER−/PgR− cancer.[14] Meanwhile, studies in Japan have indicated no association between breastfeeding and the risk of either receptor-positive or negative breast cancer.[16,17] Although our data for the risk of ER−/PgR+ cancer were based on a small sample size, our findings suggest that a longer period of breastfeeding might protect against all subtypes of breast cancer, being almost consistent with the findings of the abovementioned meta-analysis.

With regard to the risk associated with a family history of breast cancer, a meta-analysis reveals a positive association between a history of breast cancer in mother or sisters and breast cancer risk among both premenopausal and postmenopausal women.[28] Our previous study conducted in Miyagi prefecture also demonstrates such an association.[3] In the present study, the increased risk posed by a family history of breast cancer is consistently observed for all subtypes. In addition, there is a variation in the magnitude of risk among the subtypes. A higher OR for family history is found for ER−/PgR− cancer ($P_{heterogeneity}$ = 0.044). The mechanism might include genetic mutation, such as BRCA1,[29] which has been associated with a positive family history of breast cancer, and might confer susceptibility to ER−/PgR− cancer.[30]

The present study had both strengths and limitations. First, we considered comparability between cases and controls. We selected the controls from among patients admitted to the same hospital as the cases. The participation rates were high for both cases and controls. However, the distribution of risk factors for breast cancer among control subjects may have differed from that in the general population. To improve comparability between the cases and controls, statistical analyses were appropriately controlled for background characteristics, such as area of residence and referral patterns. Although persistent bias


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**Table 3. OR (95% CI) of breast cancer risk by hormone receptor status among premenopausal women**

| | Control | Premenopausal | | | | | | | $P_{heterogeneity}$ |
|---|---|---|---|---|---|---|---|---|---|
| | | ER+/PgR+ ($n$ = 260) | | | | ER−/PgR− ($n$ = 100) | | | |
| | | Case | OR | 95% CI | $P$ | Case | OR | 95% CI | $P$ | |
| Age at menarche | | | | | | | | | | |
| ≤12 | 465 | 129 | 1.00 (reference)[a] | | | 49 | 1.00 (reference)[a] | | | |
| 13 | 270 | 67 | 0.94 | 0.66–1.35 | | 22 | 0.77 | 0.45–1.32 | | |
| 14 | 183 | 41 | 0.91 | 0.60–1.40 | | 13 | 0.70 | 0.36–1.36 | | |
| ≥15 | 150 | 21 | 0.48 | 0.26–0.87 | | 16 | 1.08 | 0.54–2.18 | | |
| $P$ for trend | | | | | 0.056 | | | | 0.72 | 0.37 |
| Parity | | | | | | | | | | |
| Parous | 891 | 215 | 1.00 (reference)[b] | | | 88 | 1.00 (reference)[b] | | | |
| Nulliparous | 144 | 33 | 0.82 | 0.52–1.27 | 0.37 | 9 | 0.58 | 0.28–1.21 | 0.15 | 0.41 |
| Age at first birth[h] | | | | | | | | | | |
| ≤24 | 401 | 75 | 1.00 (reference)[c] | | | 34 | 1.00 (reference)[c] | | | |
| 25–29 | 391 | 104 | 1.28 | 0.90–1.84 | | 41 | 1.04 | 0.63–1.72 | | |
| ≥30 | 89 | 34 | 1.85 | 1.07–3.19 | | 13 | 1.57 | 0.74–3.34 | | |
| $P$ for trend | | | | | 0.027 | | | | 0.35 | 0.57 |
| Parity number[h] | | | | | | | | | | |
| 1 | 135 | 29 | 1.00 (reference)[d] | | | 11 | 1.00 (reference)[d] | | | |
| 2 | 445 | 125 | 1.38 | 0.84–2.29 | | 55 | 1.62 | 0.79–3.35 | | |
| 3 | 250 | 51 | 1.28 | 0.72–2.28 | | 18 | 0.97 | 0.41–2.29 | | |
| 4 | 47 | 8 | 1.32 | 0.52–3.34 | | 3 | 0.93 | 0.23–3.76 | | |
| ≥5 | 14 | 2 | 0.88 | 0.17–4.51 | | 1 | 0.97 | 0.10–9.39 | | |
| $P$ for trend | | | | | 0.78 | | | | 0.44 | 0.4 |
| Breastfeeding[h] | | | | | | | | | | |
| Formula only | 160 | 42 | 1.00 (reference)[e] | | | 13 | 1.00 (reference)[e] | | | |
| Mixed breastfeeding and formula | 542 | 136 | 0.92 | 0.60–1.41 | | 57 | 1.28 | 0.67–2.44 | | |
| Breastfeeding only | 188 | 36 | 0.63 | 0.36–1.09 | | 18 | 1.43 | 0.65–3.13 | | |
| Total month of breastfeeding[h] | | | | | | | | | | |
| 0–3 | 155 | 77 | 1.00 (reference)[e] | | | 16 | 1.00 (reference)[e] | | | |
| 3–12 | 120 | 37 | 0.63 | 0.39–1.03 | | 13 | 1.35 | 0.59–3.10 | | |
| 12–24 | 130 | 41 | 0.60 | 0.37–0.98 | | 12 | 0.98 | 0.41–2.36 | | |
| >24 | 108 | 34 | 0.70 | 0.40–1.22 | | 10 | 1.15 | 0.44–2.96 | | |
| $P$ for trend | | | | | | | 0.091 | | 0.88 | 0.28 |
| Quantity of breast milk secretion[h] | | | | | | | | | | |
| Poor or no | 317 | 86 | 1.00 (reference)[e] | | | 26 | 1.00 (reference)[e] | | | |
| Fair | 299 | 62 | 0.72 | 0.49–1.07 | | 32 | 1.38 | 0.78–2.42 | | |
| Good | 257 | 64 | 0.80 | 0.54–1.19 | | 26 | 1.45 | 0.80–2.64 | | |
| Family history of breast cancer in mother or sisters | | | | | | | | | | |
| No | 1046 | 239 | 1.00 (reference)[f] | | | 89 | 1.00 (reference)[f] | | | |
| Yes | 35 | 21 | 2.86 | 1.56–5.23 | 0.0007 | 11 | 4.34 | 2.07–9.07 | <.0001 | 0.31 |
| Oral contraceptives use | | | | | | | | | | |
| Never | 942 | 227 | 1.00 (reference)[g] | | | 91 | 1.00 (reference)[g] | | | |
| Ever | 99 | 25 | 1.22 | 0.74–2.01 | 0.44 | 8 | 0.91 | 0.42–1.97 | 0.8 | 0.5 |
| Use of exgenous female hormones other than oral contraceptives | | | | | | | | | | |
| Never | 943 | 228 | 1.00 (reference)[g] | | | 90 | 1.00 (reference)[g] | | | |
| Ever | 73 | 17 | 0.95 | 0.53–1.72 | 0.87 | 6 | 1.00 | 0.41–2.42 | 0.99 | 0.93 |

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), 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). [c]Additionally adjusted by family history of breast cancer, age at menarche, parity number (1, 2, 3, 4, ≥5). [d]Additionally adjusted by family history of breast cancer, age at menarche, age at first birth (≤24, 25–29, ≥30). [e]Additionally adjusted by family history of breast cancer, age at menarche, age at first birth, parity number (1, 2, 3, 4, ≥5). [f]Additionally adjusted by parity number (0, 1, 2, 3, 4, ≥5). [g]Additionally adjusted by family history of breast cancer, age at menarche, parity number (0, 1, 2, 3, 4, ≥5). [h]For parous women only. BMI, body mass index; CI, confidence interval; ER, estrogen receptor; OR, odds ratio; PgR, progesterone receptor.

might exist, it is likely that any problems with comparability have been weakened. Second, the problem of limited statistical power must be considered in the analysis of ER−/PgR+ cancer; the results for this subtype might be inconclusive because of the small number of cases. To confirm the risk for ER−/PgR+ cancer, further studies are needed. Third, we must evaluate the possibility of information bias. Self-reported information on exposure might have been vulnerable to misclassification. However, any such misclassification in reproductive factors would have been non-differential.[31] This bias

**Table 4. OR (95% CI) of breast cancer risk by hormone receptor status among postmenopausal women**

| | Control | Postmenopausal | | | | | | | | $P_{heterogeneity}$ |
|---|---|---|---|---|---|---|---|---|---|---|
| | | ER+/PgR+ ($n$ = 300) | | | | ER−/PgR− ($n$ = 157) | | | | |
| | | Case | OR | 95% CI | $P$ | Case | OR | 95% CI | $P$ | |
| Age at menarche | | | | | | | | | | |
| ≤12 | 194 | 54 | 1.00 (reference)[a] | | | 28 | 1.00 (reference)[a] | | | |
| 13 | 304 | 64 | 0.87 | 0.56–1.35 | | 35 | 0.79 | 0.46–1.37 | | |
| 14 | 375 | 61 | 0.71 | 0.46–1.10 | | 41 | 0.80 | 0.47–1.37 | | |
| ≥15 | 855 | 105 | 0.61 | 0.40–0.93 | | 43 | 0.46 | 0.26–0.80 | | |
| $P$ for trend | | | | | 0.012 | | | | 0.0056 | 0.48 |
| Age at natural menopause | | | | | | | | | | |
| ≤47 | 259 | 37 | 1.00 (reference)[b] | | | 19 | 1.00 (reference)[b] | | | |
| 48–50 | 546 | 98 | 1.31 | 0.85–2.03 | | 42 | 1.00 | 0.56–1.79 | | |
| 51–53 | 398 | 62 | 0.93 | 0.58–1.50 | | 42 | 1.23 | 0.68–2.21 | | |
| ≥54 | 167 | 42 | 1.64 | 0.97–2.76 | | 22 | 1.47 | 0.75–2.87 | | |
| $P$ for trend | | | | | 0.38 | | | | 0.17 | 0.56 |
| Parity | | | | | | | | | | |
| Parous | 1620 | 238 | 1.00 (reference)[c] | | | 136 | 1.00 (reference)[c] | | | |
| Nulliparous | 85 | 33 | 2.56 | 1.61–4.07 | <.0001 | 6 | 0.76 | 0.32–1.81 | 0.54 | 0.0095 |
| Age at first birth[i] | | | | | | | | | | |
| ≤24 | 800 | 101 | 1.00 (reference)[d] | | | 64 | 1.00 (reference)[d] | | | |
| 25–29 | 648 | 107 | 1.26 | 0.92–1.74 | | 55 | 0.90 | 0.61–1.35 | | |
| ≥30 | 117 | 22 | 1.16 | 0.65–2.09 | | 14 | 1.11 | 0.56–2.22 | | |
| $P$ for trend | | | | | 0.26 | | | | 0.96 | 0.43 |
| Parity number[i] | | | | | | | | | | |
| 1 | 131 | 28 | 1.00 (reference)[e] | | | 21 | 1.00 (reference)[e] | | | |
| 2 | 757 | 122 | 0.73 | 0.44–1.21 | | 62 | 0.47 | 0.27–0.84 | | |
| 3 | 497 | 61 | 0.66 | 0.38–1.15 | | 43 | 0.57 | 0.31–1.06 | | |
| 4 | 163 | 21 | 0.86 | 0.43–1.70 | | 8 | 0.44 | 0.18–1.09 | | |
| ≥5 | 72 | 6 | 0.64 | 0.23–1.78 | | 2 | 0.30 | 0.06–1.41 | | |
| $P$ for trend | | | | | 0.51 | | | | 0.18 | 0.48 |
| Breastfeeding[i] | | | | | | | | | | |
| Formula only | 234 | 45 | 1.00 (reference)[f] | | | 25 | 1.00 (reference)[f] | | | |
| Mixed breastfeeding and formula | 683 | 123 | 1.06 | 0.71–1.58 | | 71 | 1.06 | 0.64–1.76 | | |
| Breastfeeding only | 683 | 69 | 0.76 | 0.48–1.20 | | 40 | 0.92 | 0.51–1.64 | | |
| Total month of breastfeeding[i] | | | | | | | | | | |
| 0–3 | 226 | 65 | 1.00 (reference)[f] | | | 38 | 1.00 (reference)[f] | | | |
| 3–12 | 173 | 37 | 0.74 | 0.46–1.19 | | 19 | 0.56 | 0.30–1.04 | | |
| 12–24 | 248 | 48 | 0.70 | 0.45–1.09 | | 19 | 0.54 | 0.29–1.00 | | |
| >24 | 361 | 58 | 0.63 | 0.38–1.04 | | 26 | 0.60 | 0.31–1.17 | | |
| $P$ for trend | | | | | 0.062 | | | | 0.076 | 0.73 |
| Quantity of breast milk secretion[i] | | | | | | | | | | |
| Poor or no | 420 | 79 | 1.00 (reference)[f] | | | 44 | 1.00 (reference)[f] | | | |
| Fair | 550 | 79 | 0.93 | 0.65–1.35 | | 45 | 0.95 | 0.60–1.50 | | |
| Good | 602 | 73 | 0.78 | 0.54–1.13 | | 40 | 0.82 | 0.51–1.32 | | |
| Family history of breast cancer in mother or sisters | | | | | | | | | | |
| No | 1879 | 274 | 1.00 (reference)[g] | | | 138 | 1.00 (reference)[g] | | | |
| Yes | 84 | 26 | 1.67 | 1.01–2.76 | 0.044 | 19 | 3.23 | 1.86–5.62 | <.0001 | 0.052 |
| Oral contraceptives use | | | | | | | | | | |
| Never | 1586 | 267 | 1.00 (reference)[h] | | | 139 | 1.00 (reference)[h] | | | |
| Ever | 52 | 5 | 0.49 | 0.19–1.30 | 0.15 | 7 | 1.39 | 0.59–3.28 | 0.46 | 0.095 |
| Use of exgenous female hormones other than oral contraceptives | | | | | | | | | | |
| Never | 1571 | 262 | 1.00 (reference)[h] | | | 141 | 1.00 (reference)[h] | | | |
| Ever | 56 | 7 | 0.68 | 0.29–1.60 | 0.38 | 4 | 0.64 | 0.22–1.85 | 0.41 | 0.93 |

All models were adjusted by age, BMI (<18.5, 18.5–25, 25–30, ≥30), smoke (never, current or past, missing), alcohol (never, current or past), occupation (housewife, other), physical activity (<1 h per week, more than 1 h per week), menopausal status (natural menopause, menopause due to other reason), age at menopause (≤47, 48–50, 51–53, ≥54), year of recruitment (continuous), area (southern Miyagi Prefecture, other), 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.



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is unlikely to have distorted our present results. Fourth, it is possible that the inclusion of patients with benign tumors in the control group influenced the results, because patients with benign tumors of the gynecologic organs or breast might have a background similar to that of patients with breast cancer. Therefore, we performed additional analyses by excluding patients with benign tumors of the gynecologic organs ($n$ = 375) or breast ($n$ = 36) from the controls. However, the exclusion of these patients had no effect on the OR (data not shown).

One of the strengths of our study was the stability of menopausal status. In any prospective study, some of the premenopausal women in the original cohort may become postmenopausal by the end of follow up.[3,4,15] In contrast, any case-control study like the present one has information on menopausal status at the time of diagnosis. Another strength of our study was the low rate of missing data (8.4%) for hormone receptor status. Although missing cases were less likely to have been referred from screening, the distribution of the hormone receptor statuses in our study was roughly the same as that in a large previous study in Japan.[32] Compared with our present study, the rates of missing data in previous studies, including cohort studies, which ranged from 9% to 61%, were relatively high.[10,11,14,15,17] Cancer incidence in Japanese cohort studies has been evaluated based on population-based cancer registries.[3,33,34] However, data on hormone receptor status in population-based cancer registries are incomplete.[3,33,34] Therefore, hospital-based studies would be more suitable for assessing the risk of breast cancer by hormone receptor status.[18] From this viewpoint, the present study is considered to represent one of the most accurately conducted assessments of breast cancer risk in terms of hormone receptor status.

In conclusion, this hospital-based case-control study has clarified risk factor profiles according to breast cancer subtypes stratified by joint hormone receptor status and menopausal status. A later age at menarche is associated with a decreased risk of both ER+/PgR+ and ER−/PgR− among women overall and postmenopausal women. Nulliparity is associated with an increased risk of ER+/PgR+, but not ER−/PgR−, among postmenopausal women and women overall. A longer duration of breastfeeding is associated with a decreased risk of all subtypes among women overall. These results indicate that a later age at menarche has a protective effect against both ER+/PgR+ and ER−/PgR− cancer, but that parity might impact differently on various subtypes of breast cancer. A longer duration of breastfeeding might protect against breast cancer, irrespective of receptor type. Further studies are needed to clarify the etiology of the rare ER+/PgR− and ER−/PgR+ cancer subtypes among Japanese women.

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## Disclosure Statement

The authors have no conflict of interest to declare.

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# Oestrogen-induced genes in ductal carcinoma *in situ*: their comparison with invasive ductal carcinoma

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## Abstract

It is well known that oestrogens play important roles in both the pathogenesis and development of invasive ductal carcinoma (IDC) of human breast. However, molecular features of oestrogen actions have remained largely unclear in pure ductal carcinoma *in situ* (pDCIS), regarded as a precursor lesion of many IDCs. This is partly due to the fact that gene expression profiles of oestrogen-responsive genes have not been examined in pDCIS. Therefore, we first examined the profiles of oestrogen-induced genes in oestrogen receptor (ER)-positive pDCIS and DCIS (DCIS component (DCIS-c)) and IDC (IDC component (IDC-c)) components of IDC cases ($n=4$ respectively) by microarray analysis. Oestrogen-induced genes identified in this study were tentatively classified into three different groups in the hierarchical clustering analysis, and 33% of the genes were predominantly expressed in pDCIS rather than DCIS-c or IDC-c cases. Among these genes, the status of *MYB* (C-MYB), *RBBP7* (RBAP46) and *BIRC5* (survivin) expressions in carcinoma cells was significantly higher in ER-positive pDCIS ($n=53$) than that in ER-positive DCIS-c ($n=27$) or IDC-c ($n=27$) by subsequent immunohistochemical analysis of the corresponding genes ($P<0.0001$, $P=0.03$ and $P=0.0003$ respectively). In particular, the status of C-MYB immunoreactivity was inversely ($P=0.006$) correlated with Ki67 in the pDCIS cases. These results suggest that expression profiles of oestrogen-induced genes in pDCIS may be different from those in IDC; and C-MYB, RBAP46 and survivin may play important roles particularly among oestrogen-induced genes in ER-positive pDCIS.

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## Introduction

Breast cancer is the most common malignant neoplasm in women worldwide. In particular, the incidence of ductal carcinoma *in situ* (DCIS) has been markedly increasing possibly due to advancements in population-based mammographic screening for detection (Li *et al.* 2005), and ~20% of breast carcinoma cases actually present as pure DCIS (pDCIS) without invasive components at the time of diagnosis in many countries (Kepple *et al.* 2006, Tsikitis & Chung 2006). This pDCIS is in general considered as a precursor lesion of invasive ductal carcinoma (IDC). It has been demonstrated that approximately half of untreated pDCIS progresses to IDC with marked variability in the latency of the progression (Cuzick 2003) and up to 80% of IDC were also reported to contain at least small foci of DCIS component (DCIS-c) distinct from the IDC component (IDC-c) if carefully evaluated (Ellis *et al.* 2003). Therefore, it has become very important to examine the biological features of pDCIS to identify the possible molecular mechanisms related to the acquisition of invasive

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properties and subsequently to improve clinical outcome of early breast cancer patients.

It is well known that oestrogens play important roles in the progression of breast carcinoma through an interaction with oestrogen receptor (ER). ER is expressed in approximately two-thirds of IDC, and endocrine therapy has been administered in these patients in order to suppress the intratumoural oestrogen actions. A great majority of pDCIS was also reported to express ER in their parenchymal cells (Wiechmann & Kuerer 2008), and the results of National Surgical Adjuvant Breast Project (NSABP) B-24 trial did demonstrate that adjuvant tamoxifen therapy was clinically effective in ER-positive pDCIS and reduced the recurrence of noninvasive breast carcinomas by 27% (Cuzick 2003). Pathological and biological responses to preoperative tamoxifen therapy in ER-positive pDCIS patients has been also reported (Chen *et al.* 2009).

ER is well known to activate the transcription of various target genes in a ligand-dependent manner, and various oestrogenic functions are also characterised by expression profiles of these genes in oestrogen target cells. Various oestrogen-responsive genes have been also identified in IDC (Frasor *et al.* 2003), and an analysis of these genes can greatly contribute to the understanding of molecular functions of oestrogen actions, such as cell proliferation, anti-apoptosis, invasion, metastasis, recurrence and resistance to endocrine therapy, in IDC (Suzuki *et al.* 2012). However, expression profiles of oestrogen-responsive genes have not necessarily been examined in pDCIS to the best of our knowledge. Therefore, it has still remained unclear whether oestrogen actions and/or effectiveness of endocrine therapy in pDCIS could be the same as that in IDC.

Therefore, in this study, we first examined expression profiles of oestrogen-induced genes in carcinoma tissues of breast cancer patients and demonstrated different expression profiles of oestrogen-induced genes in ER-positive pDCIS from ER-positive DCIS-c or IDC-c following an isolation of the corresponding cells under light microscopy using laser-capture dissection. Subsequent microarray analysis indicated that *MYB* (C-MYB), *RBBP7* (retinoblastoma suppressor (Rb)-associated protein 46 (RBAP46)) and *BIRC5* (survivin) were predominantly expressed in pDCIS compared with DCIS-c and IDC-c among these oestrogen-induced genes. Therefore, we subsequently immunolocalised these gene products in ER-positive pDCIS tissues in order to further characterise their oestrogenic actions.

## Materials and methods

### Patients and tissues

Two sets of tissue specimens were used in this study. The first set is composed of eight specimens of ER-positive breast carcinoma (four pDCIS and four IDC cases) obtained from Japanese women (age: 51–77 years in pDCIS, and 49–75 years in IDC) who underwent surgical treatment from 2003 to 2008 in the Department of Surgery, Tohoku University Hospital, Sendai, Japan. One IDC patient was premenopausal, and the others were postmenopausal. All the IDC specimens used in this study contained both DCIS-c and IDC-c, and the patients did not receive chemotherapy, irradiation or hormonal therapy before the surgery. All the cases examined in this study were associated with nuclear grade 1 or 2, and their ER labelling index (LI) was ranged from 40 to 96% in pDCIS, 35 to 100% in DCIS-c and 42 to 100% in IDC-c respectively. These specimens were stored at −80 °C for subsequent microarray analysis. The second set is composed of 80 specimens of ER-positive ductal carcinoma of human breast (53 pDCIS and 27 IDC cases) obtained from Japanese female patients who underwent surgical treatment from 1995 to 2008 in the Department of Surgery, Tohoku University Hospital, Sendai, Japan. These patients also did not receive chemotherapy, irradiation or hormonal therapy before the surgery. The median age of these patients was 61 years (range 39–80 years) for pDCIS and 55 (range 32–84 years) for IDC, and all the cases of IDC contained both DCIS-c and IDC-c in this study. All the specimens were fixed in 10% formalin and embedded in paraffin wax.

The entire resected surgical specimen was sectioned into slices with 3–5 mm thickness, and all the slices were histologically evaluated by surgical pathologists. In this study, pDCIS was defined when DCIS-c was detected but no foci of stromal invasion in carcinoma were detected in all the slides of the cases evaluated. In the first set, thinner section stained with haematoxylin and eosin was prepared from the frozen specimen, and histological features of these lesions were confirmed.

Research protocols for this study were approved by the Ethics Committee at Tohoku University Graduate School of Medicine (accession no. 2009-107).

### Laser-capture microdissection/microarray analysis

Gene expression profiles of breast carcinoma cells in the first set of the specimens (four pDCIS, four DCIS-c and four IDC-c samples) were examined using microarray analysis. Laser-capture microdissection

(LCM) was conducted using the MMI Cellcut (Molecular Machines and Industries, Flughofstrase, Glattbrugg, Switzerland). Briefly, breast carcinomas were embedded in Tissue-Tek optimal cutting temperature compound (Sakura Finetechnical Co., Tokyo, Japan) and sectioned at a thickness of 10 μm. Breast carcinoma cells were dissected under the light microscopy and laser transferred from these frozen sections. The total RNA (~200 ng) was subsequently extracted from these cell fractions isolated by LCM using the RNeasy Micro Kit (Qiagen). In IDC cases, carcinoma cells were separately collected in DCIS-c and IDC-c. Whole Human Genome Oligo Microarray (G4112F (ID: 012391)), Agilent Technologies (Waldbronn, Germany), containing 41 000 unique probes, was used in this study, and sample preparation and processing were performed according to the manufacturer's protocol. In this study, we focused on the expression of 51 genes identified to be oestrogen-induced ones in MCF7 breast carcinoma cells by Frasor *et al.* (2003) (two genes corresponding *PPP2R1B* were included in this analysis). Hierarchical clustering analysis was performed using the Cluster and TreeView programs (the software copyright Stanford University 1998–1999, http://rana.stanford.edu) to generate tree structures based on the degree of similarity, as well as matrices comparing the levels of expression of individual genes in each specimens.

### Immunohistochemistry

Immunohistochemical analysis was performed in the second set (53 pDCIS and 27 IDC cases) described above. Monoclonal antibodies for ER (6F11), progesterone receptor (PR; 1A6) and Ki67 (MIB1) were purchased from NovoCastra (Newcastle upon Tyne, UK), Chemicon (Temecula, CA, USA) and DAKO (Carpinteria, CA, USA) respectively. Rabbit polyclonal antibodies for human epidermal growth factor receptor-2 (HER2; A0485) were obtained from DAKO. In addition, rabbit polyclonal antibodies for C-MYB (EPR718(2)), RBAP46 (EPR5082) and survivin (NB500-201) were purchased from Epitomics (Burlingame, CA, USA) and Novus Biologicals (Littleton, CO, USA) respectively.

A Histofine Kit (Nichirei Biosciences, Tokyo, Japan) that employs the streptavidin–biotin amplification method was used in this study. Antigen retrieval was performed by heating the slides in an autoclave at 120 °C for 5 min in antigen retrieval solution (pH 9.0; Nichirei Biosciences) for C-MYB immunostaining or citric acid buffer (2 mM citric acid and 9 mM trisodium citrate dehydrate (pH 6.0)) for immunostaining of other antibodies. Dilutions of primary antibodies used in this study were as follows: ER, 1/50; PR, 1/50; HER2, 1/100; Ki67, 1/100; C-MYB, 1/50; RBAP46, 1/1000 and survivin, 1/1000. The antigen–antibody complex was subsequently visualised with 3,3′-diaminobenzidine (DAB) solution (1 mM DAB, 50 mM Tris–HCl buffer (pH 7.6) and 0.006% $H_2O_2$) and counterstained with haematoxylin. As a positive control, human IDC tissue was used for C-MYB (McHale *et al.* 2008) and survivin (Barnes *et al.* 2006) immunostaining, and a cellblock of MCF7 breast carcinoma cells was used for RBAP46 (Creekmore *et al.* 2008). Normal rabbit IgG was used instead of the primary antibody, as a negative control in this study.

### Immunohistochemical evaluation

Immunoreactivity of ER, PR and Ki67 was detected in the nucleus, and their immunoreactivity was evaluated in counting more than 1000 carcinoma cells for each case. The percentage of immunoreactivity, i.e. LI, was subsequently determined. Cases with ER LI of more than 1% were considered ER-positive breast carcinoma in this study (Hammond *et al.* 2010). HER2 immunoreactivity was evaluated according to the grading system proposed in HercepTest (DAKO), and strongly circumscribed membrane-immunoreactivity of HER2 present in more than 30% carcinoma cells were considered positive (Wolff *et al.* 2007). Both C-MYB and RBAP46 immunoreactivities were detected in the nuclei of carcinoma cells and were evaluated by employing the H-scoring system (McCarty *et al.* 1985). Briefly, C-MYB- and RBAP46-positive carcinoma cells were classified into three groups according to immunointensity (i.e. strongly, moderately or weakly positive cells), and H scores were subsequently generated by adding together 3×% of strongly positive cells, 2×% of moderately positive cells, 1×% weakly positive cells, and 0×% of negative cells (range 0–300). Survivin immunoreactivity was detected in the cytoplasm of carcinoma cells and was semi-quantitatively evaluated by modified H-scoring system (Mehta *et al.* 2012), in which the percentage of cytoplasmic immunoreactivity was categorised as 0 (no expression), 10 (up to 10%), 20 (10–20%) until 100 (90–100%), and giving a possible range of 0–300.

### Statistical analysis

An association of various clinicopathological factors among three carcinoma components (pDCIS, DCIS-c and IDC-c) was evaluated using a Kruskal–Wallis test or a cross-table with the $\chi^2$ test. An association between C-MYB, RBAP46 and survivin immunoreactivity and

clinicopathological factors was evaluated by a cross-table using the $\chi^2$ test. An association of clinicopathological factors between two components of IDC cases was evaluated using a Wilcoxon signed-ranks test. The statistical analyses were performed using the JMP Pro version 9.02 (SAS Institute, Inc., Cary, NC, USA), and $P$ values of $<0.05$ were considered significant in this study.

## Results

### Expression profiles of oestrogen-induced genes in pDCIS compared with those of DCIS-c and IDC-c

We first surveyed expression profiles of oestrogen-induced genes in isolated carcinoma cells of pDCIS using microarray analysis which was focused on oestrogen-induced genes reported by Frasor *et al.* (2003), in order to examine the characteristics of oestrogenic actions in pDCIS. Fifty-one oestrogen-induced genes examined were tentatively classified into three groups (i.e. Groups A, B and C) depending on the hierarchical clustering analysis (Fig. 1). In addition, isolated and examined pDCIS carcinoma cells were clustered among the cases examined. Results demonstrated that the genes in Group C were predominantly expressed in pDCIS rather than in DCIS-c or IDC-c, and the genes in Group A were predominantly expressed in DCIS-c and/or IDC-c. Genes classified into Group B were expressed regardless of the carcinoma types. No significant clustering of samples was detected in association with nuclear grade, menopausal status and ER LI of the cases examined in this study.

As shown in Table 1, no significant differences of characteristics were detected between Groups A and C in this study.

### Clinicopathological features of pDCIS, DCIS-c and IDC-c

We then evaluated an association of various clinicopathological parameters among pDCIS ($n=53$), DCIS-c ($n=27$) and IDC-c ($n=27$), which were examined in this study. Nuclear grade ($P=0.68$), ER LI ($P=0.94$), PR LI ($P=0.87$) and HER2 status ($P=0.33$) were not significantly different among these three groups, but Ki67 LI was significantly ($P<0.0001$) lower in pDCIS than that in DCIS-c and IDC-c (Table 2). No significant differences of patients' age ($P=0.43$) and menopausal status ($P=0.34$) were detected between pDCIS and IDC patients in this study. HER2 positive status in our study (45% in pDCIS, 33% in DCIS-c and 30% in IDC-c) was consistent with that of a previous report (Park *et al.* 2006).

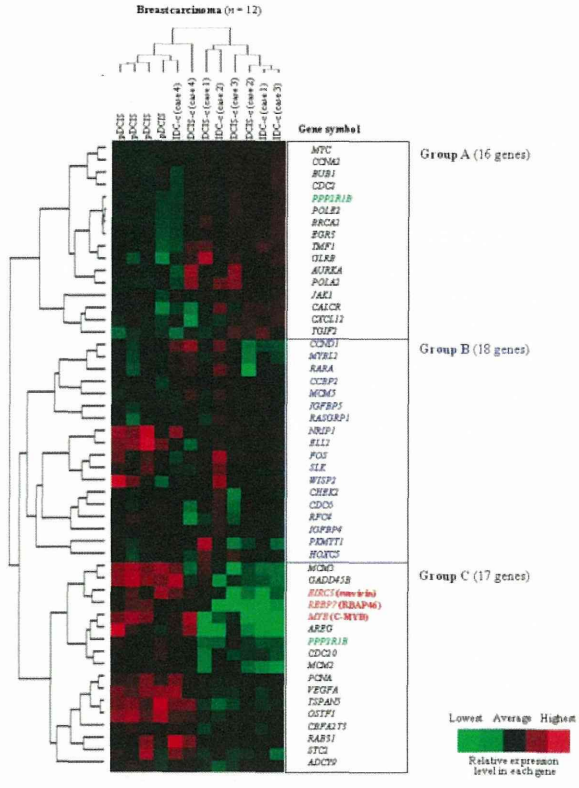


**Figure 1** Hierarchical clustering analysis of mRNA expression levels focused on oestrogen-induced genes identified by Frasor *et al.* (2003). Colour of blocks represents relative mRNA expression level of each gene compared with the average in 12 breast carcinoma samples (four pDCIS, four DCIS-c and four IDC-c). Gene symbols in each gene were listed. Gene-performed immunohistochemistry was noted in red. Two genes corresponding *PPP2R1B* were coloured green.

### Immunolocalisation of C-MYB, RBAP46 and survivin in pDCIS

Results of the microarray analysis demonstrate different expression profiles of oestrogen-induced genes in pDCIS compared with those in DCIS-c and IDC-c. We then performed immunohistochemistry for three representative oestrogen-induced genes (C-MYB (*MYB*), RBAP46 (*RBBP7*) and survivin (*BIRC5*)) in the breast carcinoma tissues in Group C towards further confirmation of the findings.

As demonstrated in Fig. 2A, C-MYB was immunolocalised in the nuclei of carcinoma cells, and its H-score was significantly ($P<0.0001$) higher in pDCIS than that in DCIS-c or IDC-c (Fig. 2B). RBAP46 immunoreactivity was also detected in the nuclei of carcinoma cells (Fig. 2C), and its immunoreactivity was significantly ($P=0.03$) higher in pDCIS (Fig. 2D).

**Table 1** Comparison of characteristics of genes between Groups A and C

| Characteristic of genes | Number of genes: Group A ($n=15$) | Number of genes: Group C ($n=16$) | *P* value |
|---|---|---|---|
| First time of significant upregulation by oestrogen | | | |
| 4 h | 7 (47%) | 11 (69%) | 0.51 |
| 8 h | 1 (7%) | 0 (0%) | |
| 24 h | 5 (33%) | 4 (25%) | |
| 48 h | 2 (13%) | 1 (6%) | |
| Major biological function | | | |
| Cell cycle and apoptosis | 6 (40%) | 5 (31%) | |
| Growth factors, cytokines and hormones | 1 (7%) | 3 (19%) | |
| Receptors and signal transduction proteins | 2 (13%) | 5 (31%) | 0.34 |
| Transcription factors and transcriptional coregulators | 6 (40%) | 3 (19%) | |

Data of characteristics of genes were taken from a report by Frasor *et al.* (2003). Data are presented as the number of cases and percentage. Two genes corresponding *PPP2R1B* were excluded in this table, because these were classified into both Groups A and C.

Survivin was immunolocalised in the cytoplasm of carcinoma cells, and some nuclei of the carcinoma cells were also immunohistochemically positive for survivin (Fig. 2E). Relative survivin immunoreactivity was significantly ($P=0.0003$) higher in pDCIS than that in DCIS-c or IDC-c (Fig. 2F).

As shown in Table 3, when we divided the cases into two groups according to several important pathological factors, such as nuclear grade, HER2 status and ER LI, C-MYB immunoreactivity was significantly higher in pDCIS than that in DCIS-c or IDC-c regardless of the status. Similar tendency was also detected in RBAP46 and survivin immunoreactivities; but *P* values did not reach significant levels in some groups.

As two genes corresponding *PPP2R1B* were classified into different groups (i.e. Groups A and C) in the microarray analysis (Fig. 1), we performed immunohistochemistry of PPP2R1B (also known as a protein phosphatase 2, regulatory subunit A, β (PP2A-Aβ)) in these cases. PPP2R1B immunoreactivity was detected in the breast carcinoma cells (Supplementary Figure S1A, see section on supplementary data given at the end of this article), but its immunointensity was generally weak and was not significantly different among the pDCIS, DCIS-c and IDC-c groups examined in this study (Supplementary Figure S1B, see section on supplementary data given at the end of this article).

## Association between C-MYB, RBAP46 and survivin immunoreactivity and various clinicopathological parameters in pDCIS

Results of both microarray and immunohistochemical analyses described earlier indicated that C-MYB, RBAP46 and survivin were abundantly expressed in pDCIS. As demonstrated in Table 4, when 53 pDCIS cases examined were tentatively classified into two different groups according to the median value of C-MYB H-score, the status of C-MYB immunoreactivity was inversely ($P=0.006$) associated with Ki67 LI in pDCIS cases. No other significant association was detected between C-MYB immunoreactivity and other clinicopathological parameters of the patients examined, such as patients' age, menopausal status, nuclear grade, comedo necrosis, ER LI, PR LI and HER2 status. The status of RBAP46 immunoreactivity was not significantly associated with any clinicopathological parameters examined (Table 5), while the status of survivin immunoreactivity was positively associated with patients' age ($P=0.002$; Table 6). Association between PPP2R1B immunoreactivity and clinicopathological parameters in pDCIS cases is summarised

**Table 2** Association of various clinicopathological parameters among pDCIS, DCIS-c and IDC-c

| Parameter | pDCIS ($n=53$) | DCIS-c ($n=27$) | IDC-c ($n=27$) | *P* value |
|---|---|---|---|---|
| Nuclear grade[a] | | | | |
| Grades 1+2 | 44 (83%) | 24 (89%) | 24 (89%) | 0.68 |
| Grade 3 | 9 (17%) | 3 (11%) | 3 (11%) | |
| ER LI (%) | 81 (12–100) | 80 (15–100) | 80 (8–100) | 0.94 |
| PR LI (%) | 40 (0–100) | 40 (0–100) | 40 (0–100) | 0.87 |
| HER2 status[a] | | | | |
| Negative | 29 (55%) | 18 (67%) | 19 (70%) | 0.33 |
| Positive | 24 (45%) | 9 (33%) | 8 (30%) | **<0.0001** |
| Ki67 LI (%) | 4 (1–12) | 8 (1–23) | 12 (1–32) | |

*P* value <0.05 was considered significant and is in boldface.
[a]Data are presented as the number of cases and percentage. All other values represent the median (min–max).

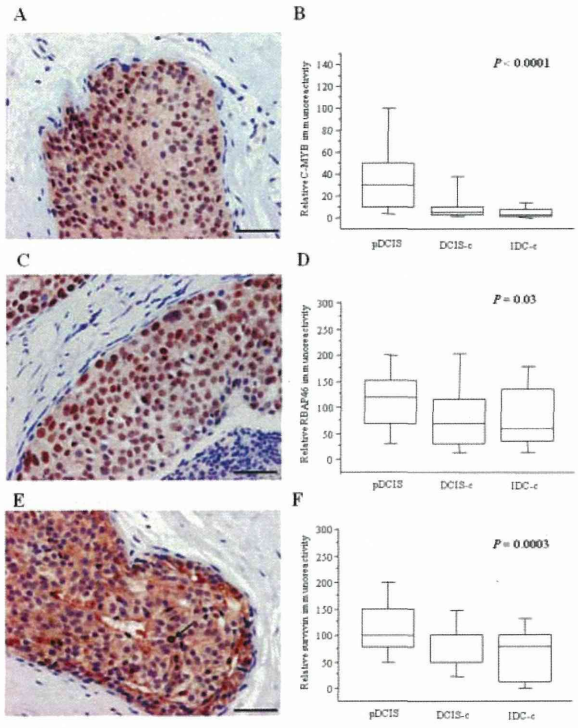


**Figure 2** Immunohistochemistry for C-MYB (A and B), RBAP46 (C and D) and survivin (E and F) in the breast cancer cases. Immunoreactivity of C-MYB (A) and RBAP46 (C) was detected in nuclei of carcinoma cells in pDCIS. Survivin was immunolocalised in the cytoplasm of carcinoma cells in pDCIS and was also positive in some nuclei of the carcinoma cells (an arrow; E). Bar = 50 μm respectively. Relative immunoreactivity of C-MYB, RBAP46 and survivin in pDCIS, DCIS-c and IDC-c was summarised in B, D and F respectively. Data are represented as box and whisker plots. Briefly, the median value is represented by a horizontal line in each box, and the 75th (upper margin) and 25th (lower margin) percentiles of the values are demonstrated. The upper and lower bars indicate the maximum and minimum values respectively. In F, the median value of relative survivin immunoreactivity in DCIS-c was 100. Statistical analysis was carried out using the Kruskal–Wallis test. *P* values $<0.05$ were considered significant and were indicated in bold letter.

in Supplementary Table S1, see section on supplementary data given at the end of this article.

### Association between clinicopathological parameters and three oestrogen-induced proteins in DCIS-c and IDC-c

As summarised in Table 7, Ki67 LI was significantly lower ($P=0.04$) in DCIS-c than that in IDC-c, but no significant differences between clinicopathological parameters of the patients and the status of immunoreactivity of C-MYB, RBAP46 and survivin were detected between DCIS-c and IDC-c of 27 IDC patients in this study.

## Discussion

pDCIS is generally considered as a precursor lesion of IDC. Two different models have been proposed to explain the possible mechanisms of transition from pDCIS to IDC, i.e. theories of linear progression or parallel disease (Wiechmann & Kuerer 2008). In the former model, low-grade pDCIS lesions are considered to progress to high-grade pDCIS lesions and then to become IDC (Carter *et al.* 1988, Bodian *et al.* 1993, Lakhani *et al.* 1999). In the latter model of hypothesis, low-grade pDCIS lesions progress to low-grade IDC and high-grade pDCIS lesions to high-grade IDC (Sontag & Axelrod 2005, Wiechmann & Kuerer 2008). Accumulating data including chromosomal-alteration studies support the parallel disease theory (Hwang *et al.* 2004, Irvine & Fentiman 2007), and the great majority of molecular alterations detected in breast carcinoma, including *ESR1* which codes for ER, can be clearly detected already in pDCIS, whether high or low grades (Nofech-Mozes *et al.* 2005, Burkhardt *et al.* 2010). In this study of ER-positive breast carcinoma, both ER and PR LIs in pDCIS were similar to those in IDC-c or DCIS-c, which is considered to be compatible with parallel disease theory of development. Shibuya *et al.* (2008) also previously demonstrated that various oestrogen-producing enzymes were abundantly expressed in pDCIS, and intratumoural oestrogen concentration was similar between pDCIS and IDC (Shibuya *et al.* 2008). Therefore, oestrogens are considered to play pivotal roles in pDCIS as well as in IDC.

Results of our present study also demonstrated that Ki67 LI was significantly lower in ER-positive pDCIS than that in ER-positive IDC. Antibody Ki67 recognises cells located in all the phases of cell cycle except for $G_0$ (resting) phase (Gerdes *et al.* 1983), and Ki67 LI is closely correlated with the cell proliferation activity of the tissues (van Diest *et al.* 2004). Ki67 was also reported as a prognostic factor in pDCIS (van Diest *et al.* 2004) as well as in IDC (de Azambuja *et al.* 2007), and increased Ki67 was associated with negative ER status of breast carcinoma (Burkhardt *et al.* 2010). All these findings suggest that oestrogen actions are more associated with cell proliferation of breast carcinoma in IDC than in pDCIS.

This is the first study to demonstrate expression profiles of oestrogen-induced genes in pDCIS compared with IDC. Results of our present microarray analysis did reveal that one-third of oestrogen-induced genes were predominantly expressed in pDCIS, while the other one-third of the genes mainly in IDC and the rest in both categories with equivalent frequency.

**Table 3** Statistical associations of C-MYB, RBAP46 and survivin immunoreactivity among pDCIS, DCIS-c and IDC-c cases according to several pathological parameters

| Parameter | C-MYB immunoreactivity | RBAP46 immunoreactivity | Survivin immunoreactivity |
|---|---|---|---|
| Nuclear grade | | | |
| Grades 1+2 | **<0.0001** | **0.04** | **0.001** |
| Grade 3 | **0.008** | 0.5 | 0.3 |
| HER2 status | | | |
| Negative | **<0.0001** | **0.02** | **0.01** |
| Positive | **0.01** | 0.73 | **0.02** |
| ER LI (%) | | | |
| 8–79 | **0.0003** | 0.06 | **0.01** |
| 80–100 | **0.0002** | 0.20 | **0.008** |

Data are presented as *P* values. *P* values <0.05 were considered significant and are in boldface.

These findings suggest that oestrogenic actions in pDCIS were different from those in IDC, even if the carcinoma cells expressed ER and intratumoural oestrogen was present at a significant level in both of these lesions. Among the genes predominantly expressed in IDC (Group A in Fig. 1), *EGR3* (early growth-responsive gene 3) was reported to play a pivotal role in the process of oestrogen-mediated invasion in breast cancer, and its expression was associated with adverse clinical outcome of the patients with ER-positive IDC (Suzuki *et al.* 2007). In addition, the kinetochore-bound protein kinase *BUB1* (budding uninhibited by benzimidazoles 1) is also considered to play possible role in the process of breast tumourigenesis (Klebig *et al.* 2009), and its mRNA expression was also reported to be positively associated with clinical recurrence in ER-positive IDC patients (Suzuki *et al.* 2012). *MYC* (C-MYC) was also reported to be associated with poor prognosis or adverse clinical outcome of ER-positive breast cancer patients (Chen & Olopade 2008). Robanus-Maandag *et al.* (2003) reported that *MYC* amplification may drive transition from pDCIS to IDC in human breast (Robanus-Maandag *et al.* 2003), although some conflicting data were reported in the literature (Burkhardt *et al.* 2010). These findings suggest that oestrogen-mediated transactivation is considered to vary among the target genes, and the genes promoting aggressive biological or clinical behaviour of breast carcinoma cells may be more efficiently induced by oestrogen in IDC. However, immunoreactivity of C-MYB, RBAP46 and survivin was not associated with ER LI in pDCIS cases in this study, and previous studies have demonstrated that the expression of these molecules was regulated by several factors (for instances, miroRNA-150 downregulated C-MYB in liver cancer stem cells (Zhang *et al.* 2012), RBAP46 functioned as a downstream target gene of WT1 (Guan *et al.* 1998), and genetic variants of the survivin promotor were associated with survivin expression (Xu *et al.* 2004)). Therefore, factors other than oestrogen may also be involved in the different expression profiles of oestrogen-induced genes in pDCIS from IDC. Our experiments serve as a starting point for clarifying the molecular features of oestrogen actions in pDCIS, and further examination is required.

We first identified C-MYB, RBAP46 and survivin as oestrogen-induced proteins predominantly expressed in pDCIS compared with IDC in this study. Among these three genes identified by gene profilings, a nuclear transcription factor C-MYB regulates differentiation and proliferation in various types of cells (Oh & Reddy 1999), and expression of *C-MYB* mRNA was

**Table 4** Association between C-MYB immunoreactivity and clinicopathological parameters in pDCIS

| | C-MYB immunoreactivity | | |
|---|---|---|---|
| Parameter | High (*n*=26) | Low (*n*=27) | *P* value |
| Patients' age | 61 (48–80) | 61 (39–80) | 0.91 |
| Menopausal status[a] | | | |
| Premenopausal | 7 (30%) | 3 (56%) | 0.14 |
| Postmenopausal | 19 (70%) | 24 (44%) | |
| Nuclear grade[a] | | | |
| Grades 1+2 | 20 (77%) | 24 (89%) | 0.25 |
| Grade 3 | 6 (23%) | 3 (11%) | |
| Comedo necrosis[a] | | | |
| Absent | 11 (42%) | 7 (26%) | 0.21 |
| Present | 15 (58%) | 20 (74%) | |
| ER LI (%) | 84 (13–100) | 80 (12–100) | 0.77 |
| PR LI (%) | 40 (6–93) | 46 (0–100) | 0.72 |
| HER2 status[a] | | | |
| Negative | 14 (54%) | 15 (56%) | 0.90 |
| Positive | 12 (46%) | 12 (44%) | |
| Ki67 LI (%) | 3 (1–10) | 6 (2–12) | **0.006** |

Fifty-three pDCIS cases were classified into two (i.e. high and low) groups according to the median value of C-MYB immunoreactivity. *P* value <0.05 was considered significant and is in boldface.
[a]Data are presented as the number of cases and percentage. All other values represent the median (min–max).

**Table 5** Association between RBAP46 immunoreactivity and clinicopathological parameters in pDCIS

| Parameter | RBAP46 immunoreactivity | | *P* value |
|---|---|---|---|
| | High ($n$=28) | Low ($n$=25) | |
| Patients' age | 65 (39–80) | 54 (49–77) | 0.06 |
| Menopausal status[a] | | | |
| Premenopausal | 4 (14%) | 6 (24%) | 0.81 |
| Postmenopausal | 24 (86%) | 19 (76%) | |
| Nuclear grade[a] | | | |
| Grades 1+2 | 21 (75%) | 23 (92%) | 0.99 |
| Grade 3 | 7 (25%) | 2 (8%) | |
| Comedo necrosis[a] | | | |
| Absent | 9 (32%) | 9 (36%) | 0.77 |
| Present | 19 (68%) | 16 (64%) | |
| ER LI (%) | 88 (12–100) | 80 (13–100) | 0.60 |
| PR LI (%) | 44 (6–100) | 40 (0–100) | 0.19 |
| HER2 status[a] | | | |
| Negative | 16 (57%) | 13 (52%) | 0.71 |
| Positive | 12 (43%) | 12 (48%) | |
| Ki67 LI (%) | 4 (1–12) | 4 (2–10) | 0.31 |

Fifty-three pDCIS cases were classified into two (i.e. high and low) groups according to the median value of RBAP46 immunoreactivity.
[a]Data are presented as the number of cases and percentage. All other values represent the median (min–max).

rapidly stimulated by oestrogen administration in the MCF7 breast carcinoma cells (Frasor *et al.* 2003). C-MYB protein was detected in ER-positive IDC and was associated with a good prognosis in the patients (Guerin *et al.* 1990, Drabsch *et al.* 2007, Deisenroth *et al.* 2010, Thorner *et al.* 2010). Immunohistochemistry for C-MYB in pDCIS has been reported only by McHale *et al.* (2008) to the best of our knowledge, in which C-MYB immunoreactivity in the breast carcinoma containing both pDCIS and IDC was significantly higher than that in normal/hyperplastic epithelium. Results of our present study first demonstrated that C-MYB immunoreactivity was significantly higher in pDCIS than in IDC and was inversely associated with Ki67 LI in pDCIS. Very recently, Thorner *et al.* (2010) reported that stable RNAi knock-down of endogenous *C-MYB* in the MCF7 cells increased tumourigenesis, both *in vitro* and *in vivo*, suggesting a tumour suppressor function in luminal breast cancer subtypes (Thorner *et al.* 2010). Results of our present study are consistent with these previously reported studies, and decreased induction of C-MYB expression by oestrogen may result in the possible acceleration of oestrogen-mediated cell proliferation of breast carcinoma in IDC.

RBAP46, a nuclear protein, was originally identified as histone-binding proteins and its components of protein complexes have been demonstrated to be involved in the process of histone deacetylation and chromatin remodelling (Zhang *et al.* 1997, Bowen *et al.* 2004). *RBAP46* mRNA expression was reported to be rapidly induced by oestrogens in MCF7 cells (Frasor *et al.* 2003). Results of previous *in vitro* studies demonstrated that RBAP46 modulated oestrogen responsiveness in MCF7 cells in a gene-specific manner through interaction with ERα (Creekmore *et al.* 2008), and RBAP46 was also reported to inhibit an oestrogen-stimulated progression of transformed breast epithelial cells (Zhang *et al.* 2007). However, immunohistochemical evaluation of RBAP46 has not been reported in breast carcinoma to the best of our knowledge. In this study, RBAP46 immunoreactivity was more frequently detected in ER-positive pDCIS than in IDC, which also indicated that RBAP46 may play an important role in the alteration of oestrogen actions in the process of transition from pDCIS to IDC.

Survivin is known as an inhibitor of apoptosis, which prevents cell death by mainly blocking activated caspases (Ryan *et al.* 2006). Survivin mRNA expression was reported to be slowly induced by oestrogen in MCF7 cells (Frasor *et al.* 2003). Immunolocalisation of cytoplasmic survivin has been reported in human breast carcinoma by several groups, with positivity ranging from 56 to 76% of pDCIS cases (Barnes *et al.* 2006, Okumura *et al.* 2008) and 17 to

**Table 6** Association between survivin immunoreactivity and clinicopathological parameters in pDCIS

| Parameter | Survivin immunoreactivity | | *P* value |
|---|---|---|---|
| | High ($n$=25) | Low ($n$=28) | |
| Patients' age | 66 (48–80) | 54 (39–80) | **0.002** |
| Menopausal status[a] | | | |
| Premenopausal | 4 (16%) | 6 (21%) | 0.61 |
| Postmenopausal | 21 (84%) | 22 (79%) | |
| Nuclear grade[a] | | | |
| Grades 1+2 | 19 (76%) | 25 (89%) | 0.20 |
| Grade 3 | 6 (24%) | 3 (11%) | |
| Comedo necrosis[a] | | | |
| Absent | 7 (28%) | 11 (39%) | 0.39 |
| Present | 18 (72%) | 17 (61%) | |
| ER LI (%) | 87 (27–100) | 80 (12–100) | 0.25 |
| PR LI (%) | 47 (0–100) | 40 (7–100) | 0.58 |
| HER2 status[a] | | | |
| Negative | 12 (48%) | 17 (61%) | 0.35 |
| Positive | 13 (52%) | 11 (39%) | |
| Ki67 LI (%) | 4 (1–12) | 4 (1–12) | 0.80 |

Fifty-three pDCIS cases were classified into two (i.e. high and low) groups according to the median value of survivin immunoreactivity. *P* value <0.05 was considered significant and is in boldface.
[a]Data are presented as the number of cases and percentage. All other values represent the median (min–max).

**Table 7** Association of clinicopathological parameters and three oestrogen-induced proteins between DCIS-c and IDC-c in 27 IDC patients

| Parameter | DCIS-c | IDC-c | *P* value |
|---|---|---|---|
| Nuclear grade[a] | | | |
| Grades 1+2 | 24 (33%) | 24 (25%) | 0.99 |
| Grade 3 | 3 (17%) | 3 (17%) | |
| ER LI (%) | 80 (15–100) | 80 (8–100) | 0.97 |
| PR LI (%) | 40 (0–100) | 40 (0–100) | 0.56 |
| HER2 status[a] | | | |
| Negative | 18 (67%) | 19 (70%) | 0.77 |
| Positive | 9 (33%) | 8 (30%) | |
| Ki67 LI (%) | 8 (1–23) | 12 (1–32) | **0.04** |
| C-MYB immunoreactivity | 5 (0–70) | 3 (0–70) | 0.13 |
| RBAP46 immunoreactivity | 69 (0–250) | 60 (0–230) | 0.80 |
| Survivin immunoreactivity | 100 (0–220) | 80 (0–150) | 0.19 |

[a]Data are presented as the number of cases and percentage. All other values represent the median (min–max). *P* value $<0.05$ was considered significant and is in boldface.

71% of IDC cases (Tanaka *et al.* 2000, Kennedy *et al.* 2003, Barnes *et al.* 2006, Sohn *et al.* 2006, Al-Joudi *et al.* 2007, Hinnis *et al.* 2007, Kleinberg *et al.* 2007). In particular, Barnes *et al.* (2006) reported that cytoplasmic survivin immunoreactivity was significantly ($P=0.0001$) frequent in pDCIS compared with IDC, which is consistent with results of this study. In addition, Barnes *et al.* also reported that the status of survivin immunoreactivity was significantly correlated with pDCIS recurrence and suggested that survivin was involved particularly in an early event of breast carcinoma development. Therefore, anti-apoptotic effects of oestrogen may play an important role also in pDCIS. Results of our present study also demonstrated a positive association between the status of survivin immunoreactivity and patients' age in pDCIS cases (Table 6). Considering a previous report that polymorphisms in survivin promotor were associated with the age of onset of ovarian cancer (Han *et al.* 2009), some factors other than oestrogen may be involved in the development of pDCIS, but it awaits further investigations for clarification.

Amari *et al.* (1999) examined the loss of heterozygosity in tumours derived from 23 patients, which harboured synchronous lesions of atypical ductal hyperplasia (ADH), DCIS and IDC, and reported that genetic alterations accumulate during cancer progression from ADH to DCIS and finally to IDC (Amari *et al.* 1999). However, several groups reported a close association of molecular features between DCIS-c and IDC-c (Done *et al.* 1998, Half *et al.* 2002, van der Groep *et al.* 2009, Burkhardt *et al.* 2010). In this study, various clinicopathological features and three oestrogen-induced proteins examined were not significantly different between DCIS-c and IDC-c in ER-positive IDC cases. Therefore, alterations of oestrogenic actions may mainly occur at the possible transition from pDCIS to IDC, rather than the intraductal to invasive growth of cancerous cells. Further examinations are required to clarify molecular features of oestrogen actions in pDCIS, which may also contribute to improved histopathological diagnosis of pDCIS through definitive differentiation from DCIS-c of IDC in the biopsy specimen of human breast.

In summary, we examined the expression profiles of oestrogen-induced genes in pDCIS using microarray analysis to characterise molecular features of oestrogen actions in pDCIS. Results demonstrated that one-third of the genes examined were predominantly expressed in pDCIS rather than DCIS-c or IDC-c of IDC cases. Among these pDCIS-associated genes, *C-MYB*, *RBAP46* and survivin immunoreactivity was significantly higher in pDCIS than that in DCIS-c or IDC-c by subsequent immunohistochemical analysis. In particular, C-MYB immunoreactivity was inversely associated with Ki67 LI in pDCIS cases. These results suggest that expression profiles of oestrogen-induced genes in pDCIS are different from those in IDC, and C-MYB, RBAP46 and survivin may play important roles to characterise the oestrogen actions in pDCIS.

## Supplementary data

This is linked to the online version of the paper at http://dx.doi.org/10.1530/ERC-11-0345.

## Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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# Body mass index and survival after breast cancer diagnosis in Japanese women

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## Abstract

**Background:** Body mass index (BMI) may be an important factor affecting breast cancer outcome. Studies conducted mainly in Western countries have reported a relationship between higher BMI and a higher risk of all-cause death or breast cancer-specific death among women with breast cancer, but only a few studies have been reported in Japan so far. In the present prospective study, we investigated the associations between BMI and the risk of all-cause and breast cancer-specific death among breast cancer patients overall and by menopausal status and hormone receptor status.

**Methods:** The study included 653 breast cancer patients admitted to a single hospital in Japan, between 1997 and 2005. BMI was assessed using a self-administered questionnaire. The patients were completely followed up until December, 2008. Hazard ratios (HRs) and 95% confidence intervals (CIs) were estimated according to quartile points of BMI categories, respectively: <21.2, ≥21.2 to <23.3 (reference), ≥23.3 to <25.8 and ≥25.8 kg/m$^2$.

**Results:** During the follow-up period, 136 all-cause and 108 breast cancer-specific deaths were observed. After adjustment for clinical and confounding factors, higher BMI was associated with an increased risk of all-cause death (HR = 2.61; 95% CI: 1.01–6.78 for BMI ≥25.8 vs. ≥21.2 to <23.3 kg/m$^2$) among premenopausal patients. According to hormonal receptor status, BMI ≥25.8 kg/m$^2$ was associated with breast cancer-specific death (HR = 4.95; 95% CI: 1.05–23.35) and BMI <21.2 kg/m$^2$ was associated with all-cause (HR = 2.91; 95% CI: 1.09–7.77) and breast cancer-specific death (HR = 7.23; 95% CI: 1.57–33.34) among patients with ER + or PgR + tumors. Analysis by hormonal receptor status also showed a positive association between BMI and mortality risk among patients with ER + or PgR + tumors and with BMI ≥21.2 kg/m$^2$ (p for trend: 0.020 and 0.031 for all-cause and breast cancer-specific death, respectively).

**Conclusions:** Our results suggest that both higher BMI and lower BMI are associated with an increased risk of mortality, especially among premenopausal patients or among patients with hormonal receptor positive tumors. Breast cancer patients should be informed of the potential importance of maintaining an appropriate body weight after they have been diagnosed.

**Keywords:** Breast cancer, Survival, Body mass index, Hormone receptor, Menopausal status

## Background

Many previous epidemiologic studies have demonstrated that higher body mass index (BMI) is associated with an increased risk of postmenopausal breast cancer, whereas it is associated with a reduced risk of premenopausal breast cancer [1]. Furthermore, some studies conducted mainly in Western countries have found associations between higher BMI and a higher risk of all-cause death [2-10] or breast cancer-specific death [6,11,12] among women with breast cancer, although other studies have found no such association [13-16]. As various inconsistencies have been reported across menopausal status between BMI and survival among premenopausal [2,4,8,12,17-21] and postmenopausal women [5,8,11,12,21], it is important to stratify menopausal status in order to adequately assess the relationship between BMI and mortality of breast cancer patients.

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In adipose tissue, conversion of androgens to estrogens by aromatase occurs [22]. Estrogen accelerates breast tumor growth via the estrogen receptor. Breast tumors have estrogen or progesterone receptors, and tumor subtypes defined by these receptors may represent biologically different entities [23,24] and influence the survival of patients. Therefore it seems important to consider tumor subtypes when evaluating the relationship between BMI and mortality due to breast cancer, and in fact several studies have already investigated the effects of tumor subtype in terms of hormone receptor status [2,4,9,10,13,14,20].

In Japan, two previous studies have assessed the relationship between BMI and survival in breast cancer patients [25,26]. However, those studies were small in scale and controlled for only a few known risk factors. Only one previous study has addressed this issue in terms of menopausal status [26], but no attempt has yet been made to do so in terms of hormone receptor status.

In the present study, therefore, we investigated the relationship between BMI and the risk of all-cause death and breast cancer-specific death among breast cancer patients in terms of menopausal status and also hormone receptor status using a hospital-based prospective cohort study. Some known risk factors, tumor stage, and data on the therapy used for breast cancer were taken into account as covariates. Analyses stratified according to menopausal and hormone receptor status were performed, along with analysis of the patients overall.

## Methods

### Study subjects

Between January 1997 and December 2005, 718 female patients aged 29 years or over were newly diagnosed as having breast cancer at the Miyagi Cancer Center Hospital (MCCH). All of these patients were requested to complete a questionnaire upon initial admission. After diagnosis, their details were entered into the hospital-based cancer registry and the patients were followed up. This cancer registry recorded clinical and pathological findings and information on antineoplastic treatments for all patients with cancer admitted to the MCCH. The MCCH is located in Natori City, situated in the southern part of Miyagi Prefecture. It has 383 administrative beds, and functions as both a general hospital and a comprehensive research institute for both all types of cancer and benign diseases.

Among the 718 newly diagnosed breast cancer patients, 664 (92.5%) completed the questionnaire. After excluding 7 patients with a history of cancers other than breast cancer, the 657 remaining patients were included in the present study, which was approved by the ethical review board of Miyagi Cancer Center.

### Questionnaire and clinical information

In January 1997, we began a survey in connection with the present study. Information on lifestyle and personal history was collected from all patients using a self-administered questionnaire, which was distributed to patients on the day of their reservation for initial admission to the MCCH, i.e., 10–15 days before admission, and collected by nurses on the actual admission day. Details of the questionnaire survey have already been described elsewhere [27,28].

The questionnaire covers items on demographic characteristics, current height and weight, family histories of cancer and other diseases, general lifestyle factors before the development of current symptoms including history of smoking, menopausal status, and comorbidity of other diseases.

Clinical information including tumor stage and treatment, such as chemotherapy, radiation therapy and endocrine therapy, was obtained from the MCCH hospital-based cancer registry. Information on hormone receptor status, i.e. expression of the estrogen receptor (ER) and progesterone receptor (PgR), was extracted from medical records. To measure ER and PgR status, enzyme immunoassay (EIA) was used in the early period of the study. After mid-2003, immunohistochemistry (IHC) was conducted. 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 [29]. The concordance between the two assays was 94.3% for ER and 100% for PgR in the laboratory of the MCCH [29]. Receptor status was unknown for ER in 69 cases (10.5%), PgR in 80 (12.2%) cases, and both in 69 (10.5%) cases. 392 (59.7%) cases were ER + and 318 (48.4%) were PgR + .

### Ascertainment of exposures and follow-up

At the MCCH, initial therapy is administered after admission in principal. Therefore, data on weight and height collected using the questionnaire was considered to be pretreatment data. BMI was calculated as weight divided by the square of current height (kg/m$^2$). Height and weight were measured by medical staff in a subsample (n = 315) of our study at the time of initial hospital admission. The self-reported height and weight data were highly correlated with the measured data (correlation coefficient: 0.94 for height and 0.96 for weight). Four patients for whom BMI values were missing were excluded, leaving a final total of 653 patients for analysis. We stratified the patients according to BMI quartile points: <21.2 kg/m$^2$, ≥21.2 kg/m$^2$ to <23.3 kg/m$^2$, ≥23.3 kg/m$^2$ to <25.8 kg/m$^2$ and ≥25.8 kg/m$^2$. The BMI category ≥21.2 kg/m$^2$ to <23.3 kg/m$^2$ was selected as the reference.

Follow-up was performed by reference to the MCCH Cancer Registry up to December 31, 2008. Active follow-up was conducted by accessing hospital visit records, resident registration cards and permanent domicile data. Information on the dates and causes of death was obtained with permission from the Ministry of Justice. During the study period, no subject was lost to follow-up.

### Statistical analysis

The end point of our analysis was all-cause death and breast cancer-specific death according to the International Classification of Disease for Oncology, Tenth Edition (ICD-10). Survival time was calculated for each patient from the date of diagnosis to the date of death or the end of follow-up (December 31, 2008).

The Cox proportional hazards model was used to estimate hazard ratios (HRs) and 95% confidence intervals (CIs) for all-cause death and breast cancer-specific death in relation to BMI [30]. Tests for trend were employed in the Cox model for all BMI categories and for ≥21.2 kg/m$^2$ respectively, because we expected the overall relationship of BMI to mortality to be U-shaped rather than linear (i.e., we expected women with BMI <21.2 kg/m$^2$ have higher mortality than the reference category). We considered the following variables to be potential confounders: age, tumor stage (in situ or localized, local invasion, lymph node metastasis, distant metastasis), hormone receptor status (ER + or PgR+, ER-/PgR-), radiation therapy (no, yes), chemotherapy (no, yes), endocrine therapy (no, yes) and comorbidities (no, yes). Comorbidities included hypertension, ischemic heart disease, stroke and diabetes mellitus. Smoking (current, past, never), family history of breast cancer in mother or sister (no, yes), and physical activity (almost no, more than one hour per week, missing), some of which have already been established as risk factors for breast cancer, were also considered to be adjusted for [31-33]. Missing values for confounders were treated as an additional variable category, and included in the model.

Separate analyses were conducted after dividing the patients according to premenopausal or postmenopausal status, along with analysis of the patients overall. Stratification according to hormonal receptor status was also performed. To evaluate heterogeneity of the associations between BMI and all-cause death and breast cancer-specific death across menopausal status (premenopausal vs. postmenopausal) and hormone receptor status (ER + or PgR + vs. ER-/PgR-), interaction terms (BMI * menopausal status, BMI * hormone receptor status) were tested. Likelihood ratio tests were used to assess the significance of heterogeneity by comparing the model including the interaction term to the main-effects model. 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, patients 44–57 years of age (defined as the mean age at natural menopause ±2 SD) were regarded as having unknown menopausal status.

Results were regarded as significant if the two-sided P values were <0.05. All statistical analyses were performed using the SAS software package (version 9.2; SAS Institute, Cary, NC).

## Results

During a median follow-up period of 5.85 years, 136 all-cause and 108 breast cancer-specific deaths were observed. The characteristics of the patients at the time of breast cancer diagnosis are shown in Table 1. Heavier patients tended to have hormonal receptor-positive tumors. With regard to hormone receptor status, 410 (62.8%) cases were ER + or PgR+, and 174 (26.6%) were ER-/PgR-. Women with higher BMI were more likely to be older, to be postmenopausal, to exercise more, to have more comorbidities, and to have hormone receptor-positive tumors.

Table 2 shows the association of BMI with all-cause death. Compared to women with BMI ≥21.2 to <23.3 kg/m$^2$, those with BMI <21.2 kg/m$^2$ were shown to have a higher risk of death by age-adjusted analysis (HR = 1.73, 95% CI: 1.07–2.80), but not by multivariate-adjusted analyses (1.60, 0.97–2.63). No dose–response relationship was observed between BMI and all-cause death (multivariate-adjusted p for trend = 0.59). Analysis limited to women with BMI ≥21.2 kg/m$^2$ also demonstrated no dose–response relationship (multivariate-adjusted p for trend = 0.11). Stratification by menopausal status yielded inconsistent results. BMI had no significant association with all-cause death among postmenopausal women, whereas a significantly increased risk of all-cause death was found among premenopausal obese women (BMI ≥25.8 kg/m$^2$) in both age-adjusted (2.49, 1.03–6.03) and multivariate-adjusted analyses (2.61, 1.01–6.78). For premenopausal women with BMI ≥21.2 kg/m$^2$, trend test demonstrated a marginal dose–response relationship between BMI and all-cause death (multivariate-adjusted p for trend = 0.059). The trends were not significantly different between premenopausal and postmenopausal women with BMI ≥21.2 kg/m$^2$ (P for heterogeneity of trends = 0.11).

With regard to breast cancer-specific death, age-adjusted analysis and multivariate-adjusted analysis showed that women with BMI <21.2 kg/m$^2$ were not at higher risk (Table 3). No dose-response relationship between BMI and breast cancer-specific death was found.

Analysis stratified by hormonal receptor status demonstrated differences in the risk of death across strata for