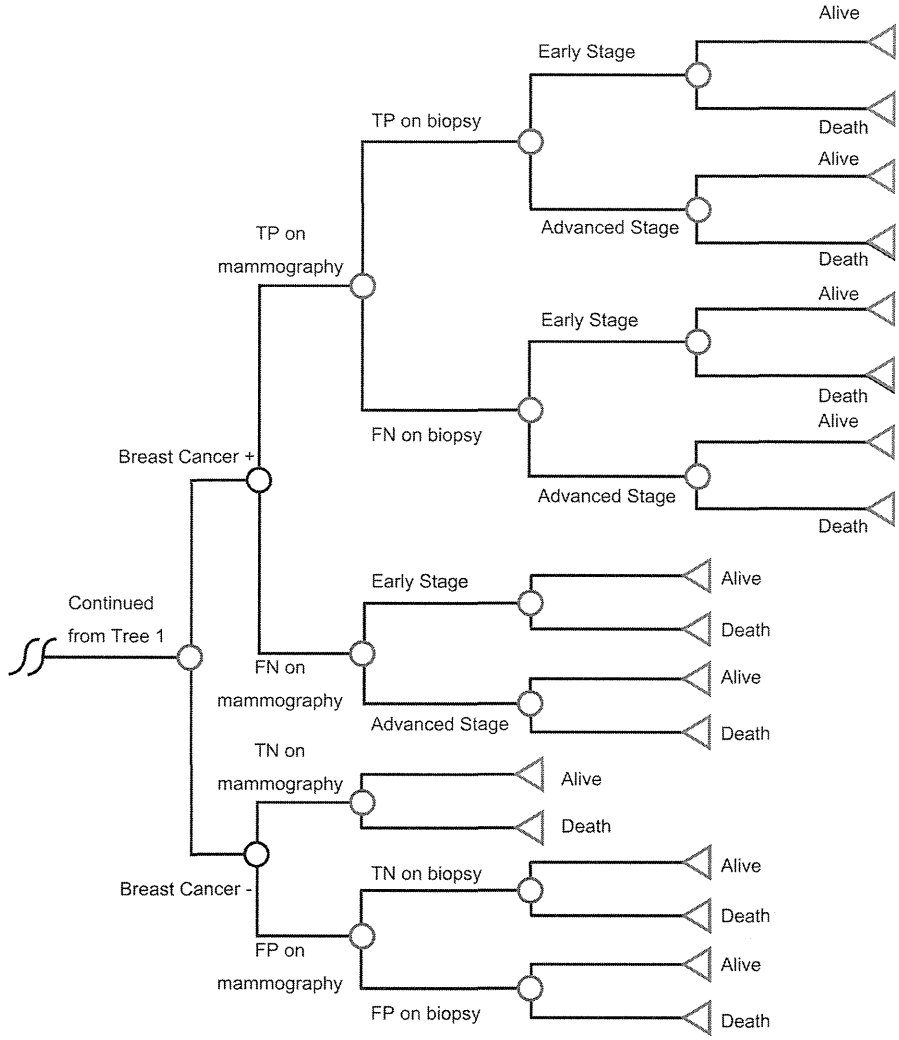

Fig. 2 The analysis model, Tree 2. Continued model from Tree 1 on the health state of women with non-breast cancer history. *TP* true positive, *FP* false positive, *TN* true negative, *FN* false negative. Alive continues to the next health state according to health condition

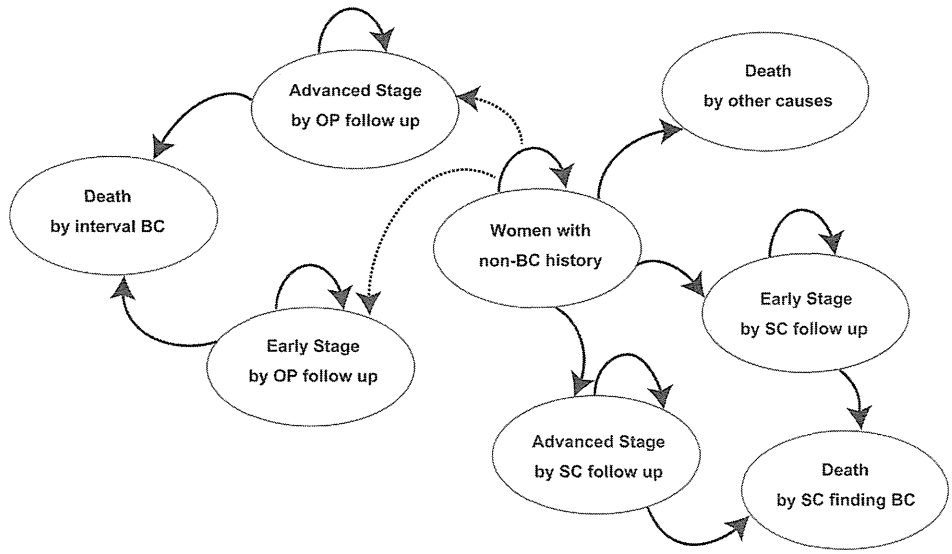

Fig. 3 Markov diagram. The six health states were defined as women with non-breast cancer (BC) history, early stage by screening (SC) follow-up, advanced stage by SC follow-up, early stage by outpatient visit (OP) follow-up, advanced stage by OP follow-up and death. There are three deaths in the diagram, yet all are considered to be the same absorb state as death. Health states move to another or stay in the same state following the *arrows* in each cycle (2 years). *Dashed arrows* are interval breast cancers at outpatient visit with symptoms even though recent screening resulted in breast cancer negative

Table 1 Base case input data—probabilities

	Probabilities	Years
Epidemic data		
Breast cancer prevalence [18]	0.0028	1997–2002
Screening found breast cancer[a]		
Early stage (0–I)	0.830	
Advanced stage (II–IV)	0.170	
Interval breast cancer[a]		
Early stage (0–I)	0.560	1997–2002
Advanced stage (II–IV)	0.440	
Sensitivity and specificity		
Mammography double reading [18]		
Sensitivity	0.858	1997–2002
Specificity	0.907	
Mammography single + CAD [19]		
Sensitivity	0.908	1990–2010
Specificity	0.927	
Biopsy[a]		
Sensitivity	0.956	1997–2002
Specificity	1.000	
Mortality rates		
Women with non-BC history[b]	0.00194	2010
Screening found breast cancer[a]		
Early stage (0–I)	0.011	1997–2002
Advanced stage (II–IV)	0.111	
Interval breast cancer[a]		
Early stage (0–I)	0.028	1997–2002
Advanced stage (II–IV)	0.281	

[a] Miyagi Cancer Society

[b] Abridged life table in 2010, Ministry of Health, Labour and Welfare

Table 2 Correspondence table of TNM and neoplasm staging

		N0	N1	N2	N3	
			a, b, c	a, b	a, b	c
Tis		CIS[a]	–	–	–	–
T0		Localized[b]	Regional lymph node involvement			Distant metastasis
T1T2T3	Pectoral invasion (−)	Localized[b]				
	Pectoral invasion (+)	Adjacent organ invasion				
T4						
M1		Distant metastasis				

The table was translated from Gan-joho.jp. http://ganjoho.jp

[a] Carcinoma in situ: invasion (−) or Paget without cancer

[b] No primary tumor

from referring doctors and final tissue diagnosis by MPCR. Stage prevalence of breast cancer is also from MPCR in the form of neoplasm staging data, which we translated to TNM as described in Table 2. We could not find clarified CAD data for screening mammography in Japan and instead used a systematic review for 2011 [19].

Expected costs

Costs in this study include only breast cancer-related costs and CAD installation costs (Table 3). The screening cost, including mammography, palpation and the first reading cost, is the average screening commission fees from Sendai and 28 other cities, towns and villages in Miyagi to screening providers. The second reading cost is mammography reading commission fees from screening providers to physicians. For the outpatient visit fees for interval breast cancer, we summarized receipt data for 30 breast cancer patients from the database at our hospital. Since there are many treatment choices, we used the medical fee simulation function on the website to calculate eight treatment variations for early stage and 24 for advanced stage, including total extirpation, breast-conserving therapy, radiation therapy and chemotherapy [20].

We researched the marketing cost for CAD with four device makers, but the CAD installation cost is quite ambiguous. We defined the price with the cost of two 5-M monitors, a 3-T server and full-maintenance fees, and determined that 40 % of the full price is the actual marketing cost by consulting the seller. The CAD lifetime is 6 years with a 50 % depreciation rate by the declining-balance method. We needed the installation cost per person and thus divided the cost by the number of examinees screened at Miyagi Cancer Society between 1997 and 2002.

Life expectancy

Quality-adjusted life years (QALYs) are a useful factor for evaluating cost-effectiveness. However, Japan does not have much quality-of-life research for cancer directly translatable to QALYs. Moreover, quality of life may vary too much according to cultural background to be cited from international publications, and thus we simply used life expectancy in this study.

The mortality rate insert is important for calculating life expectancy in the Markov model. We used the life table from the national statistics for BC− and calculated the mortality of BC+ from the 5-year survival rate from MPCR.

Cost-effectiveness analysis

After inputting all the data, roll back calculation was performed to evaluate differences in expected costs and life

Table 3 Base case input data—costs

	Costs (JPY)	Years
Mammography + first reading[a]	8,257	2012
Second reading[a]	138	
Biopsy[b]	9,290	2012
CAD installation cost[c]	337	2012
Diagnosis costs at outpatient[d]	42,404/visit	2012
Treatment costs of early stage (0–I) [20]	2,432,673/first 2 years	2012
Treatment costs of advanced stage (II–IV) [20]	4,291,761/first 2 years	
Follow-up medical costs of early stage (0–I) [20]	391,812/one cycle	
Follow-up medical costs of advanced stage (II–IV) [20]	1,584,099/one cycle	

[a] The average screening commission fees and reading fees for Sendai city and 28 communities in Miyagi. The cost includes mammography, palpation and the first reading cost

[b] The first outpatient visit fee + biopsy = 2,700 + 6,590 at our hospital

[c] CAD installation cost including maintenance, two monitors (5 M) and storage server (3 T) costs was 49,003,873 yen. The marketing cost is 40 % of the full price. The cost was depreciated by the declining-balance method with a 50 % depreciation rate and 6 years' lifetime, and divided by the number of examinees

[d] Average receipt data from our hospital

years between the two methods. When single + CAD resulted in higher costs and longer life years than double reading, the incremental cost-effectiveness ratio (ICER) is useful for evaluating cost-effectiveness. The ICER is calculated by dividing the incremental costs by the incremental effectiveness of a new method over a conventional method, reflecting the increased medical cost for gaining one life year under the CAD-based methodology compared with double reading in this study [21]. We set 6,000,000 yen as a threshold based on Ohkusa and Sugawara [22].

Sensitivity analysis

Sensitivity analysis was performed to evaluate the robustness of the model and input data.

One-way and multi-way sensitivity analyses

Most of our probability data came from Miyagi Cancer Society and MPCR, which have high-accuracy databases (DCN 10.4 %, DCO 2.23 % in 2007) and our cost data from actual receipt data from our university hospital. However, several data were unavailable from these sources, and we instead used international publications or averaged small data samples; for example, sensitivity and specificity of single + CAD were data from outside of Japan, and the CAD installation cost was from one medical device maker. The number of annual examinees, screening cost and double reading cost were also changeable depending on the facility setting. For this uncertainty, we performed one-way sensitivity analysis with those parameters.

We also performed multi-way sensitivity analysis using parameters that had a large impact on incremental costs, effectiveness or ICER in the one-way sensitivity analysis.

Scenario sensitivity analysis

The recommended age for mammography screening is still controversial from the perspective of benefits to the examinee. We performed scenario sensitivity analysis to obtain results when the hypothetical population comprised 40-, 50- and 60-year-old women. Breast cancer prevalence, sensitivity and specificity of double reading and biopsy, and the stage prevalence of breast cancer were adjusted for each demographic and roll back calculations performed to compare the differences in incremental costs, effectiveness and ICER.

Results

Cost-effectiveness analysis

The expected cost and life expectancy for double reading were 458,488 yen and 23.8274 years, and 461,192 yen and 23.8361 years for single + CAD. Single + CAD increased the expected cost by 2,704 yen and extended life expectancy by 0.0087 years over double reading (Table 4). The ICER was 310,805 yen/life year gained, which was lower than our threshold, so the base case result was still

Table 4 Cost-effectiveness analysis

Strategy	Cost (JPY)	Incremental cost (JPY)	Effectiveness (years)	Incremental effectiveness (years)	Incremental cost-effectiveness ratio (ICER = JPY/life year gained)
Double reading	458,488		23.8274		
Single + CAD	461,192	2,704	23.8361	0.0087	310,805

1 USD = 79.80 JPY, PPP = 106 JPY in 2011

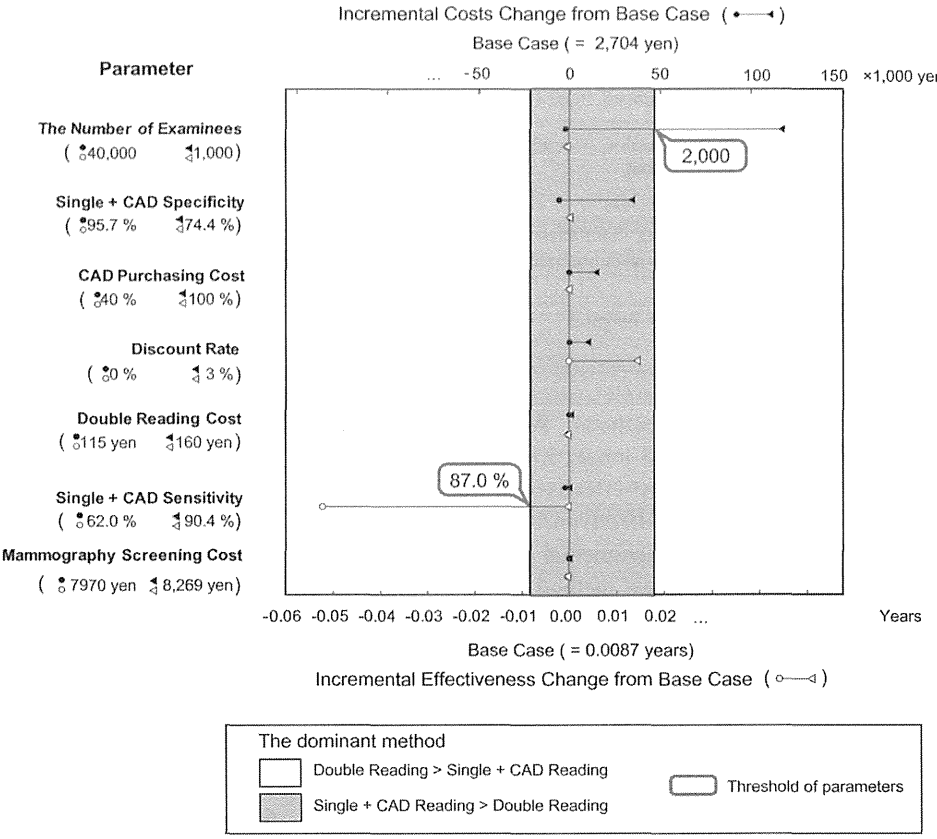

Fig. 4 One-way sensitivity analysis. The incremental cost and effectiveness changes for seven parameters. The base case was 2,704 yen and 0.0087 years and is defined as 0 in the chart. Single + CAD is the dominant method in the *grey area*, while double reading is dominant in the *white area*. Incremental cost was quite sensitive to the number of examinees and single + CAD specificity. Incremental effectiveness was sensitive only to the single + CAD sensitivity and discount rate. When the number of examinees was lower than 2,000 per year or sensitivity of single + CAD was lower than 87.0 %, the ICERs exceeded the threshold, and double reading became the dominant method

cost-effective even though single + CAD increased costs compared to double reading.

Sensitivity analysis

One-way and multi-way sensitivity analysis

The analysis result is summarized in Fig. 4. The parameter that had the greatest impact on expected cost was the examinee volume. When the number of examinees changed from 1,000 to 40,000, the incremental cost increased from 1,111 to 92,278 yen. The second parameter that affected the result was specificity of screening mammography on single + CAD. The incremental cost changed from 55,100 to −5,885 yen within the parameter range of 74.4–95.7 %. Changing the CAD purchasing cost from 40 to 100 % of full price increased the incremental cost from 2,704 to 8,565 yen. Changing the discount rate from 0 to 3 % decreased it from 7,881 to 2,704 yen. The other parameters had less impact on the result.

Changing the sensitivity of screening mammography on single + CAD within the range 62.0–90.4 % resulted in life expectancy increasing from −0.0518 to 0.0079 years. Changing the discount rate from 0 to 3 % decreased life expectancy from 0.0228 to 0.0087 years. The other parameters had no impact on the result according to the one-way sensitivity analysis.

Using the base case result and ICER threshold, we focused on parameters for which the result exceeded the threshold or had negative incremental effectiveness. The incremental cost exceeded the threshold when the number of examinees was less than 2,000, and the incremental effectiveness exceeded the threshold when sensitivity of single + CAD was less than 87.0 %. The relationship

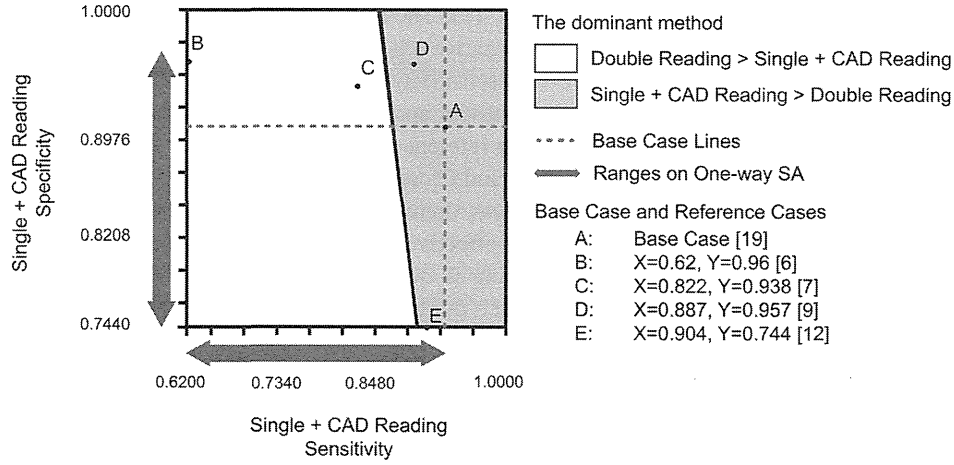

Fig. 5 Two-way sensitivity analysis. The graph shows the correlation between double reading and single + CAD for our model. Single + CAD was dominant in plot A with 90.8 % sensitivity and 92.7 % specificity by single + CAD reading. B, C, D and E were reference cases from reading test reports in Japan

between sensitivity and specificity of single + CAD can be seen in the result of the two-way sensitivity analysis summarized in Fig. 5.

Scenario sensitivity analysis

When we changed the breast cancer prevalence, sensitivity and specificity of double reading and biopsy, and the stage prevalence of breast cancer by initial age of screening, the incremental costs were 1,851, 2,704 and 3,068 yen at the ages of 40, 50 and 60, respectively. The incremental effectiveness was 0.0246 years for the 40-year-old population, 0.0087 years for the 50-year-old population and 0.0062 years for the 60-year-old population. The ICERs were 75,241 yen/life year gained, 310,805 yen/life year gained and 494,834 yen/life year gained at the ages of 40, 50 and 60, respectively.

Discussion

Simulation and analysis of the cost-effectiveness of breast cancer screening showed that a single reading with CAD increased lifetime medical costs and life years when compared with the conventional double reading method in the hypothetical setting. The ICER was below the threshold so CAD use in screening mammography was cost-effective. Sensitivity analyses proved that the result was quite sensitive to changes in input data, especially the number of examinees and sensitivity and specificity of the single mammography reading with CAD.

In the present work, we built the model with decision trees and a Markov model to analyze cost-effectiveness between two breast cancer screening methods. This model is applicable for other screening procedures such as ultrasonography, CT or MRI with appropriate input data for each modality. Also, data collection for other breast cancer screening situations, such as targeting a high-risk breast cancer population or some other demographic, may give further healthcare policy suggestions from the cost-effectiveness perspective. While there have been cost-effectiveness analysis reports in the medical field before, few focus on modeling breast cancer screening with CAD [3, 14]. Lindfors et al. [14] also reported a detailed modeling analysis of CAD use with a Markov model, but our methodologies of comparison and the medical cost setting in Japan are quite different from theirs. Ohnuki et al. [3, 23] similarly presented a breast cancer screening model with a continuous decision tree in 2006, but the structure does not include complete check up branches after the screening, and moreover their model did not consider changes in the input data with population aging. Our combined decision tree and Markov model with its detailed diagnosis process and Markov simulation include population aging, which is important for accurate breast cancer screening evaluation.

Our cost-effectiveness analysis focusing on CAD use for breast cancer screening in Japan may be the first such report and thus considered a pilot study for evaluating the economic and social benefits of two alternative screening methodologies. This cost-effectiveness evidence suggests the one reader with the CAD method could be used as a substitute for the double reading method in Japan's current healthcare setting. However, we used relatively high averaged sensitivity and specificity of a single reading with CAD from a systematic review because there is little research on CAD reading evaluation targeting screening mammography in Japan [18]. The sensitivity and specificity of CAD vary depending on the type of region being detected, such as calcification, mass, FAD or distortion. We performed sensitivity analysis within the range of the minimum and maximum data for four recent reading

examination publications in Japan [6, 7, 9, 12]. The result varied widely within this range and was negative when the sensitivity was less than 87 %. Two-way sensitivity analysis expressed the detailed effects of sensitivity and specificity of single + CAD (Fig. 5), with four reference cases (B, C, D, F) plotted in the graph. Cases B and C converted our analysis result, and double reading was dominant in both cases, which describes that the cost-effectiveness of introducing CAD to breast cancer screening is quite sensitive to sensitivity and specificity. Moreover, our base case had about 16,000 annual examinees, but to accommodate other screening facilities, we changed this number to between 1,000 and 40,000. The incremental cost was greatly impacted by these changes, and in particular if it was less than 2,000 examinees per year, the ICER exceeded our threshold.

Even though we have developed a detailed model of breast cancer screening and treatment, using the method in the medical field required us to simplify the model with several definitions and assumptions. First, we assumed that the rescreening rate was 100 % 2 years after the initial screening in the Markov model. However, the actual rate is lower (Miyagi Cancer Society: 50.3 %, average rate between 2002 and 2006) [24]. If lower rates of rescreening were considered in the model, then both methods would have more interval cancers, resulting in greater medical costs and shorter life years. However, the incremental cost and life years would be constant because of the similar impact on both methods. Second, the scenario sensitivity analysis showed that the result was always cost-effective for 40–60-year-old women with an ICER lower than the threshold, but with better cost-effectiveness for younger examinees than older. However, the analysis was limited by data collection in the single + CAD method. The data we used for sensitivity analysis did not have the age-specific sensitivity and specificity of a single reading with CAD. Therefore, we used fixed values even though they may vary with age. It was obvious from the sensitivity analysis that the variation in sensitivity and specificity of single reading with CAD could affect the cost-effectiveness. Further studies are needed to address this issue.

In conclusion, our cost-effectiveness analysis on breast cancer screening comparing a single reading using CAD and double reading for Japanese women found that a single reading with CAD increased costs by 2,704 yen and extended life years by 0.0087 years over double reading. The ICER was 310,805, which is below our threshold. The result is quite sensitive to some parameter changes, such as the number of examinees and sensitivity and specificity of a single reading with CAD. Our research, however, suggests that the high accuracy of CAD would make it an effective tool as a second reader for screening mammography, especially when there is a high volume of examinees.

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The Correlation between Body Mass Index and Breast Cancer Risk or Estrogen Receptor Status in Okinawan Women

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Dietary changes resulting from the post-World War II occupation of Okinawa by the US military have been largely deleterious, resulting in a marked increase of obesity among Okinawan residents. In this study, we examined the association between BMI and the risk of developing breast cancer according to the menstruation status and age, and the correlation between BMI and expression of estrogen receptor (ER). Breast cancer cases were 3,431 females without any personal or family history of breast cancer. Control subjects were 5,575 women drawn from the clinical files of Nahanishi Clinic. We found that women, who were overweight or obese, regardless of menopausal stage, had a higher risk of breast cancer compared to women with normal weight and this difference was statistically significant ($p < 0.001$, respectively). This risk was especially apparent in older (> 40 years) overweight or obese women. The women who were overweight or obese during postmenopausal ages were at higher risk of ER-positive breast cancer compared to women with normal weight. Results of our present study clearly indicate that increased BMI was associated with increased risk of developing breast cancer in Okinawan women, regardless of menopausal status. In addition, there was statistically significant correlation between BMI and ER expression in the postmenopausal period. Given the obesity epidemic associated with the extreme sociological and dietary changes brought about by the post-war occupation of Okinawa, the present study provides essential guidelines on the management, treatment and future breast cancer risk in Okinawa.

Keywords: body mass index; breast cancer risk; estrogen receptor; menstruation status; obesity
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Introduction

Breast cancer is the most common cancer in women worldwide (Tamaki et al. 2011, 2012; Youl et al. 2011). In Japan, the most predominant cancer in women at this juncture is that of the breast with incident rates increasing (Matsuda et al. 2012). This continuous increase of breast cancer incidence in Japan has resulted in widespread public health concern, with much attention being devoted to the importance of body mass index (BMI) and more specifically obesity as one of the most important risk factors (Harris et al. 2011a).

Obesity is a growing health problem globally (Berg et al. 2005) and its prevalence among the younger generations has increased continuously in Japan (Matsushita et al. 2008). Of note, this trend has been especially marked among individuals living in Okinawa compared to individuals living in mainland Japan (Matsushita et al. 2008). In Okinawa, the longevity advantage has been well documented (Matsushita et al. 2008) in older generations. This advantage is attributed to lifestyle, in particular, diet rather than ethnicity. The traditional diet of Okinawa consists of foods low in calories but rich in nutritional value, particularly in terms of vitamins, minerals, and phytonutrients in the form of antioxidants and flavonoids (Suzuki et al. 2001; Willcox et al. 2007) and importantly delicious in flavor. However, the post-World-War-II US military occupation of Okinawa had largely deleterious health effects, with Okinawan people developing higher rates of obesity corresponding to these post-war years (Todoriki et al. 2004; Willcox 2005; Tamaki et al. 2013) and a loss of the previous longevity advantage associated with the Okinawan life-

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style (Todoriki et al. 2004; Willcox 2005; Tamaki et al. 2013). The main change in this period, and simplest and most plausible explanation for this change was the introduction of an US lifestyle and diet to the Okinawan people. The US diet of the 1950s characterized by historically unprecedented amounts of readily available junk foods characteristically high in sugars and fats with minimal nutrition, combined with a low physical activity of US lifestyle might have contributed to the current high prevalence of overweight and/or obese individuals in Okinawa (Matsushita et al. 2008).

Importantly BMI is known to have a multifaceted mechanistic connection with breast cancer risk, although limited evidence suggests the risks associated with an increased BMI may be dependent upon menopausal status (Harris et al. 2011b). The majority of studies report an increased risk of higher BMI in both pre and post-menopausal breast cancers (Cauley et al. 1989; Sonnenschein et al. 1999; Key et al. 2003; Barlow et al. 2006; Tian et al. 2007), while one study suggests an inverse association between BMI in early adulthood and breast cancer incidence (Harris et al. 2011b). Despite the studies above, to the best of our knowledge, no studies have reported on the correlation between BMI and breast cancer risk for Okinawan people and in particular increased risks associated with the marked increases in BMI since US military occupation. Therefore, we examined the correlation between BMI and breast cancer of both premenopausal and postmenopausal women. To try and develop some mechanistic insight, we examined interactions between BMI and expression of estrogen receptor (ER), as these have been previously suggested to be associated in breast cancer patients (Feigelson et al. 2006; Millikan et al. 2008; Kwan et al. 2009; Suzuki et al. 2011; Sueta et al. 2012).

Methods

Study population

This study was conducted based upon Nahanishi Clinic Data Base System, Nahanishi Clinic, Okinawa, Japan. Table 1 summarizes the relevant information of the patients enrolled in our present study. This was a single institution study drawn from patients who visited the Nahanishi Clinic between May 1996 and March 2013. Case subjects were 3,431 female breast cancer patients without any personal or family breast cancer history. Control subjects were 5,575 women who visited the clinic but did not have detectable breast cancer; namely, the control subjects were defined as those who visited the clinic for breast cancer screening or non-malignant breast cancer growths. The median ages were 53 years old (20-99) of breast cancer group and 44 years old (20-90) of control group. The study protocol was approved by the Ethics Committee at Nahanishi Clinic Okinawa, Naha, Japan (NNCEC2013005). Results of their weights and heights were self-reported. As a relative indicator of body weight, BMI was calculated as weight in kilograms divided by the square of the height in meters (kg/m^2). Based on the criteria of World Health Organization classification, $18.5 < BMI$ was defined as underweight, $25 \leq BMI < 30$ as overweight and $30 \leq BMI$ as obese. The presence of ER was determined by distinctive nuclear immunoreactivity and the scores were graded from 0 to 8 using the Allred score, with positive case defined as the score of more than 3 (Allred et al. 1998). We had incomplete information regarding menstruation status (8,163 out of 9,006 subjects) and ER status (3,049 out of 3,431 breast cancer patients) leaving us with variable numbers when examining these factors in relation to breast cancer risk. We also examined the correlation between BMI and ER expression during premenopausal and postmenopausal status in 3,049 patients. Statistical analyses were performed using StatMate IV for Windows (ATMS, Tokyo, Japan). Breast cancer risk and ER expression were estimated by computation of the odds ratios and their 95% confidence intervals (CIs). Odds ratios were considered significant when 1.0 was not included in the 95% CI. Results were considered significant at $P < 0.05$. In all odds ratios calculations women falling in the normal BMI range of 18.5-25 were used as the reference grouping.

Results

The correlation between BMI and breast cancer risk according to menstruation in Okinawan Women

The effects of BMI on breast cancer development risk during premenopausal and postmenopausal periods were assessed using odds ratios. Table 2 summarized the results of the correlation between BMI and breast cancer risk

Table 1. Patients' characteristics.

	Cancer	Control
Total number	3,431	5,575
Age	53 (20-99)	44 (20-90)
BMI		
BMI < 18.5 (underweight)	152	500
18.5 ≤ BMI < 25 (normal weight)	1,969	3,803
25 ≤ BMI < 30 (overweight)	1,022	1,023
30 ≤ BMI (obesity)	288	249
Premenopause	996	3,711
Postmenopause	1,604	1,852
Uncertain menstruation	831	12

Table 2. The correlation between BMI and breast cancer risk according to menstruation.

	Cancer	Control	Reference Cancer	Reference Control	*P* Value	Odds Ratio	95% CI
Total: 8,163							
Premenopausal	996	3,711					
Underweight	81	432	915	3,279	0.002	0.67	0.52-0.86
Obesity	59	140	937	3,571	0.004	1.61	1.17-2.20
Overweight + Obesity	238	634	758	3,077	< 0.001	1.52	1.29-1.80
Postmenopausal	1,604	1,852					
Underweight	38	68	1,566	1,784	0.034	0.63	0.43-0.95
Obesity	181	109	1,423	1,743	< 0.001	2.03	1.59-2.61
Overweight + Obesity	800	635	804	1,217	< 0.001	1.91	1.66-2.19

according to the status of menstruation. In premenopausal women being underweight was associated with a statistically significant reduction in odds ratio while being overweight or obese was associated with a statistically significant increase in odds ratio and hence risk of breast cancer. Similar statistically significant patterns were seen in the group of postmenopausal women.

The correlation between BMI and breast cancer risk according to the age of women

Table 3 summarizes the results of the correlation between BMI and breast cancer risk stratified by the age of the patients. The women who were greater than 40 years in age and were either overweight or obese had a significantly higher risk of breast cancer development compared to the women in a normal weight range. The same tendency was also detected in 30-40-year-old women i.e., the women who were either overweight or obese tended to have higher incidence of breast cancer than those not. There were no statistically significant differences between underweight and non-underweight in any of the age groups.

The correlation between BMI and ER expression according to menstruation

Table 4 summarizes the results of the correlation between BMI and ER status divided into premenopausal and postmenopausal groups. There were no statistically significant differences between BMI and ER status in the premenopausal period. However, women who were overweight or obese during postmenopausal ages were at higher rates of ER-positive breast cancer when compared to normal weight women ($p = 0.002$ and $p < 0.001$, respectively). There was no statistically significant correlation between BMI and ER status in underweight women in premenopausal or postmenopausal women.

Discussion

We examined the correlation between BMI and breast cancer of both premenopausal and postmenopausal women born, brought up and living in Okinawa. Results of our present study of Okinawan women are quite unique in terms of recent drastic and enormous changes of diet and lifestyle in one single community. To the best of our knowledge, this is the first study to examine whether BMI is associated with risk of breast cancer development or not in this population.

Results of previous meta-analysis studies suggested that high BMI during premenopausal period may protect against breast cancer development in later life, although the association did not reach statistical significance (Cheraghi et al. 2012). This trend has been also reported in several other studies showing a significant inverse association between body weight and breast cancer incidence in premenopausal women (Harris et al. 2011a, b). In contrast, the same meta-analysis demonstrated that increased BMI during postmenopausal period could significantly increase the risk of breast cancer development (Cheraghi et al. 2012). The increased risk in postmenopausal women associated with being overweight and/or obese is due to the fact that adipose tissue is the major source of estrogen following menopause (Cauley et al. 1989; Key et al. 2003). While the mechanism of protection by obesity in premenopausal women is unknown, the observation that young overweight women are more likely to have anovulatory cycles with less cumulative exposure to endogenous estrogen (Stoll 1994) or the greater clearance of estrogen by the liver in young overweight women (Potischman et al. 1996) resulting in a reduction in circulating hormones have been proposed as potential mechanisms.

Results of our present study demonstrated that the Okinawan women who were overweight and/or obese during premenopausal and postmenopausal ages had a statistically significant higher risk of breast cancer development compared to normal weigh women (BMI 18.5-25). This was particularly true in women who fell into age group greater than 40 years. These results clearly indicate that increased BMI was associated with increased risk of breast cancer in Okinawan women regardless of menopausal status.

This trend was inverted in underweight women who exhibited lower breast cancer risk. Though previous meta-analyses and international evaluation showed that being

Table 3. The correlation between BMI and breast cancer risk according to age.

	Cancer	Control	Reference Cancer	Reference Control	*P* Value	Odds Ratio	95% CI
Total: 9,006							
20 ≤ age < 30	32	688					
Underweight	7	155	25	533	0.897	0.96	0.41-2.27
Obesity	0	17	32	671	0.718		
Overweight + Obesity	3	60	29	628	0.884	1.08	0.32-3.65
30 ≤ age < 40	335	1,414					
Underweight	31	184	304	1,230	0.073	0.68	0.46-1.02
Obesity	22	53	313	1,361	0.032	1.80	1.08-3.01
Overweight + Obesity	63	204	272	1,210	0.055	1.37	1.01-1.88
40 ≤ age < 50	1,031	1,442					
Underweight	64	98	967	1,344	0.616	0.91	0.66-1.26
Obesity	52	56	979	1,386	0.196	1.31	0.89-1.93
Overweight + Obesity	276	298	755	1,144	< 0.001	1.40	1.16-1.69
50 ≤ age < 60	892	1,112					
Underweight	24	38	868	1,074	0.421	0.78	0.47-1.31
Obesity	79	53	813	1,059	< 0.001	1.96	1.37-2.81
Overweight + Obesity	368	323	524	789	< 0.001	1.72	1.42-2.07
60 ≤ age < 70	665	620					
Underweight	12	18	653	602	0.263	0.61	0.29-1.29
Obesity	70	50	595	570	0.156	1.34	0.92-1.96
Overweight + Obesity	345	250	320	370	< 0.001	1.60	1.28-1.99
70 ≤ age	476	299					
Underweight	14	7	462	292	0.784	1.20	0.50-3.17
Obesity	65	20	411	279	0.004	2.21	1.31-3.72
Overweight + Obesity	255	137	221	162	0.043	1.36	1.02-1.82

Table 4. The correlation between BMI and ER expression.

	ER+	ER−	Reference ER+	Reference ER−	*P* Value	Odds	95% CI
Total: 3,049							
Premnopause	954	269					
Underweight	19	8	935	261	0.463	0.66	0.29-1.53
Obesity	43	17	911	252	0.232	0.67	0.37-1.20
Overweight + Obesity	213	73	741	196	0.118	0.77	0.57-1.05
Postmenopause	1,256	325					
Underweight	38	8	1,218	317	0.723	1.24	0.57-2.68
Obesity	160	25	1,096	300	0.002	2.05	1.30-3.22
Overweight + Obesity	671	138	585	187	< 0.001	1.55	1.21-1.99

underweight is a risk factor among premenopausal women (Renehan et al. 2008), in our study being underweight was associated with lower breast cancer risk. However this requires further study to fully understand underlying mechanistic factors.

Many previous studies demonstrated that the association between BMI and risk of postmenopausal breast cancer is stronger for ER-positive breast cancer than ER-negative breast cancer (Feigelson et al. 2006; Suzuki et al. 2011; Sueta et al. 2012). However some previous studies demonstrated that premenopausal ER-negative breast cancer patients were more likely to be overweight and/or obese at

diagnosis compared with ER-positive breast cancer patients (Millikan et al. 2008; Kwan et al. 2009). The results of this study demonstrated that there was statistically significant correlation between BMI and ER expression in the post-menopausal, but not premenopausal period.

Residents of the Okinawa archipelago historically have had one of the highest longevity rates in the world, attributable at least in part to a traditional diet that is low in calories but delicious and nutritionally balanced as described above (Suzuki et al. 2001; Todoriki et al. 2004; Willcox 2005; Willcox et al. 2007). However, coincident with the huge socio-cultural changes of the last 70 years, many of which are associated with the post-World-War-II American occupation, Okinawa now has the dubious privilege of having the highest rates of obesity within Japan (Takasu et al. 2007; Matsushita et al. 2008), with obesity currently the most serious public health problem in Okinawan women. It is therefore imperative to have more evidence on whether obesity is associated with increased risks of breast cancer development in these populations (Lee and Yee 1995; Pollack 1998; Yee 1998), and to provide clear information as to the other health hazards of overweight/obesity to women living in Okinawa islands. While the present study aimed to address this issue, it has several limitations. One is the selection bias because this study was conducted in a single institution. Therefore, results obtained in our present study may not represent the features of Okinawa residents as a whole. In addition, the data of environmental factors such as physical status and diabetes history were not necessarily available in this study. Therefore, we could not evaluate the environmental factors, BMI and others and this awaits further investigations for clarification.

In conclusion, this study provides the first evidence to suggest the association between increased breast cancer risk and BMI in an Okinawan population. Given the obesity epidemic associated with the extreme sociological and dietary changes brought about by the post-war occupation of Okinawa, this information provides essential guidelines on the management, treatment and future breast cancer risk in the Okinawan islands.

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Conflict of Interest

The authors declare no conflict of interest.

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Increased androgen receptor activity and cell proliferation in aromatase inhibitor-resistant breast carcinoma

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ABSTRACT

Aromatase inhibitors (AI) are commonly used to treat postmenopausal estrogen-receptor (ER)-positive breast carcinoma. However, resistance to AI is sometimes acquired, and the molecular mechanisms underlying such resistance are largely unclear. Recent studies suggest that AI treatment increases androgen activity during estrogen deprivation in breast carcinoma, but the role of the androgen receptor (AR) in breast carcinoma is still a matter of controversy. The purpose of this study is to examine the potential correlation between the AR- and AI-resistant breast carcinoma. To this end, we performed immunohistochemical analysis of 21 pairs of primary breast carcinoma and corresponding AI-resistant recurrent tissue samples and established two stable variant cell lines from ER-positive T-47D breast carcinoma cell line as AI-resistance models and used them in *in vitro* experiments. Immunohistochemical analysis demonstrated that the expression of prostate-specific antigen (PSA) and Ki-67 were significantly higher and ER and progesterone receptor (PR) were lower in recurrent lesions compared to the corresponding primary lesions. Variant cell lines overexpressed AR and PSA and exhibited neither growth response to estrogen nor expression of ER. Androgen markedly induced the proliferation of these cell lines. In addition, the expression profile of androgen-induced genes was markedly different between variant and parental cell lines as determined by microarray analysis.

These results suggest that in some cases of ER-positive breast carcinoma, tumor cells possibly change from ER-dependent to AR-dependent, rendering them resistant to AI. AR inhibitors may thus be effective in a selected group of patients.

1. Introduction

Breast cancer is one of the most common malignancies in women worldwide. The estrogen receptor (ER) is expressed in approximately two-thirds of breast carcinomas, and a great majority of ER-positive cases respond well to endocrine therapy. Aromatase inhibitors (AI), such as anastrozole, exemestane, and letrozole, potently block estrogen biosynthesis from androgens;

Abbreviations: AI, aromatase inhibitor; AR, androgen receptor; ARE, androgen-response element; DDC, L-DOPA decarboxylase; DHT, dihydrotestosterone; ER, estrogen receptor α; ERE, estrogen-response element; E_2, estradiol; GFP, green fluorescent protein; LI, labeling index; PR, progesterone receptor; PSA, prostate-specific antigen; TS, testosterone.

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these agents have shown better clinical outcomes than anti-estrogen tamoxifen in several clinical trials [1–3]. Nevertheless, in some cases, acquired resistance has been reported after initial successful AI treatment [4]. The molecular mechanisms underlying AI resistance have been examined by several groups [5–9] but still remain largely unclear.

In contrast, androgens are considered to predominantly exert antiproliferative effects in breast carcinoma [10]. Recent meta-analysis showed that androgen receptor (AR) status was associated with better clinical outcomes in breast carcinoma patients [11]. However, the involvement of AR in promoting proliferation, especially in ER-negative cases [12,13], has been reported. Hickey et al. [14] have suggested that the AR rather than the ER plays an oncogenic role due to AR activation when estrogen activity is reduced.

Biologically active androgen, dihydrotestosterone (DHT), is produced locally in breast carcinoma. Aromatase acts as a negative regulator of *in situ* DHT production by decreasing its precursor [15]. Intratumoral DHT concentration was significantly higher in breast carcinoma treated with AI compared to untreated controls [16]. AI therapy is suggested to increase local androgen actions besides inducing estrogen deprivation [15,16]. Therefore, it is possible that intratumoral androgen activity may increase in recurrent breast carcinoma under estrogen deprivation caused by AI treatment, thus playing an important role in AI resistance. However, androgen activity has not been examined in recurrent breast carcinoma following AI therapy. In this study, we first examined the immunohistochemical features of recurrent breast carcinoma lesions during AI treatment and showed an increment of AR activity compared to the corresponding primary lesions. Moreover, we established AI-resistant model cell lines from ER-positive breast carcinoma cell line, T-47D, and further characterized biological roles of AR in the AI-resistant cell lines.

2. Materials and methods

2.1. Patients and tissues

ER-positive breast carcinoma specimens ($n = 21$) were obtained from postmenopausal women who underwent surgical treatment between 2002 and 2009 at: Tohoku University Hospital, Sendai, Japan ($n = 7$); Tohoku Kosai Hospital, Sendai, Japan ($n = 5$); Miyagi Cancer Center Hospital, Natori, Japan ($n = 5$); and Iwate Prefectural Central Hospital, Morioka, Japan ($n = 4$). The patient characteristics are summarized in Table 1. All patients received oral aromatase inhibitors after surgery and had asynchronous recurrence during this treatment. The median duration of treatment with AI was 34 months. 3 of the 17 patients who received anastrozol initially switched their treatment from anastrozol to exemestane because of an incidence of recurrence or side effect of anastrozol. The corresponding recurrent breast carcinoma lesions were available for examination in all cases. All specimens were fixed in 10% formalin and embedded in paraffin wax.

Our research protocol was approved by the Ethics Committee at Tohoku University School of Medicine and other institutional review boards.

2.2. Immunohistochemistry

Mouse monoclonal antibodies for AR (AR441) and Ki-67 (MIB1), and rabbit polyclonal antibody for prostate-specific antigen (PSA; IR514/IS514) were purchased from DAKO (Carpinteria, CA, USA). Amplification was performed using the Histofine Kit (Nichirei Biosciences, Tokyo, Japan), which employs the streptavidin–biotin amplification method. The antigen–antibody complex was visualized with 3,3′-diaminobenzidine (DAB) solution (1 mM DAB, 50 mM Tris–HCl buffer (pH 76), and 0.006% H_2O_2) and counterstained with hematoxylin.

Table 1
Clinicopathological characteristics of 21 patients in the present study.

	Number of patients	Median (min-max)
Patient age at the surgery[a]		63 (48-72)
Stage		
I	5	
II	9	
III	7	
Histological type		
Invasive ductal carcinoma	19	
Invasive lobular carcinoma	2	
Intrinsic subtype[b]		
Luminal A	16	
Lumbinal B	5	
Histological grade		
1	3	
2	15	
3	3	
Chemotherapy received		
Neoadjuvant Chemotherapy	3	
Adjuvant chemotherapy	7	
Not received	12	
Type of AI received after the surgery		
Anastrozole	17	
Exemestane	6	
Letrozole	1	
Time from the surgery to recurrence[a] (months)		53 (7-76)
Duration of AI-treatment[a] (months)		34 (7-70)
Recurrent lesions examined		
Lymph node	11	
Chest wall	5	
Lung	4	
Bone	1	

[a] Data represent the median (min–max), and all other values are presented as the number of cases.[b]Intrinsic subtype was defined according to 2011 St. Gallen surrogate definition [45].

Immunohistochemistry to detect expression of the ER (CONFIRM anti-ER (SP1), Roche Diagnostics Japan, Tokyo, Japan) and progesterone receptor (PR; CONFIRM anti-PgR (1E2), Roche Diagnostics, Japan) was performed using the Ventana Benchmark XT instrument (Roche Diagnostics, Japan). Immunohistochemical analysis of HER2 expression was performed using HercepTest™ (DAKO).

2.3. Scoring of immunoreactivity

ER, PR, AR, and Ki-67 immunoreactivity was detected in the nuclei of breast carcinoma cells, and the percentage of immunoreactive cells, *i.e.*, labeling index (LI), was determined. PSA immunoreactivity was considered positive if any cytoplasmic staining was observed in the carcinoma cells [17]. HER2 immunoreactivity was evaluated according to a grading system proposed in HercepTest™, and specimens with a score of 3+ were considered positive. Moreover, *HER2* gene amplification was investigated by fluorescence *in situ* hybridization (FISH) in score 2+ cases.

2.4. Cells and reagents

T-47D breast carcinoma cells were stably transfected with the estrogen response element (ERE)-green fluorescent protein (GFP)

reporter plasmid as reported previously [18] (Supplementary Fig. S1A). T-47D cells were cultured in RPMI-1640 medium (Sigma–Aldrich, St. Louis, MO, USA) supplemented with 5% or 10% fetal calf serum (FCS; Tissue Culture Biogicals, Turale, CA, USA). Phenol red-free RPMI (PRF-RPMI; Gibco BRL, Grand Island, NY, USA) supplemented with 5% or 10% dextran-coated charcoal-treated FCS (DCC-FCS) was used as the steroid-depleted medium in each experiment. T-47D cells were obtained from the American Type Culture Collection (Manassas, VA, USA), and authenticated using a PowerPlex® 16 STR system on January 25, 2013.

Estradiol, testosterone, DHT, bicalutamide, fulvestrant, and NSD-1015 (3-hydroxybenzylhydrazine dihydrochloride) were purchased from Sigma–Aldrich.

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2.5. Real-time PCR

Total RNA was extracted using ISOGEN (Nippon Gene, Toyama, Japan), and the extracted RNA was converted to cDNA using a

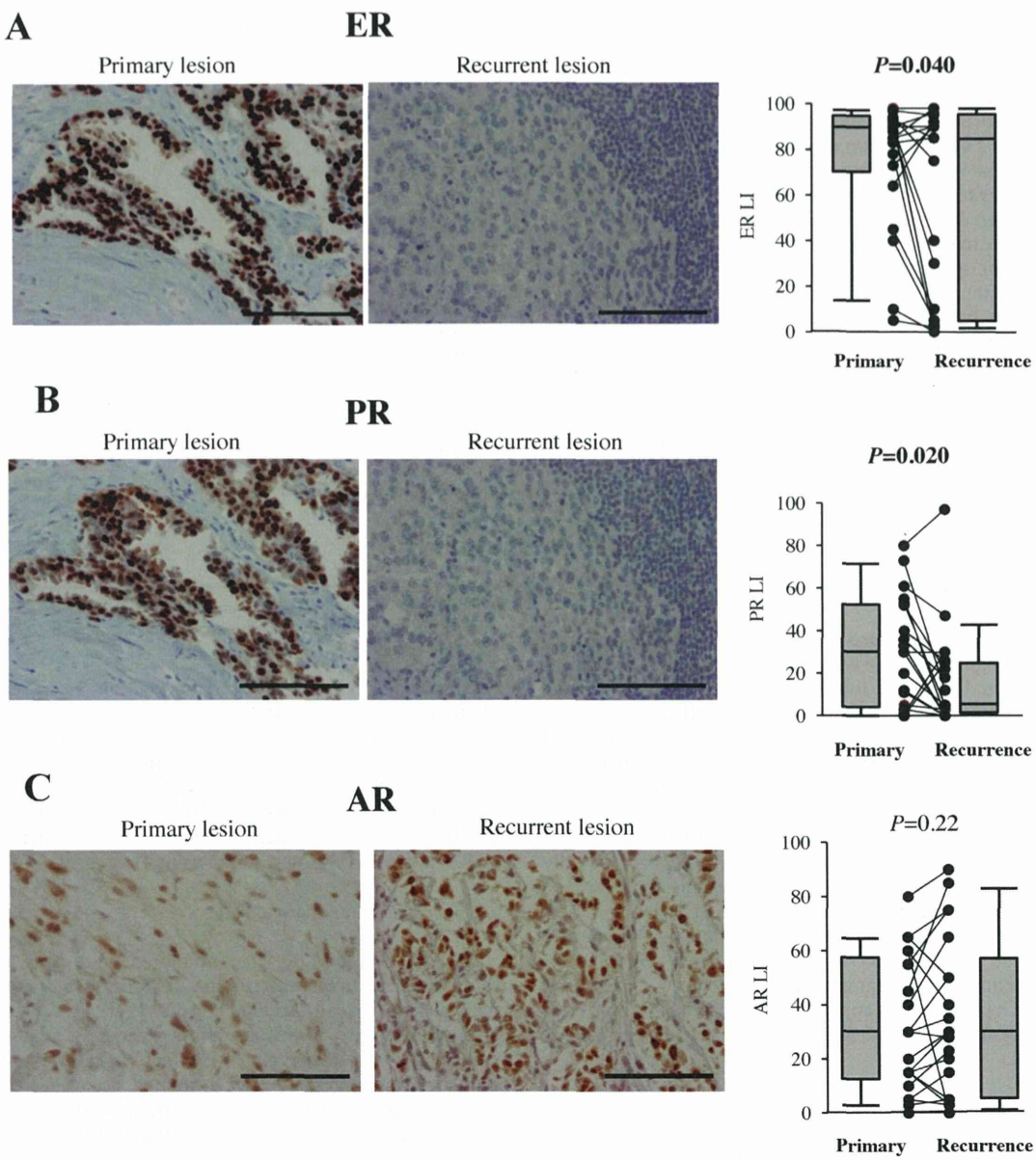

Fig. 1. Immunohistochemical analysis in AI-resistant breast carcinoma. Left panels show immunoreactivity of ER (A), PR (B), AR (C), PSA (D), and Ki-67 (E) in primary lesions, and middle panels show the same in corresponding recurrent lesions from the same patients during AI treatment. Left and middle panels of A and B show the same area. Bar = 100 μm. Right panels summarize changes in immunoreactivity in 21 paired breast carcinoma tissues obtained from primary and recurrent lesions. Each value is indicated by a slid circle, with lines connecting paired values from the same patient. The grouped data are represented as box-and-whisker plots. In A–C and E, the median value is shown by a horizontal line in the box plot, and the gray box denotes the 75th (upper margin) and 25th percentiles of the values (lower margin). The upper and lower bars indicate the 90th and 10th percentiles, respectively. Statistical analyses were performed using a Wilcoxon signed-rank test, and $P < 0.05$ (bold) was considered significant.

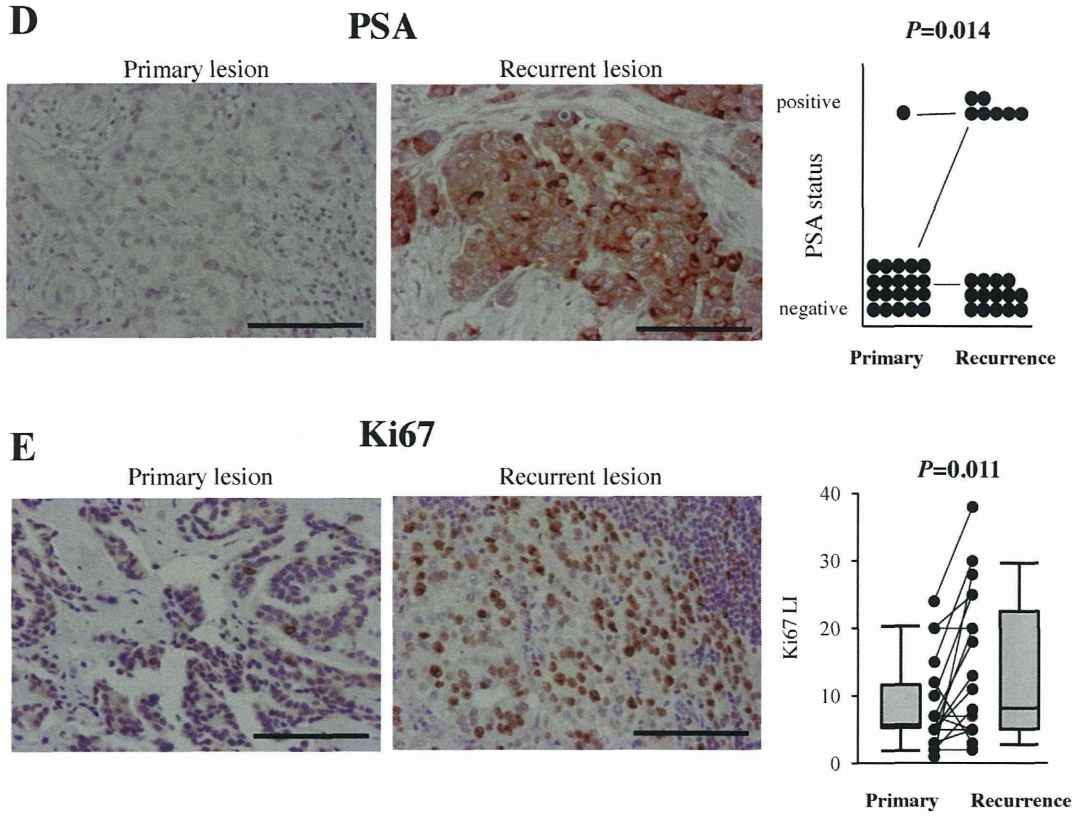

Fig. 1. (Continued)

QuantiTect reverse transcription kit (QIAGEN, Mississauga, Ontario, Canada). A 2-μl aliquot was used as a template for real-time PCR, which was performed according to the manufacturer's protocol using the Applied Biosystems Step One Real-time PCR System (Life Technologies Corporation, Carlsbad, CA, USA). The expression of the target gene relative to the *RPL13A* internal control was calculated. The primer data are summarized in Supplementary Table S1.

Supplementary material related to this article found, in the online version, at http://dx.doi.org/10.1016/j.jsbmb.2014.08.019.

2.6. Immunoblotting

Proteins were extracted using Complete Lysis-M (Roche, Indianapolis, IN, USA). Protein extracts (10 μg) were subjected to SDS-PAGE (Super Sep Ace 10%, Wako Pure Chemical Industries, Osaka, Japan) and transferred onto a membrane (Amersham Hybond-P PVDF Membrane, GE Healthcare, Buckinghamshire, UK). The primary antibodies were anti-AR (#3202), anti-PSA (#5365), and anti-β-tubulin (#2146) (Cell Signaling Technology, Tokyo, Japan) and anti- ER antibody (sc-7207; Santa Cruz Biotechnology, Santa Cruz, CA, USA). The secondary antibody, alkaline phosphatase-conjugated goat anti-rabbit, was obtained from Bio-Rad Laboratories (Hercules, CA, USA). Antibody–protein complexes were detected using Immun-Star™ AP substrate (Bio-Rad Laboratories), and the protein bands were visualized using the ImageQuant™ LAS 4000 image analyzer (GE Healthcare Bio-Sciences AB, Uppsala, Sweden).

2.7. Luciferase reporter assay

The estrogen response element reporter plasmid, ERE-tk-Luci, was used as described previously [19]. The androgen response element (ARE) reporter plasmid, pGLPSAp5.8 [20] , containing the PSA–ARE was kindly provided by Dr. Mizokami (Kanazawa University, Kanazawa, Japan). The control vector pRL-TK (Promega, Madison, WI, USA) was used as an internal control for transfection efficiency. The luciferase assay was performed according to a previous report [19] with some modifications. Cells were cultured in a steroid-depleted medium for 3 days before the transfection using TransIT LT-1 reagent (Mirus, Madison, WI, USA), and luciferase activity was measured using the Dual-Luciferase Reporter Assay System (Promega).

2.8. Cell proliferation assay

After 3 days in steroid-depleted medium, cells were seeded in 24-well culture plates at a density of 20,000 cells/well with drugs and hormones for 4 days. Cells were then harvested and counted using a Sysmex CDA-500 automated cell counter (Sysmex, Kobe, Japan).

2.9. Microarray analysis

Whole Human Genome DNA Microarray 4 × 44 K ver. 2.0 (Agilent Technologies, Santa Clara, CA, USA) was used in this study. Cells were cultured in steroid-depleted medium for 3 days, followed by a treatment with 1 nM DHT with or without 10 μM

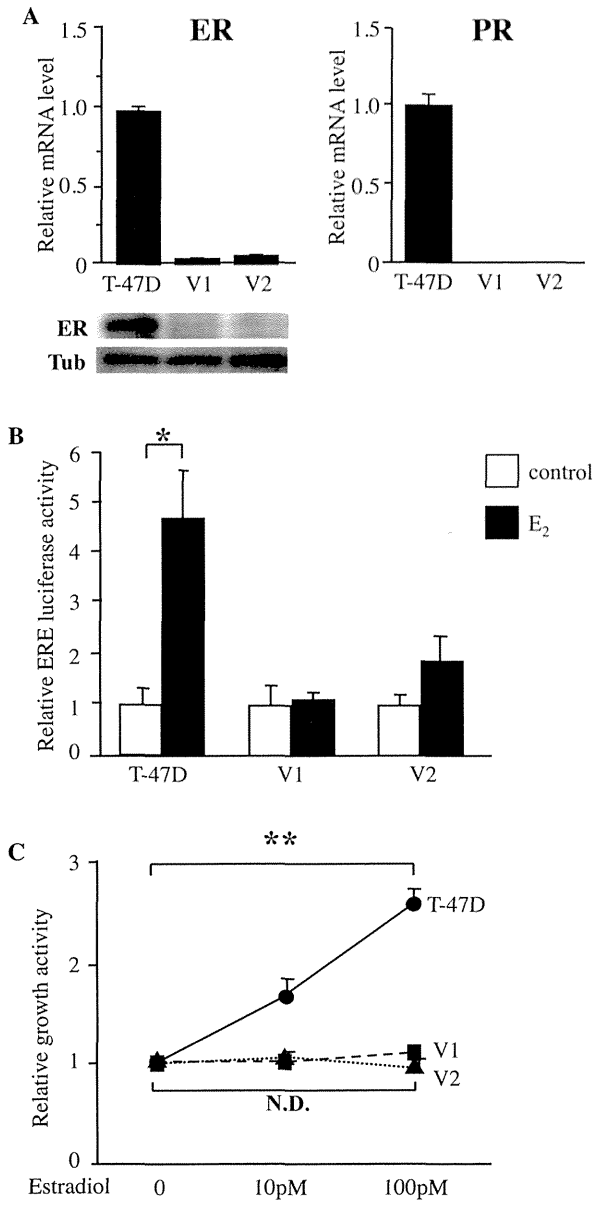

Fig. 2. ER function in AI-resistant breast carcinoma cells. A: ER (left upper panel) and PR (right upper panel) mRNA expression in V1, V2, and T-47D cells as determined by real-time PCR. Relative expression levels are expressed as a ratio compared to those in T-47D cells (left bar). Data are presented as mean ($n=2$). Lower panel demonstrates ER immunoreactivity in cells by immunoblotting. β-tubulin (Tub) immunoreactivity is shown as an internal control. B: ER activity in these cells by ERE- luciferase reporter assays. The cells were treated with 100 pM estradiol (closed bar) or vehicle control (ethanol; open bar) for 2 days. The values relative to vehicle control are shown as mean ± S.D. ($n=3$). C: Estrogen-mediated cell proliferation. V1, V2, and T-47D cells were treated with the indicated concentrations of estradiol or vehicle control (ethanol) for 4 days. The relative proliferative activity is the ratio compared to the vehicle control, presented as mean ± S.D. ($n=3$). $^{*}P<0.05$; $^{**}P<0.01$.

bicalutamide for 24 h. Subsequently, total RNA was extracted, amplified, and labeled using the Low Input Quick Amp Labeling Kit (Agilent Technologies). The relative levels of gene expression were calculated by global normalization, and scatter plot analysis of the microarray data was performed using GeneSpring 12.5 (Agilent Technologies). Microarray data are available in the ArrayExpress database (www.ebi.ac.uk/arrayexpress) under the accession number EMBL: E-MTAB-1933. The biological functions and interactions of each gene were identified by ingenuity pathway analysis (Ingenuity® Systems, www.ingenuity.com).

2.10. Statistical analysis

The Wilcoxon signed rank test and Student's *t*-test were used in the immunohistochemical analyses and *in vitro* experiments, respectively, and $P<0.05$ was considered significant.

3. Results

3.1. Immunohistochemical features in recurrent breast carcinoma during AI treatment

We first compared the immunohistochemical features of recurrent breast carcinoma lesions during AI treatment with those of the corresponding primary lesions. As shown in Fig. 1 and Table S2, LIs of the ER (Fig. 1A) and PR (Fig. 1B) were significantly lower in the recurrent lesions ($P=0.040$ and $P=0.020$, respectively). In the recurrent lesions, 2 out of 21 cases had lost the ER expression completely. In contrast, the AR LI was higher in recurrent lesions than primary lesions from the same patients in 13 out of 21 cases (62%) (Fig. 1C), although this difference did not reach a significance ($P=0.22$). Immunohistochemical analysis of primary lesions for the androgen-induced protein PSA [21] revealed only one positive case, while recurrent lesions were positive in 7 out of 21 cases. This result indicates that the PSA status was markedly increased in patients with AI-resistant recurrence ($P=0.014$) (Fig. 1D). The Ki-67 LI was also significantly higher in the recurrent lesions ($P=0.011$) (Fig. 1E), but that of HER2 was not significantly different between the primary and the recurrent lesions ($P=0.50$).

Supplementary material related to this article found, in the online version, at http://dx.doi.org/10.1016/j.jsbmb.2014.08.019.

3.2. ER-dependent cell proliferation is absent in variant cell lines established to model AI resistance

To facilitate further studies of androgen activity in recurrent tumors, we next established AI- resistance breast carcinoma cell lines from T-47D cells that expressed both the ER and AR [22]. We cultured T-47D cells stably transfected with ERE-GFP for 3 months in an androgen-supplemented steroid-depleted medium, thus reflecting the hormonal conditions under AI treatment (Supplementary Fig. S1). The cells gradually lost GFP fluorescence with the progression of passages under these conditions. Finally, none of them had vivid fluorescence. We picked two colonies as variant cell lines, temporarily named them V1 and V2 and cultured them under the same conditions (androgen-supplemented steroid-depleted medium). Disappearance of ERE–GFP fluorescence indicated a loss of the ER activity in variant cell lines. As shown in Fig. 2A, the ER mRNA expression level was much lower in variant cell lines (0.048-fold in V1 and 0.07-fold in V2) compared to parental T-47D cells (upper left panel), and the ER immunoreactivity was undetectable in these cells (lower panel). The expression of PR mRNA, encoding a known estrogen-induced protein [23], was undetectable in V1 and V2 cells (upper right panel). The ERE–luciferase reporter assays revealed that the ER transcriptional activity was not significantly changed by an estradiol treatment in V1 or V2 cells, differing from that of the T-47D cells (Fig. 2B). The proliferation of T-47D cells was significantly induced by estradiol in a dose-dependent manner but V1 and V2 cells did not proliferate in response to estradiol

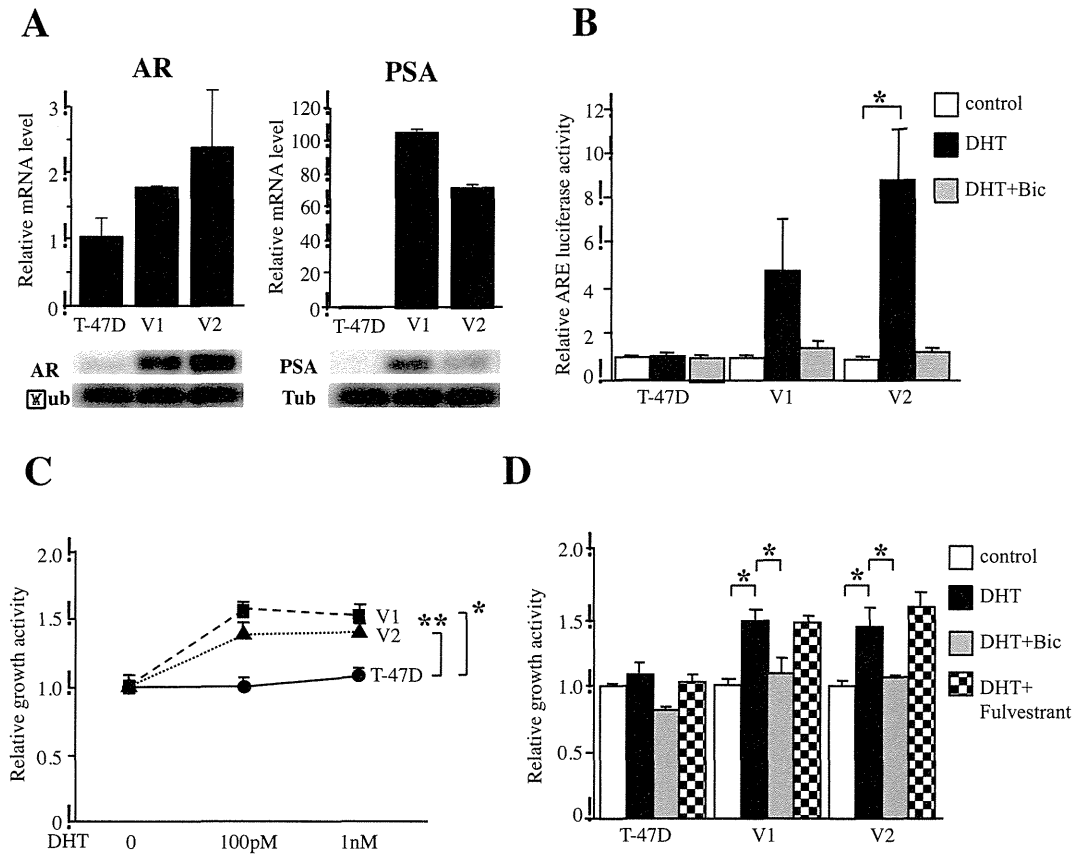

Fig. 3. AR function in AI-resistant breast carcinoma cells. A: AR (upper left panel) and PSA (upper right panel) mRNA expression in V1, V2, and T-47D cells as determined by real-time PCR. The relative expression level is the ratio compared to that of T-47D cells (left bar), and is presented as mean ($n = 2$). Lower panel summarizes immunoblotting for AR (lower left panel) and PSA (lower right panel) in these cells. β-tubulin (Tub) immunoreactivity is shown as an internal control. B: AR activity by ARE-luciferase reporter assays. Cells were treated with 1 nM DHT alone (closed bar), 1 nM DHT and 10 μM bicalutamide (gray bar), or vehicle control (ethanol; open bar) for 2 days. The values relative to the vehicle control are shown and data are presented as mean ± S.D. ($n = 3$). C: Androgen-mediated cell proliferation. Cells were treated with the indicated concentrations of DHT or vehicle control (ethanol) for 4 days. The relative proliferation activity is the ratio compared to the vehicle control and are presented as mean ± S.D. ($n = 3$). D: Effects of the AR and ER antagonists on androgen-meditated cell proliferation. V1, V2, and T-47D cells were treated with 1 nM DHT alone (closed bar), DHT with 10 μM bicalutamide (gray bar), or 100 nM fulvestrant (checkered bar) for 4 days. The relative proliferation activity is the ratio compared to the vehicle control (ethanol alone; open bar), and data are presented as mean ± S.D. ($n = 3$). $^{*}P < 0.05$; $^{**}P < 0.01$.

(Fig. 2C). These results suggest that the variant cells do not depend on estrogen-mediated signals to proliferate after long-term exposure to estrogen-depleted and androgen-supplemented conditions.

3.3. Variant cell lines exhibit androgen receptor-mediated proliferation

We next examined the response of variant cell lines to androgen. As shown in Fig. 3A, AR mRNA expression was higher (1.7-fold in V1 and 2.3-fold in V2) in the variant cell lines than the T-47D (left upper panel), and the level of AR protein was markedly higher in variant cell lines (left lower panel). The PSA mRNA expression was higher in V1 (114-fold) and V2 (78-fold) than T-47D (upper right panel), and similar results were observed for protein expression (lower right panel). ARE–luciferase reporter assays revealed that the level of AR transcription induced by DHT was higher in the variant cell lines (V1, 4.7-fold, $P = 0.07$; V2, 8.7-fold, $P < 0.05$) than the T-47D cells (1.0-fold; $P = 0.95$); AR transcription was potently inhibited by the addition of the AR-antagonist bicalutamide (Fig. 3B).

Variant cell lines demonstrated dose-dependent DHT-mediated cell proliferation that was significantly higher in V1 (1.4-fold; $P < 0.05$) and V2 (1.3-fold; $P < 0.05$) cells than T-47D cells under treatment with 1 nM DHT (Fig. 3C). DHT-mediated proliferation of variant cells was significantly inhibited by bicalutamide but was not affected by the ER-antagonist fulvestrant (Fig. 3D). These results suggest that the variant cell lines acquired an androgen receptor-mediated proliferation activity.

3.4. Expression profile of androgen-induced genes differs between variant and parental cell lines

To further examine the molecular effects of androgens on variant cells, gene expression profiles of V1 and T-47D cells were assessed by microarray analysis. We defined "androgen-induced genes" as those demonstrating greater than 2.5-fold higher expression in cells treated with DHT alone compared to those treated with DHT plus bicalutamide. A total of 390 androgen-induced genes were identified (Fig. 4A). Of these, 116 (30%) were induced only in V1 cells and 262 (67%) were induced only in T-47D cells. Only 12 genes (3%) were induced in both the cell lines. Comparison of expression levels of the 390 androgen-induced genes in cells treated with DHT alone by scatter plot revealed that 100 genes (26%) were predominantly expressed in V1 cells (Group A; V1/T-47D ratio >2.0), while 185 genes (47%) were predominantly expressed in T-47D cells (Group B; ratio <0.5)

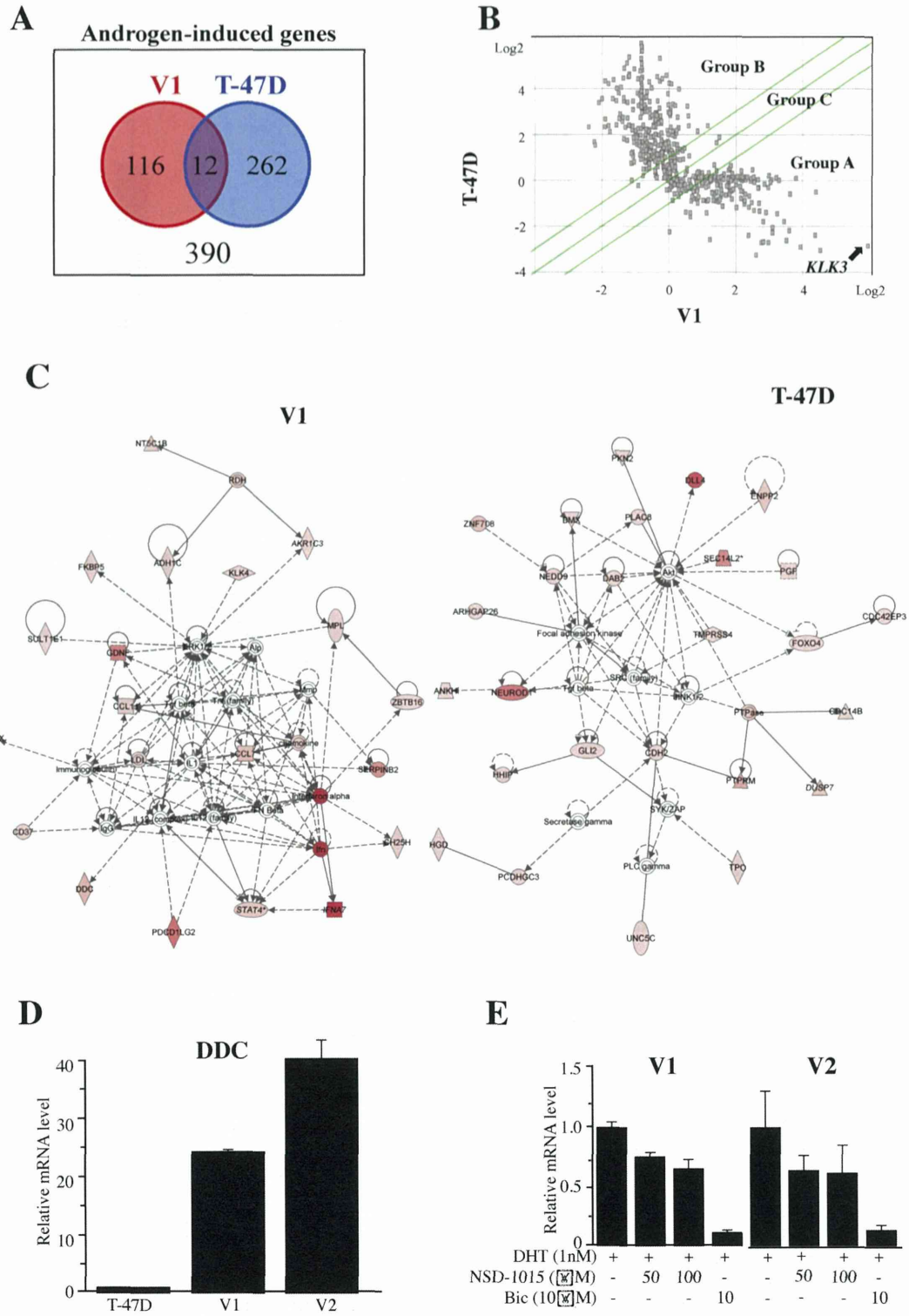

Fig. 4. Expression profile of androgen-induced genes in AI-resistant cells. A: Venn diagrams showing the numbers of androgen-induced genes identified in V1 and/or T-47D cells using microarray analysis. B: Scatter plot analysis of microarray data for 390 DHT-induced genes in V1 and T-47D cells. The position of each dot corresponds to the normalized average signal intensity ($\log_2$ scale) of a single gene. The middle line indicates values that represent the V1/T-47D ratio of 1.0, and the outer lines represent the V1/T-47D ratio of 2.0 (lower line) and 0.5 (upper line). Genes were classified by V1/T-47D ratio as follows: with a ratio of >2.0, group A; <0.5, group B; and 0.5–2.0, group C. The location of KLK3 (PSA) is marked. C: Networks of top-ranked androgen-induced genes in V1 (left panel) and T-47D (right panel) cells. The intensity of the grey node indicates the degree of up-regulation. D: Expression of DDC mRNA in V1, V2, and T-47D cells as determined by real-time PCR. Relative expression levels are the ratios in V1 and V2 cells compared to those in T-47D cells (left bar) and are presented as mean ($n = 2$). E: Effects of NSD-1015 on androgen-induced PSA mRNA expression in V1 and V2 cells. Cells were treated with 1 nM DHT and the indicated concentration of NSD-1015 or bicalutamide (Bic) for 24 h. The relative expression level is the ratio compared to that of cells treated with DHT alone (left bar), and are presented as mean ($n = 2$).