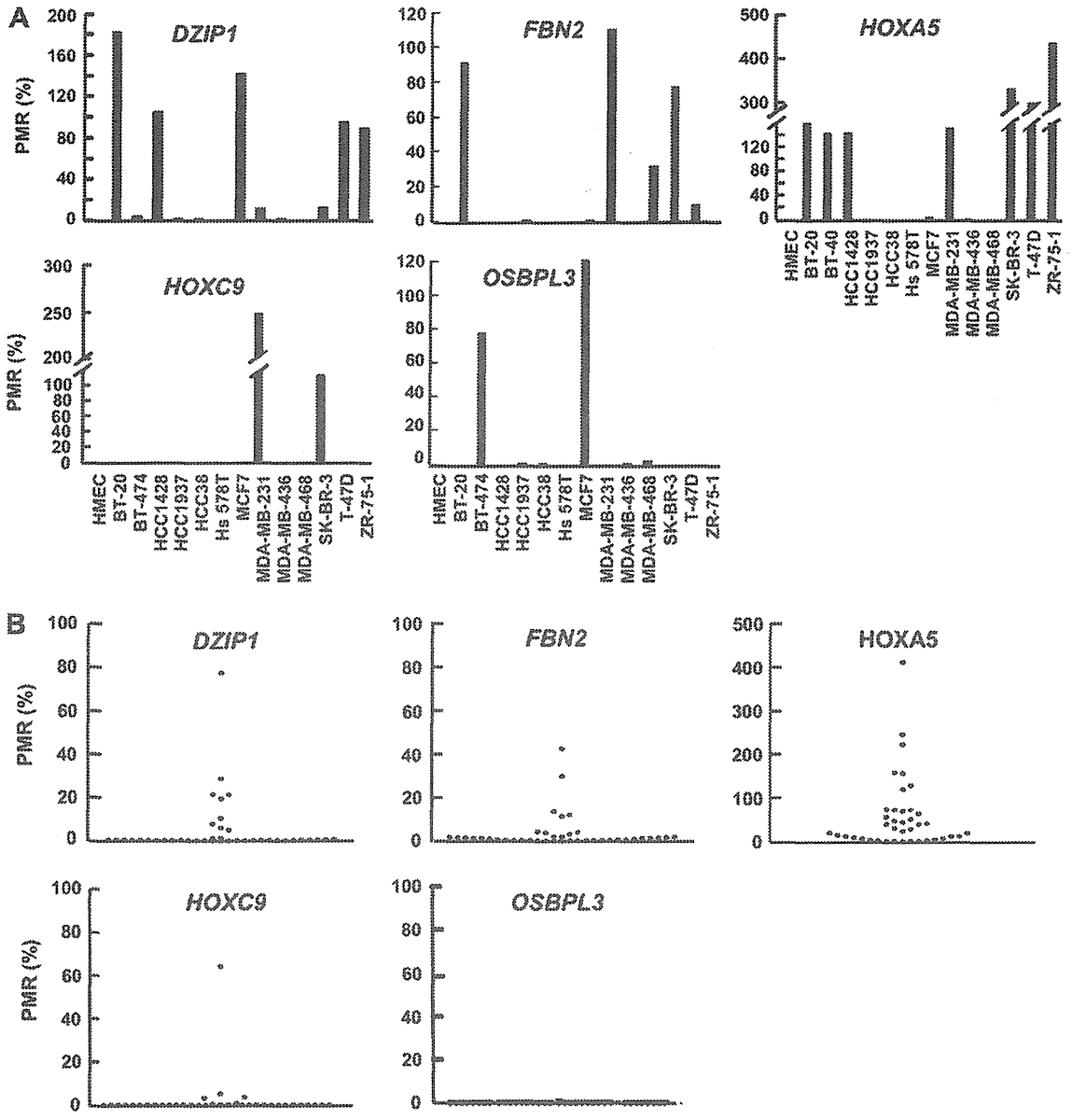

Fig. 3. DNA methylation levels of the outlier promoter CGIs in breast cancer cell lines and primary breast cancer samples. (A) Methylation levels of the outlier promoter CGIs in the HMECs and 13 breast cancer cell lines analyzed by qMSP. Five outliers, *DZIP1*, *FBN2*, *HOXA5*, *HOXC9*, and *OSBPL3*, showed high methylation levels (PMR higher than 100%) in one or more breast cancer cell line. Since the copy number of the target CGI was normalized to the copy number of the *Alu* repeat sequence, it was possible that PMR reached more than 100% when the locus containing the target CGI had an increased copy number. (B) DNA methylation levels of *DZIP1*, *FBN2*, *HOXA5*, *HOXC9*, and *OSBPL3* in primary breast cancer samples. Methylation of each CGI was analyzed in 40 primary breast cancer samples by qMSP. *DZIP1*, *FBN2*, *HOXA5*, and *HOXC9* showed aberrant methylation in primary breast cancer samples.

breast cancer and renal cell carcinoma, respectively [37,39]. These results indicated that searching for outliers might be an efficient way to identify TSGs.

3.4. Silencing of DZIP1 by aberrant methylation of its promoter CGI

Excluding known TSGs, *HOXA5* and *FBN2*, we focused on *DZIP1* and *HOXC9*. First, an association between aberrant DNA methylation and loss of expression was analyzed in normal cells and cancer cell lines (Fig. 4A). *DZIP1* was expressed both in normal cells and in cancer cell lines without its aberrant methylation, except for BT-474 and SK-BR-3, and was not expressed in cancer cell lines with its aberrant methylation. In contrast, *HOXC9* was not expressed or had only very low expression levels both in normal cells and in all cancer cell lines, regardless of their methylation levels. Since a knockdown experiment could not be performed for *HOXC9*, it was excluded from further analyses.

Methylation-silencing of *DZIP1* was confirmed by observing its re-expression after treatment with 5-aza-dC and TSA using T-47D cells that had methylated promoter CGIs among the 13 breast cancer cell lines and did not express *DZIP1*. First, T-47D cells were treated with 0, 10, and 20 μM of 5-aza-dC to obtain a dose that inhibited cell growth to 50–70% of non-treated cells. The growth of T-47D cells was inhibited to 50% at 20 μM (Supplementary Fig. 3). The presence of demethylated DNA molecules was confirmed at this dose. A combination of 5-aza-dC with TSA induced *DZIP1* re-expression while TSA only did not (Fig. 4B). These results indicated that *DZIP1* was silenced by aberrant DNA methylation.

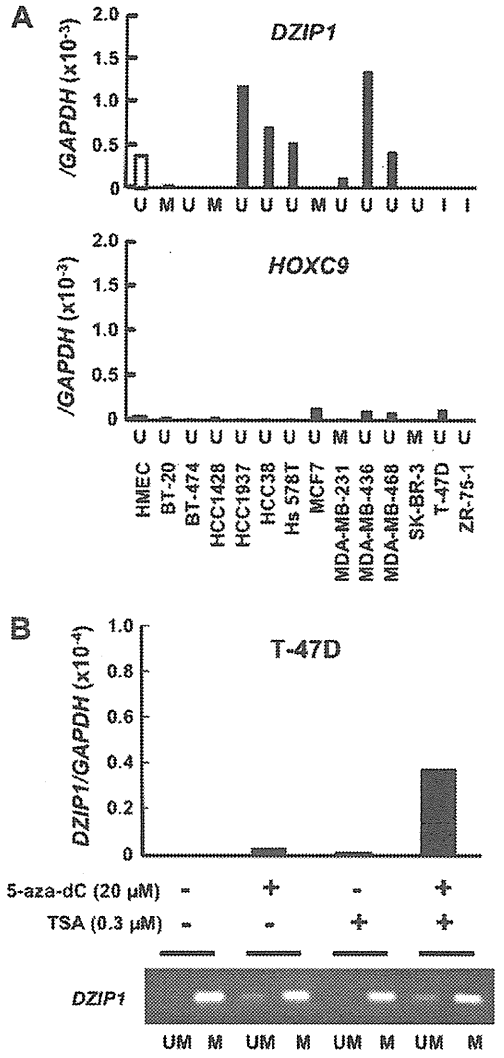

Fig. 4. Evaluation of methylation-silencing of two outliers, *DZIP1* and *HOXC9*. (A) An association between DNA methylation and loss of expression (analyzed by qRT-PCR). *DZIP1* was expressed in cell lines without its aberrant methylation and was silenced in cell lines with its aberrant methylation. In contrast, *HOXC9* was not expressed in any cell lines regardless of its low methylation. M, PMR > 100%; I, PMR 20–100%; U, PMR < 20%. (B) Re-expression of *DZIP1* by 5-aza-dC and TSA treatment. Expression levels were analyzed by qRT-PCR, and methylation statuses were analyzed by MSP. Demethylation of *DZIP1* was induced in T-47D cells by treatment with 20 μM of 5-aza-dC, and re-expression of *DZIP1* was observed after 5-aza-dC and TSA treatment. UM, unmethylated DNA; M, methylated DNA.

3.5. Tumor-suppressive function of DZIP1

To analyze whether *DZIP1* has tumor-suppressive function in breast cancer cells, *DZIP1* was knocked down by two different shRNAs (*DZIP1* shRNA1 and shRNA2) in two breast cancer cell lines that had the highest expression levels of *DZIP1*. Treatment of HCC1937 cells with shRNA1 and shRNA2 reduced the expression level of *DZIP1* mRNA to 20% and 60%, respectively, of cells with control shRNA. Treatment of MDA-MB-436 cells with shRNA1 and shRNA2 reduced the *DZIP1* expression levels to 40% of cells with control shRNA (Fig. 5A). HCC1937 cells with shRNA1 showed approximately 2-fold higher cell growth rate than HCC1937 cells with control shRNA. Likewise, MDA-MB-436 cells with shRNA1 or shRNA2 showed 1.5 to 2-fold higher cell growth rates than MDA-MB-436 cells with control shRNA (Fig. 5B). These results showed that *DZIP1* is a candidate novel TSG in breast cancer.

3.6. Comparison between epigenome-based and expression-based outlier approaches

To analyze the difference between the epigenome-based outlier approach and the expression-based outlier approach, we analyzed whether or not promoter CGIs identified by these approaches overlapped. First, to identify outliers expressed at high levels in normal cells, the expression status in the HMECs [14] was utilized. Among the 280 promoter CGIs susceptible to methylation induction during breast carcinogenesis, 21 promoter CGIs had downstream genes expressed at high levels in the HMECs (Supplementary Table 5).

Their overlap with the promoter CGIs identified by the epigenome-based outlier approach was then analyzed. Since two of the 14 promoter CGIs identified by the epigenome-based outlier approach were shared by two genes (Table 1), the remaining 12 promoter CGIs (and thus genes) were used for the analysis. Among the 12 promoter CGIs identified by the epigenome-based approach, only four promoter CGIs overlapped with those identified by the expression-based outlier approach (Fig. 6). Three and six TSGs were included in the 12 and 21 promoter CGIs, respectively, identified by the epigenome-based approach and the expression-based approach, respectively. While one TSG overlapped between the two approaches, *DZIP1* could be identified only by the epigenome-based approach. These results indicated that the epigenome-based approach could identify a different set of TSGs from the expression-based approach.

4. Discussion

We here showed (i) that a significant fraction of known TSGs silenced by aberrant DNA methylation in breast and colon cancer were outliers, and could be classified into TSGs with active Pol II and those with stalled Pol II, and (ii) that a different set of TSGs could be identified by an epigenome-based outlier approach or by an expression-based outlier approach. The epigenome-based outlier approach is established for the first time in this study. It is also applicable to obtain information on individual methylated genes whether they are TSGs or not. Epigenome information in normal cells is now being compiled by an international collaborative effort [40], and the epigenome-based outlier approach is expected to become more useful when this effort is completed.

Most genes identified by the epigenome-based and/or expression-based outlier approaches were unique. Among the five known TSGs that were outliers, only *MASPIN* had high expression, and the other four genes (*BRCA1*, *HOXA5*, *MLH1*, and *RASSF1A*) had stalled Pol II, indicating that they can be identified only by the epigenome-based approach. Among the 29 outliers identified by the genome-wide analyses, eight and 17 were uniquely identified by the epigenome-based and expression-based approaches, respectively. Genes identified only by the epigenome-based approach had stalled Pol II and thus low expression levels. Genes identified only by the expression-based approach were considered to have high levels of Pol II in gene bodies but not in promoter regions, which we analyzed in this study. It is reported that genes with active Pol II tends to have lower Pol II levels in promoter regions than those with stalled Pol II [41].

Using the epigenome-based outlier approach, *DZIP1* was identified as a candidate novel TSG. *DZIP1* is known to be involved in the regulation of hedgehog (Hh) signaling pathway [42,43]. Hh signaling pathway is activated in several types of human cancers, such as breast, esophageal, gastric, pancreatic, lung, and prostate cancers [44–51]. Although it is unknown how *DZIP1* is involved in the dysregulation of the Hh signaling pathway in mammals, in zebrafish embryo, a loss-of-function mutation of *Dzip1* is known to lead to ectopic expression of the downstream genes of the Hh signaling

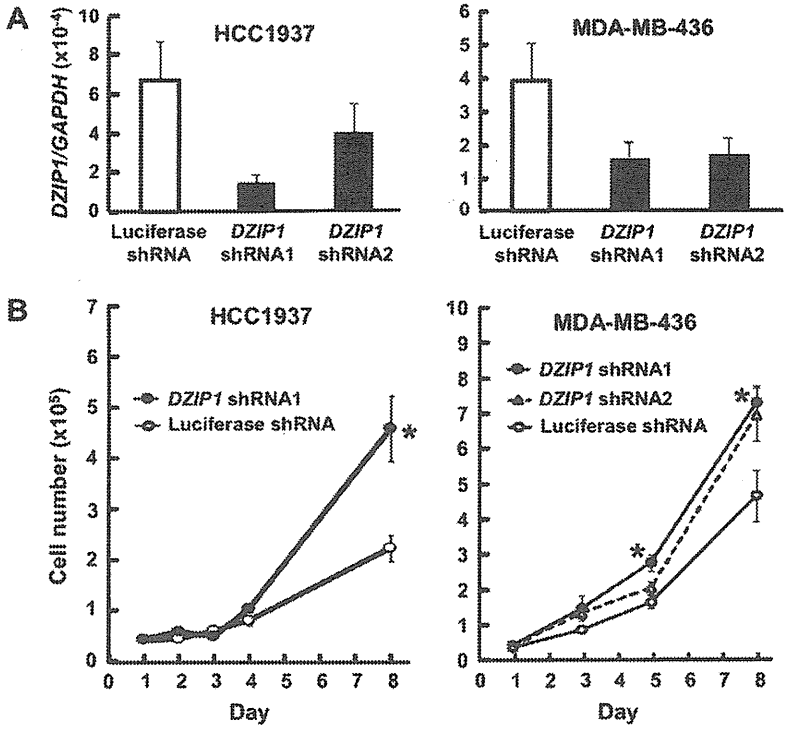

Fig. 5. Suppression of cell growth by *DZIP1*. (A) Knockdown of *DZIP1* in HCC1937 and MDA-MB-436 cells. The expression level of *DZIP1* mRNA in HCC1937 cells was reduced to about 20% and 60% of that of HCC1937 cells with control shRNA by shRNA1 and shRNA2, respectively. The expression level in MDA-MB-436 cells was reduced to about 40% of that in MDA-MB-436 cells with control shRNA by both shRNA1 and shRNA2. The mean ± SE values of three independent experiments of *DZIP1* expression levels are shown. (B) Increased growth of the HCC1937 and MDA-MB-436 cells by *DZIP1* knockdown. Cell numbers were counted at time points designated in the panels, and the mean ± SE values of three independent experiments of cell number are shown. Differences of cell growth were tested by the Student's *t* test. $^{*}p < 0.05$.

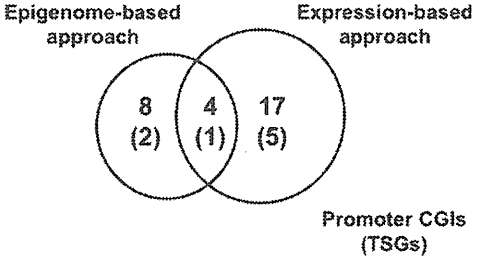

Fig. 6. The overlap of promoter CGIs identified by the epigenome-based outlier approach and those by the expression-based outlier approach. Among the 12 promoter CGIs with unique downstream genes identified by the epigenome-based approach, four promoter CGIs overlapped with those identified by the expression-based approach. *DZIP1* could be identified only by the epigenome-based approach.

pathway [42,52]. Therefore, there is a possibility that methylation-silencing of *DZIP1* induces abnormal expression of the downstream genes of the Hh signaling pathway during human carcinogenesis. Further investigations into *DZIP1* functions are necessary. *DZIP1* was also repressed in breast cancer cell lines such as BT-474 and SK-BR-3 that had unmethylated promoter CGIs of *DZIP1*. As possible causes of this repression, involvement of repressive histone modifications and defects in signaling pathways that regulate *DZIP1* expression were considered.

Among the seven known TSGs initially analyzed, *BRCA1*, *HOXA5*, and *MASPIN* were outliers in breast cancer, and *MLH1* and *RASSF1A* were in colon cancer. In contrast, *CDKN2A*, *RASSF1A*, *RBP1* in breast cancer and *CDKN2A* in colon cancer were not outliers. Especially, *RASSF1A* was an outlier in colon cancer, but not in breast cancer. This difference might explain the different incidence of aberrant DNA methylation of *RASSF1A* between breast cancers that show 50–60% incidence [44,53] and colon cancers that show 20–45% incidence [54,55].

Among the TSGs confirmed as outliers in the initial analysis, *HOXA5* was identified by the following genome-wide screening, but *BRCA1* and *MASPIN* were not. This was because the microarray used in this study did not have probes in the NFRs of *BRCA1* and *MASPIN*, and these genes cannot be identified as frequently methylated genes using the microarray used here.

Cancer cell lines were used to obtain DNA methylation-susceptible genes in this study. However, some TSGs, such as *BRCA1*, are reported to be frequently methylated in primary breast cancer tissues [56–58], but infrequently in cancer cell lines [59]. By use of primary cancer samples, identification of such types of TSGs will be facilitated.

In summary, we showed that a significant fraction of TSGs are outliers to the general rule of genes methylated in cancer cells, and that a different set of TSGs could be identified by the epigenome-based outlier approach compared to the expression-based outlier approach.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.canlet.2012.03.016.

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ORIGINAL ARTICLE

Loss of heterozygosity on chromosome 16q suggests malignancy in core needle biopsy specimens of intraductal papillary breast lesions

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Abstract It is often difficult to make a definitive diagnosis of papillary breast lesions using core needle biopsy (CNB) specimens. We studied loss of heterozygosity (LOH) on chromosome 16q in order to assess its diagnostic use for papillary breast lesions in CNB specimens. Of 25 patients with intraductal papillary breast tumors, we extracted DNA from paired samples of tumor cells from CNB specimens and non-tumor cells from subsequent excision specimens and analyzed LOH at the D16S419 and D16S514 loci on chromosome 16q. LOH analysis results were compared with final diagnoses based on pathological features of the resected specimens. On the CNB specimens, 21 tumors were histologically diagnosed as indeterminate or suspicious for malignancy, while four tumors were unambiguously malignant. Of the 21 indeterminate or suspicious tumors, 11 were finally diagnosed as benign and ten as malignant, and on these, LOH analyses were informative for 8 of the 11 benign tumors and 7 of the 10 malignant tumors. LOH was also informative on two of the four tumors unambiguously malignant on CNB. None of the eight informative benign tumors showed LOH on 16q. Six of the eleven informative malignant tumors showed LOH on 16q. LOH on 16q was significantly different between CNB specimens of benign and malignant intraductal papillary tumors (P=0.007). Analysis of LOH on 16q may be helpful in making a definitive diagnosis in cases of papillary breast lesions, in both excised and CNB specimens.

Keywords Loss of heterozygosity · Breast · Papilloma · Papillary carcinoma · Core needle biopsy

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Introduction

Preoperative diagnosis of intraductal papillary tumors of the breast is challenging because of the difficulty of differentiating intraductal papillary carcinoma from intraductal papilloma. It is very difficult to diagnose the biological nature of these tumors based on mammography and ultrasonography, unless there is evidence of massive tumor invasion or rapid growth. Although image-guided core needle biopsy (CNB) is a highly reliable method of diagnosing breast lesions, it is often difficult to differentiate between intraductal papillary lesions based on routine pathological examination of CNB specimens. This difficulty arises because intraductal papillary carcinomas tend to be well differentiated, and CNB specimens do not always include a section with pathognomonic features. Therefore, a final diagnosis

can often be made only by histological examination of the surgically resected specimen.

A number of genetic and chromosomal alterations have been identified in sporadic breast carcinomas, and their clinical implications have been investigated. Loss of heterozygosity (LOH) on chromosomes 16q and 17p are frequent in both invasive carcinoma and ductal carcinoma in situ (DCIS), irrespective of differences in the histological types and grades [1–8]. Several studies have reported a striking difference in the incidence of LOH on 16q between DCIS and intraductal papilloma [1, 5, 7] and have suggested that analysis of LOH on chromosome 16q could be helpful in the differential diagnosis of intraductal papillary tumors. In a previous study, we used Southern blot analysis to examine LOH on 16q in intracystic papillary tumors using DNA isolated from frozen, paired, surgically resected samples of tumor and non-tumor tissues [7]. More recently, we reported a polymerase chain reaction (PCR)-based LOH analysis technique using DNA isolated from paraffin-embedded tumor samples [9, 10]. In the study we report here, we used this PCR-based approach to assess its diagnostic utility on CNB specimens of indeterminate or suspicious intraductal papillary breast lesions.

Materials and methods

Samples

We selected tumor samples of 25 women with a preoperative diagnosis of intraductal papillary breast tumor by image-guided CNB, who had undergone surgical resection between 2005 and 2008, from the pathology computer database at the National Cancer Center Hospital, Japan. Image-guided CNB had been performed under sonographic guidance using either a 14-gauge needle or an 11-gauge vacuum-assisted biopsy probe. Twenty-one tumors had been diagnosed as indeterminate or suspicious for malignancy based on the pathological features of the CNB specimens and the lesions had been surgically resected for definitive histological diagnosis. The remaining four tumors had been unambiguously diagnosed as DCIS. The research protocol was approved by the Ethics Committee of the National Cancer Center Hospital, Japan. All patients gave written informed consent for use of their specimens in the study.

Histological criteria of intraductal papillary tumors

The diagnosis of intraductal papillary tumor was based on the presence of epithelial proliferations supported by fibrovascular stalks, with or without an intervening myoepithelial cell layer [11, 12]. All of the hematoxylin and eosin (H&E)-stained slides of the CNB and resected specimens were retrieved and reviewed for diagnostic consistency by the authors using published criteria.

The Japanese reporting form for cytology and core needle biopsy [13] was used to review the CNB specimens. This reporting form records findings and a judgment of whether the specimen is adequate or inadequate. Adequate specimens are categorized as normal or benign, indeterminate, suspicious for malignancy, or malignant.

Intraductal papillary tumors were diagnosed as benign or malignant using the following histological criteria of cytological and structural features [11, 14]. Papillomas or benign papillary tumors were diagnosed in cases showing an arborescent structure composed of fibrovascular stalks covered by a layer of myoepithelial cells with overlying epithelial cells. Intraductal papillary carcinomas or malignant papillary tumors were usually large papillary lesions (mean 2 cm, range 0.4–10 cm) located within a large cystic duct, with thin fibrovascular stalks devoid of a myoepithelial cell layer and a neoplastic epithelial cell population with characteristics of low-grade DCIS. Cases of "papilloma with atypia" with focal atypical epithelial proliferation and low-grade nuclei [15] were categorized as indeterminate in CNB specimens and as benign in resected specimens. For cases in which it was difficult to distinguish between benign and malignant tumors, the diagnosis was made by assessing the architectural features and visualizing the myoepithelial cell layer with immunohistochemical staining. Final diagnosis was made by pathological examination of the excision specimens.

Microdissection of paraffin-embedded tissues and DNA extraction

For all 25 patients, we extracted DNA from paired samples of intraductal papillary tumor cells from CNB specimens and non-tumor cells (normal mammary glands or lymph nodes) from surgically resected specimens, as previously described [9, 10]. Formalin-fixed and paraffin-embedded tissue sections, 5 to 10 μm thick, were cut using a microtome. Sections mounted on PEN foil slides were deparaffinized in xylene for 5 min (twice) and rehydrated using a descending series of ethanol concentrations as follows: 100% for 30 s (twice), 95% for 30 s (twice), 70% for 10 s, and distilled water for 10 s. The sections were stained with Meyer's hematoxylin, washed with water, and then stained with eosin for 1 min (H&E stain). The slides were dehydrated with 100% ethanol, placed in xylene for 10 min, and air-dried. Specific cells of interest were microdissected and selected using a Leica LMD 6000 system in accordance with the manufacturer's instructions (Leica, Narishige Micromanipulator, Wetzlar, Germany). The microdissected cells were placed in 50 μl proteinase K solution (5 mg/ml proteinase K in 10 mM Tris–HCl, 1 mM EDTA, pH 8.0, 1% Tween 20) and incubated for 36–48 h at 55°C. The

proteinase K was inactivated by incubating the samples at 95°C for 10 min, and then subjected to standard phenol-chloroform extraction and ethanol precipitation in the presence of glycogen. The pellets were resuspended in distilled water and the concentration was adjusted to 0.01 μg/μl. The extracted DNA samples were stored at 4°C until further use.

Selection of polymorphic markers

The chromosomal regions and markers used were D16S419 (16q12.2) and D16S514 (16q21). The following primer sequences were used for PCR amplification:

D16S419	Forward	5′-ATT TTT AAG GAA TGT AAA GNA CAC A-3′
	Reverse	5′-GAC GTT AGA CCA GGA GTC AG-3′
D16S514	Forward	5′-CTA TCC ACT CAC TTT CCA GG-3′
	Reverse	5′-TCC CAC TGA TCA TCT TCT C-3′

We selected polymorphic markers located on chromosome 16q based on the following criteria: (1) the markers were localized to regions with frequent DNA polymorphisms and with frequent LOH events reported in intraductal papillary carcinomas, notably low-grade DCIS [1–5, 7, 16], and (2) the amplified fragments were <250 bp, indicating that they could be successfully amplified using DNA from formalin-fixed tissues. Forward and reverse primer pairs for oligonucleotide polymorphic markers corresponding to the sequences retrieved from the UniSTS database (http://www.ncbi.nlm.nih.gov/unists) were synthesized and purchased from Perkin-Elmer (Applied Biosystems, Foster City, CA, USA). The 5′ ends of the forward primers were labeled with 6-carboxyfluorescein (6-FAM).

PCR

Genomic DNA was PCR amplified in a 25-μl reaction mixture containing 2 μl DNA solution corresponding to 20 ng genomic DNA, 0.4 pmol/μl of each primer, and 1× TaqMan Universal PCR Master Mix (Applied Biosystems) using a GeneAmp® PCR system 9600 (Applied Biosystems). The typical PCR cycling conditions included 2 min incubation at 50°C and 10 min denaturation at 95°C, followed by 40 cycles at 95°C for 15 s and 60°C for 1 min. An

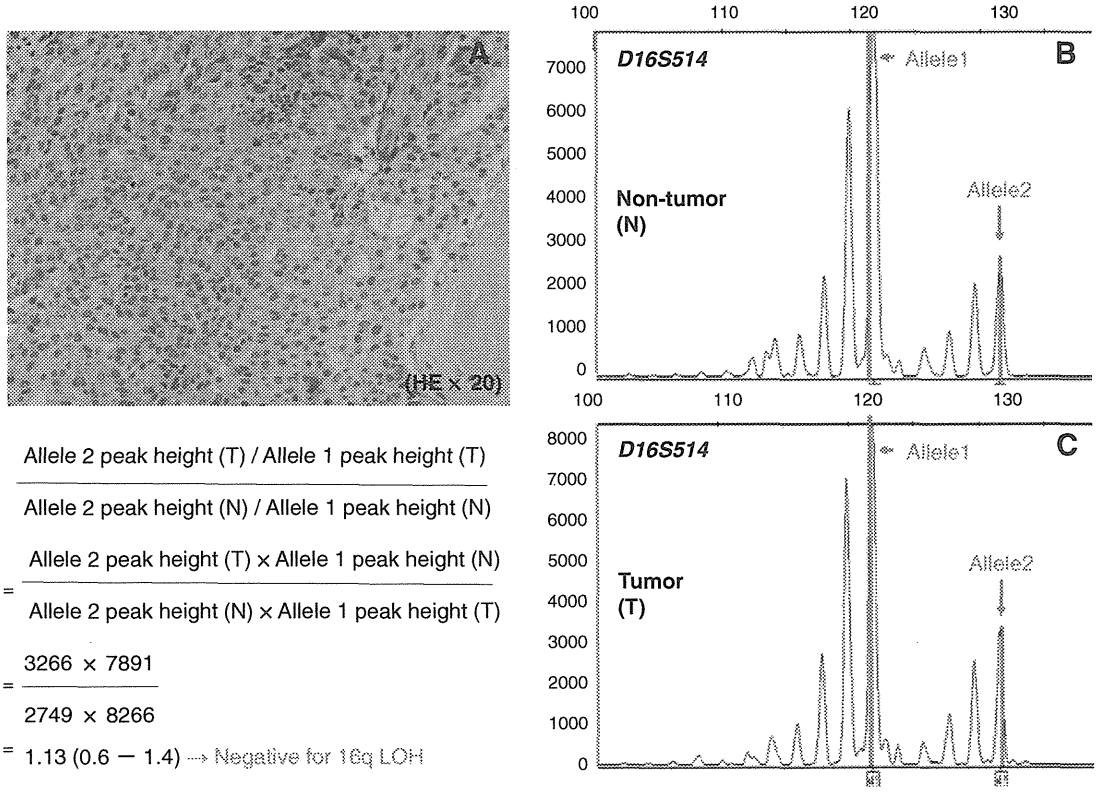


Fig. 1 Analysis of loss of heterozygosity (LOH) in an intraductal papillary tumor (case 4). **a** Based on the pathological features of the excised specimen, the tumor was diagnosed as intraductal papilloma. **b** Electrophoretogram showing constitutional heterozygosity (alleles 1 and 2) at the D16S514 locus in non-tumor DNA. The horizontal axis indicates the size of the DNA fragments (bp), and the vertical axis indicates signal intensity. **c**. Electrophoretogram showing retention of heterozygosity (alleles 1 and 2) at the D16S514 locus in tumor DNA. The axes are the same as in **b**

elongation step at 72°C for 10 min was added to the final cycle. Aliquots of the PCR products were then mixed with size standard and formamide, denatured, and run on an ABI 3130 automated capillary electrophoresis DNA sequencer (Applied Biosystems). The quantity and the quality of the DNA fragments amplified by PCR were confirmed by agarose gel electrophoresis. As a positive control, we used DNA isolated from formalin-fixed, paraffin-embedded tissues of five breast carcinomas in which LOH on 16q had already been detected by Southern blot analysis of fresh frozen tissues [17]. As a negative control, PCR was performed without template DNA.

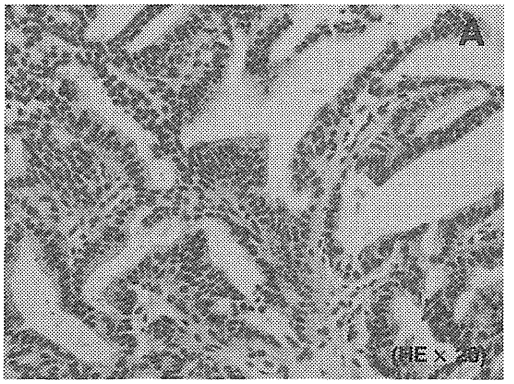
Assessment of allele loss

The amplified products were assessed for peak height and area using Gene Mapper software (version 3.7; Applied Biosystems). Non-cancerous DNA samples with two different amplified bands were defined as informative cases for LOH analysis. The presence of LOH was determined in accordance with the manufacturer's criteria. LOH was considered to exist if the ratio of the peak heights, which was calculated with the following formula, was <0.6 or >1.4: [peak height of the affected allele (allele A) of the tumor× peak height of the unaffected allele (allele B) of normal cells]/[peak height of allele A of normal cells×peak height of allele B of tumor cells] (Figs. 1 and 2) [17]. If the ratio of the peak height was 0.6 and 1.4 according to the formula, the case was judged to have retention of heterozygosity or absence of LOH.

When the results were questionable, PCR amplification and LOH analysis were performed at least twice to obtain equivalent results. Results were considered non-informative when the normal tissue was constitutionally homozygous and were not evaluated when the tissue lysates were not amplified, that is, PCR was unsuccessful. When either D16S419 or D16S514 showed LOH, the tumor was considered to have LOH. The LOH analysis results were compared with the final diagnoses based on the pathological features of the surgically resected specimens.

Statistical analyses

The χ^2 test was used to determine differences between the benign and malignant groups of intraductal papillary tumors. Differences of $P<0.05$ were considered statistically



$$\frac{\text{Allele 2 peak height (T)} / \text{Allele 1 peak height (T)}}{\text{Allele 2 peak height (N)} / \text{Allele 1 peak height (N)}}$$

$$= \frac{\text{Allele 2 peak height (T)} \times \text{Allele 1 peak height (N)}}{\text{Allele 2 peak height (N)} \times \text{Allele 1 peak height (T)}}$$

$$= \frac{760 \times 8161}{4052 \times 8530}$$

$$= 0.18\ (< 0.6, 1.4 <) \rightarrow \text{Positive for 16q LOH}$$

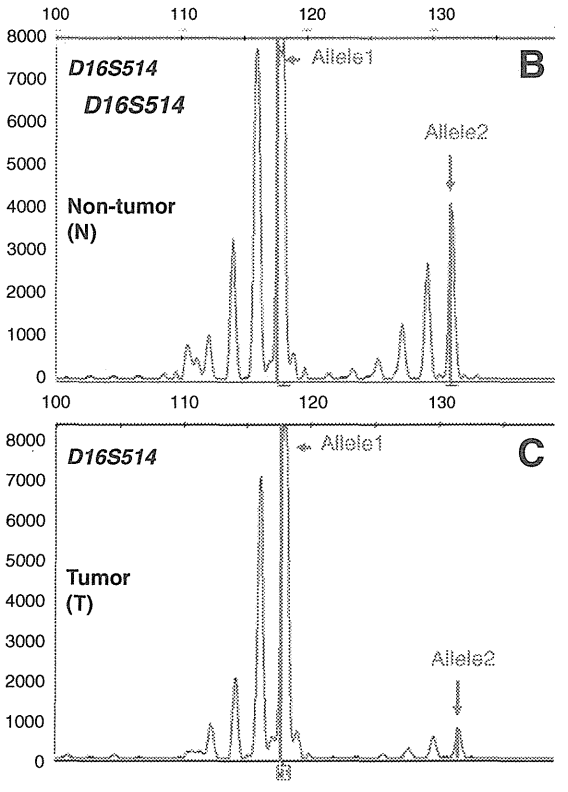


Fig. 2 Analysis of loss of heterozygosity (LOH) in an intraductal papillary carcinoma (case 15). **a** Based on the pathological features of the excised specimen, the tumor was diagnosed as intraductal papillary carcinoma. **b** Electrophoretogram showing constitutional heterozygosity (alleles 1 and 2) at the D16S514 locus in non-tumor DNA. **c** Electrophoretogram showing loss of heterozygosity (loss of allele 2) at the D16S514 locus in tumor DNA

significant. PASW statistics 17 (SPSS, Inc., Chicago, IL, USA) was used for all statistical analyses.

Results

Of the 21 indeterminate or suspicious intraductal papillary tumors, 11 were finally diagnosed as benign and 10 as malignant by microscopic examination of surgically resected specimens (Table 1). The first clinical sign was nipple discharge in 8 (38%) and a palpable mass in 4 (19%) of the 21 cases. Sonographic findings of the papillary lesions included a well-defined solid mass in nine cases (43%), a cystic lesion with solid components in five (24%), and duct dilatation with solid components in seven (33%). Multiple papillary lesions were found in seven cases (33%). The median tumor size on imaging was 1.9 cm (range 0.6–4.0 cm). There were no significant differences in clinical or imaging findings between lesions finally diagnosed as malignant on excisional biopsy specimens and those finally diagnosed as benign (Table 1). Thirteen (62%) of the 21 lesions were biopsied using a 14-gauge needle, and 8 (38%) were biopsied using an 11-gauge vacuum-assisted biopsy probe. The type of percutaneous biopsy was not correlated with postoperative conversion of histopathological diagnosis.

Table 2 shows the final histological diagnoses and 16q LOH results of CNB specimens for each of the 25 intraductal papillary tumors. Eight of the 11 benign tumors were informative, and none of these cases showed LOH on 16q. Nine of the 14 malignant tumors were informative, and these showed frequent LOH on 16q. Out of the total of 25 papillary tumors, seven were considered non-informative (constitutional homozygosity) and one was not evaluated after PCR was unsuccessful. As representative results, case 4 in which 16q LOH was negative is shown in Fig. 1 and case 15 in which 16q LOH was positive is shown in Fig. 2. Case 4 was finally diagnosed as papilloma based on the pathological features of the resected specimen. Figure 1b, c show two peaks of alleles in both the non-tumor and tumor DNA. The ratio of allele 2 peak height to allele 1 peak height in the tumor DNA divided by the ratio in the normal DNA was 1.13.

Table 1 Clinical and imaging findings in papillary breast lesions

	Total (n=21)	Final histological diagnosis		P value
		Benign (n=11)	Malignant (n=10)	
First clinical sign				
Nipple discharge	4 (19%)	1	3	0.14
Palpable mass	8 (38%)	4	4	
None	9 (43%)	6	3	
Sonographic findings				
Well-defined solid mass	9 (43%)	4	5	0.31
Cystic lesion with solid components	4 (19%)	2	2	
Duct dilatation with solid components	8 (38%)	5	3	
Mean tumor size on imaging (cm)	1.9±1.0 (0.6–4.0)	1.8±1.0 (0.6–3.0)	2.1±1.1 (0.6–4.0)	0.49
Number of lesions on imaging				
Multiple	7 (33%)	2	5	0.14
Solitary	14 (67%)	9	5	
Method of percutaneous biopsy				
Core needle biopsy (14-gauge)	13 (62%)	8	5	0.27
Vacuum-assisted biopsy (11-gauge)	8 (38%)	3	5	

Table 2 Final histological diagnoses of surgically resected specimens and 16q loss of heterozygosity (LOH) analysis results in core needle (CNB) specimens of papillary breast lesions

Case no.	Final histological diagnosis	Retained alleles on 16q	
		D16S419	*D16S514*
1	Benign	□	□
2	Benign	□	□
3	Benign	□	NI[a]
4	Benign	□	□
5	Benign	NE[b]	NI
6	Benign	□	□
7	Benign	□	NI
8	Benign	NE	□
9	Benign	NE	NI
10	Benign	□	NI
11	Benign	MSI[c]	NI
12	Malignant	■	■
13	Malignant	NI	NI
14	Malignant	NI	NI
15	Malignant	NI	■
16	Malignant	□	■
17	Malignant	□	□
18	Malignant	NI	MSI
19	Malignant	■	□
20	Malignant	□	□
21	Malignant	NI	□
22	Malignant (positive control)	NE	NE
23	Malignant (positive control)	NI	■
24	Malignant (positive control)	NI	■
25	Malignant (positive control)	NI	NI

Filled square loss of heterozygosity (LOH); *empty square* constitutional heterozygosity

NI[a]: not informative (constitutional homozygosity) NE[b]: not evaluated (PCR was unsuccessful)

MSI[c]: microsatellite instability

Therefore, this tumor was considered negative for LOH on 16q. On the other hand, case 15 (Fig. 2) was histologically diagnosed as low-grade DCIS or intraductal papillary carcinoma in the surgically resected specimen. Figure 2b, c shows a difference in the allele 2 peak heights between the normal and tumor DNA, and the ratio of allele 2 peak height to allele 1 peak height in the tumor DNA divided by the ratio in the normal DNA was 0.18. Therefore, this tumor was considered positive for LOH on 16q.

As shown in Table 3, 6 of the 11 (55%) informative malignant tumors showed LOH on 16q, whereas LOH was not detected in benign tumors. The incidence of 16q LOH in CNB specimens of intraductal papillary tumors was significantly different between benign and malignant tumors (P=0.007). Of three malignant tumors which were negative for LOH on 16q, two were histologically diagnosed as intraductal papillary carcinoma associated with papilloma in the surgically resected specimens.

Discussion

The aim of this study was to evaluate the use of LOH on chromosome 16q to make a final diagnosis in case of an indeterminate or suspicous intraductal papillary tumor in a CNB specimen. We found a statistically significant difference in the incidence of 16q LOH between of benign and malignant intraductal papillary tumors on CNB specimens. The results of the present study suggest that analysis of LOH on 16q may be helpful for making a definitive diagnosis of an indeterminate or suspicious papillary breast lesion in CNB and surgically resected specimens.

In our previous studies, we examined LOH on 16q in intracystic papillary tumors by Southern blot analysis using frozen tissue samples [3, 5] and determined that the incidence of LOH on 16q is strikingly different between cases of DCIS and papilloma [1, 7]. In the present study, we performed PCR-based LOH analysis using DNA isolated from formalin-fixed, paraffin-embedded samples from CNB specimens of intraductal papillary tumors. Although we used a different technique and different type of samples than in previous studies, we show that the incidence of 16q LOH is significantly different between CNB specimens of benign and malignant intraductal papillary tumors.

In the present study, LOH was detected at either16q12.2 or 16q21 in 6 of 11 malignant tumors (55%), whereas LOH was not detected in histologically benign tumors. Similarly, our previous data on intracystic papillary breast tumors showed that 12 of 17 intracystic papillary adenocarcinomas (71%) had LOH on 16q, whereas none of 11 intraductal papillomas had this genetic alteration [1]. Di Cristofano et al. [5] documented LOH at locus 16q23.1–16q24.1 in 7 of 11 malignant samples (63.6%), whereas none of the four informative benign samples appeared to be altered. Taken together, LOH on 16q has high specificity and positive predictive value for the diagnosis of malignancy in intraductal papillary tumors of the breast.

None of the benign papillary lesions we examined in any of our studies, including the eight papillomas in the present study, revealed LOH on 16q. In contrast, Di Cristofano et al. [5] found LOH on 16q in benign papillary lesions, with LOH at locus 16q21.1–16q22.2 detected in both malignant and benign lesions, and at 16q23.3–16q24.1 detected only in malignant lesions. Based on these results, the authors concluded that these differences might be due to the use of the novel molecular marker D16S310 which targets 16q21.1–16q22.2, which putatively contains a tumor suppressor gene involved in the genesis/progression of breast carcinomas.

We propose that the differences between results can be explained by the cellular heterogeneity of the intraductal papillary lesions. Atypical proliferative breast lesions are thought to be precursors of breast carcinomas and have frequently been shown to have LOH on 16q [18, 19].

Table 3 Incidence of loss of heterozygosity (LOH) on 16q in core needle biopsy (CNB) specimens of papillary breast lesions

Final histological diagnosis	Number of cases (%)					
	Chromosome 16q				Total	P-value
	LOH		Constitutional heterozygosity		(Informative)	
Benign	0	(0)	8	(100)	8	0.007
Malignant	4	(57)	3	(43)	7	
Malignant (positive control)	2	(100)	0	(0)	2	

Atypical proliferative lesions and carcinomas are considered to be clones and probably originated from a field within these clones [19]. "Atypical papilloma" or "papilloma with atypia" is defined as papilloma with a proliferation of epithelial cells that have cytological and architectural features consistent with atypical ductal hyperplasia (ADH). Page et al. [15] further refined these terms and used atypical papilloma when the ADH focus involved 3 mm or less of the papillary lesion and the term minor DCIS lesion when the atypical focus involved more than 3 mm of the papillary lesion. These definitions were applied to the surgically resected specimens in the present study. In contrast, Tavassoli [20] suggested using the term atypical papilloma if the area of ADH occupies less than 33% of the papillary lesion, and the term carcinoma arising in a papilloma when the area of ADH occupies 33–90% of the papillary lesion. The ratio of atypical epithelial cells to total epithelial cells may have influenced the LOH analysis results.

Papillary lesions in CNB specimens are diagnosed as benign, atypical (indeterminate), suspicious for malignancy, or definitely malignant based on their pathologic features. Papillary lesions which are histologically diagnosed as definitely malignant must be treated as breast carcinomas. Papillary lesions with atypia, i.e., lesions that are histologically diagnosed as indeterminate or suspicious for malignancy in CNB specimens, need to be resected to determine if there is a more significant lesion [21]. Based on the results of our study, we propose that papillary lesions in CNB specimens that are histologically diagnosed as indeterminate or suspicious for malignancy and show LOH on 16q should also be treated as carcinoma. However, absence of LOH on 16q occurred in both papillomas and papillary carcinomas, and the predictive value of absence of LOH for a benign lesion was only 73%. In lesions in CNB judged as indeterminate or suspicious for malignancy, absence of LOH on 16q therefore has no diagnostic significance.

It is still controversial whether lesions diagnosed as papilloma without atypia by CNB need to be resected. From a pathological review of 19 papillary lesions with postoperative conversion from nonmalignant to malignant, Cheng et al. [22] concluded that the causes of diagnostic conversion were borderline atypical lesions (47%), sampling problems (32%), interpretation errors (16%), and an inadequate sample (5%). Based on the results of the present study, we cannot give clear guidelines for the management of papillomas without atypia based on LOH on 16q, but we consider that analysis of LOH on 16q in CNB specimens with an adequate amount of tumor tissue could reduce interpretation errors and be helpful in determining whether a papilloma without atypia needs to be resected.

The following limitations of the present study are worth discussing. First, results of analysis of LOH on 16q are not sufficiently sensitive for detection of malignancy. Absence of LOH cannot guarantee a benign lesion. Second, the number of cases examined in the present study is small. Third, we did not consider the possibility of intratumor heterogeneity, e.g., cases of carcinoma arising within papilloma. To our knowledge, this is nevertheless the first report which confirms that the incidence of LOH on 16q is significantly different between CNB specimens of benign and malignant intraductal papillary tumors. In conclusion, analysis of LOH on 16q may be helpful in making a definitive diagnosis in cases of papillary breast lesions, in both excised and CNB specimens.

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Conflict of interest The authors declare that they have no conflicts of interest.

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Neoadjuvant anastrozole versus tamoxifen in patients receiving goserelin for premenopausal breast cancer (STAGE): a double-blind, randomised phase 3 trial

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Summary

Background Aromatase inhibitors have shown increased efficacy compared with tamoxifen in postmenopausal early breast cancer. We aimed to assess the efficacy and safety of anastrozole versus tamoxifen in premenopausal women receiving goserelin for early breast cancer in the neoadjuvant setting.

Methods In this phase 3, randomised, double-blind, parallel-group, multicentre study, we enrolled premenopausal women with oestrogen receptor (ER)-positive, HER2-negative, operable breast cancer with WHO performance status of 2 or lower. Patients were randomly assigned (1:1) to receive goserelin 3·6 mg/month plus either anastrozole 1 mg per day and tamoxifen placebo or tamoxifen 20 mg per day and anastrozole placebo for 24 weeks before surgery. Patients were randomised sequentially, stratified by centre, with randomisation codes. All study personnel were masked to study treatment. The primary endpoint was best overall tumour response (complete response or partial response), assessed by callipers, during the 24-week neoadjuvant treatment period for the intention-to-treat population. The primary endpoint was analysed for non-inferiority (with non-inferiority defined as the lower limit of the 95% CI for the difference in overall response rates between groups being 10% or less); in the event of non-inferiority, we assessed the superiority of the anastrozole group versus the tamoxifen group. We included all patients who received study medication at least once in the safety analysis set. We report the primary analysis; treatment will also continue in the adjuvant setting for 5 years. This trial is registered with ClinicalTrials.gov, number NCT00605267.

Findings Between Oct 2, 2007, and May 29, 2009, 204 patients were enrolled. 197 patients were randomly assigned to anastrozole (n=98) or tamoxifen (n=99), and 185 patients completed the 24-week neoadjuvant treatment period and had breast surgery (95 in the anastrazole group, 90 in the tamoxifen group). More patients in the anastrozole group had a complete or partial response than did those in the tamoxifen group during 24 weeks of neoadjuvant treatment (anastrozole 70·4% [69 of 98 patients] *vs* tamoxifen 50·5% [50 of 99 patients]; estimated difference between groups 19·9%, 95% CI 6·5–33·3; p=0·004). Two patients in the anastrozole group had treatment-related grade 3 adverse events (arthralgia and syncope) and so did one patient in the tamoxifen group (depression). One serious adverse event was reported in the anastrozole group (benign neoplasm, not related to treatment), compared with none in the tamoxifen group.

Interpretation Given its favourable risk–benefit profile, the combination of anastrozole plus goserelin could represent an alternative neoadjuvant treatment option for premenopausal women with early-stage breast cancer.

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Introduction

For premenopausal women with oestrogen receptor (ER)-positive or progesterone receptor (PgR)-positive breast cancer, treatment options include ablative surgery, radiotherapy, or cytotoxic chemotherapy. Endocrine treatments include the ER antagonist tamoxifen, and luteinising hormone releasing hormone (LHRH) agonists such as goserelin, which offer the potential for reversible ovarian ablation. Goserelin has shown efficacy for the treatment of premenopausal breast cancer, with equivalent disease-free survival to cyclophosphamide, methotrexate, and fluorouracil (CMF) chemotherapy in those patients with ER-positive disease.[1] Although extended goserelin treatment is associated with a known reduction in bone mineral density,[2] it offers a more favourable safety profile than does cytotoxic chemotherapy.[3] The combination of tamoxifen plus goserelin has shown improved progression-free survival compared with goserelin alone;[4] however, a report[5] suggested that the combination of tamoxifen with goserelin was not better than either drug alone (although patients also received concomitant cytotoxic chemotherapy). Present guidelines suggest that tamoxifen alone or with ovarian function suppression are standard treatment options for premenopausal women with ER-positive breast cancer.[6]

Based on the efficacy shown in postmenopausal women with early breast cancer,[7–9] aromatase inhibitors in combination with ovarian suppression are now being assessed for the treatment of premenopausal women with early-stage breast cancer.

Early clinical data in premenopausal women have suggested that the combination of anastrozole and

goserelin results in a greater reduction in mean oestradiol concentrations than does the combination of tamoxifen plus goserelin,[10] and data from the Austrian Breast and Colorectal Cancer Study Group Trial 12 (ABCSG-12)[11] have shown that 3-year adjuvant therapy with anastrozole plus goserelin is associated with similar disease-free survival to that associated with adjuvant tamoxifen plus goserelin therapy.[11]

The period before surgery offers an important treatment window to downstage breast tumours, which might allow for breast-conserving surgery rather than mastectomy.[12] This window provides the potential for an improved cosmetic outcome together with a reduction of surgical morbidity.[13,14] Aromatase inhibitors have shown to be effective and well tolerated neoadjuvant treatments in postmenopausal women with early breast cancer.[15] Therefore, the role of aromatase inhibitors plus goserelin for premenopausal breast cancer is of interest.

In this Study of Tamoxifen or Arimidex, combined with Goserelin acetate, to compare Efficacy and safety (STAGE), we aimed to compare anastrozole plus goserelin versus tamoxifen plus goserelin in the neoadjuvant setting (24 weeks of presurgical therapy) in premenopausal Japanese women with ER-positive early breast cancer.

Methods

Study design and patients

This phase 3, double-blind, randomised, parallel-group, multicentre study compared the efficacy and safety of anastrozole with that of tamoxifen in the neoadjuvant setting in premenopausal women with operable breast cancer receiving concomitant goserelin treatment.

We enrolled premenopausal women aged 20 years or older with ER-positive and HER2-negative breast cancer (ER-positive defined by ≥10% nuclear staining by immunohistochemistry; HER2-positive defined by immunohistochemistry 3 positivity or fluorescence in-situ hybridisation positivity, determined by each individual site) and with histologically confirmed operable and measurable lesions (T [2–5 cm], N0, M0). Locally advanced, with palpable supraclavicular nodes, or inflammatory breast cancers were deemed inoperable. Patients had to have a WHO performance status of 2 or lower. Patients were excluded if they had: necessity for concomitant chemotherapy; previous radiotherapy, chemotherapy, or hormone therapy for breast cancer; or history of systemic malignancy within 3 years. All patients provided written informed consent. The study was approved by the institutional review board for every trial centre and was done in accordance with the Declaration of Helsinki and Good Clinical Practice, the applicable local regulatory requirements, and the AstraZeneca policy on bioethics.

Randomisation and masking

Participants were enrolled by the study investigators, and eligible patients were assigned to treatment groups at random, stratified by centre, with computer-generated randomisation codes (permuted block method) that were generated sequentially at a central patient registration centre. All study personnel were masked to the randomised treatment until all data had been obtained and the primary analysis carried out. The study was of a double-dummy design, whereby the placebo tablets of anastrozole and tamoxifen were indistinguishable in their appearance and packaging from the corresponding active tablets. Breaking of the randomisation code was only to be allowed in medical emergencies that necessitated knowledge of the treatment randomisation, although this did not happen.

Procedures

Patients were randomly assigned (1:1) to receive either anastrozole 1 mg daily orally with a tamoxifen placebo plus a subcutaneous depot injection of goserelin 3·6 mg every 28 days or tamoxifen 20 mg daily orally with anastrozole placebo plus a subcutaneous injection of goserelin 3·6 mg every 28 days. Treatment continued for 24 weeks before surgery or until any criterion for discontinuation was met. Treatment will also continue in the adjuvant setting for both treatment groups for a period of 5 years.

We did tumour measurements using calliper and ultrasound every 4 weeks, and MRI or CT at day 0, week 12, and week 24. We determined objective tumour response with every measurement method and assessed according to modified Response Evaluation Criteria In Solid Tumors criteria (RECIST).[16] We measured serum concentrations of oestrone and oestradiol from blood samples taken every 4 weeks. We measured breast-tumour tissue concentrations of oestrone and oestradiol from core needle biopsy samples taken at day 0 and from samples obtained from excised tumours at surgery.

We measured bone mineral density using dual-energy X-ray absorptiometry at day 0 and at week 24 and the bone turnover markers serum bone-alkaline phosphatase (BAP) and serum crosslinked N-telopeptide of type 1 collagen (NTX) at day 0, week 12, and week 24. We identified BAP using either an enzyme immunoassay (EIA) or a chemiluminescent EIA (CLEIA). We measured NTX by EIA.

We defined histopathological response as the proportion of patients whose tumours were classified as grade 1b, 2, or 3, where grade 0 corresponds to no response; grade 1a to mild changes in cancer cells regardless of the area, or marked changes seen in less than a third of cancer cells; grade 1b to marked changes in a third or more cancer cells but less than two-thirds of cancer cells; grade 2 to marked changes in two-thirds or more of cancer cells; grade 3 to necrosis or disappearance of all cancer cells, and replacement of all cancer cells by granuloma-like or fibrous tissue, or both.[17] The pathologist at each individual site assessed histopathological effects by comparing of histopathological samples obtained at baseline and surgery.

Ki67 was stained with an antibody for MIB-1 at a central laboratory (SRL Inc, Tokyo, Japan) for assessment by a

central review board. Ki67 index was calculated as the ratio of Ki67 positive cells to total cells.

We assessed quality of life with patient-reported completion of the Functional Assessment of Cancer Therapy-Breast (FACT-B) questionnaire[18] (version 4), together with an Endocrine Subscale (ES) questionnaire.[19] The FACT-B endpoints assessed were the subscales of emotional wellbeing and social and family wellbeing and trial outcome index (TOI).

Adverse events were recorded at every patient visit and assessed according to Common Terminology Criteria for Adverse Events version 3.0.

The primary endpoint was best overall tumour response (complete response or partial response), assessed with calliper, during the 24-week neoadjuvant treatment period. Secondary endpoints were histopathological response, change in Ki67 expression, changes in serum and breast-tumour tissue concentrations of oestrone and oestradiol, quality of life, and tolerability.

Statistical analysis

We planned a sample size of 97 patients per group (194 in total) to show, with 80% power, the non-inferiority of anastrozole versus tamoxifen. This calculation was based on a two-sided 95% CI for the difference in tumour response between treatment groups, by use of calliper measurement, with a non-inferiority margin of 10%.

For best overall tumour response and histopathological response, we calculated the estimated difference between anastrozole and tamoxifen together with 95% CIs. Non-inferiority of anastrozole versus tamoxifen was to be concluded if the lower limit for the 95% CI was 10% or less. Superiority of anastrozole versus tamoxifen was to be assessed if non-inferiority was established. We also did an exploratory analysis of best overall tumour response using a logistic regression model, adjusted for PgR status (positive, negative), tumour grade (≤2, >2, missing, or unknown), and the longest breast tumour measurement at baseline (≤3 cm, >3 cm). We estimated the difference between treatment groups in changes from baseline in quality of life, together with 95% CI, using an analysis of covariance model, including treatment and baseline as covariates. We used SAS version 8.2 for all analyses.

We summarised Ki67 index, serum and breast tumour tissue concentrations of oestrone and oestradiol, laboratory test values, bone mineral density, and bone turnover markers using descriptive statistics. We summarised adverse events by system organ class and preferred term.

All analyses of efficacy and quality of life were based on the intention-to-treat population (all randomised patients). Where patients discontinued treatment, we used assessments up to discontinuation to determine the best overall tumour response. We included all patients who received study medication at least once in the safety analysis set.

This trial is registered with ClinicalTrials.gov, number NCT00605267.

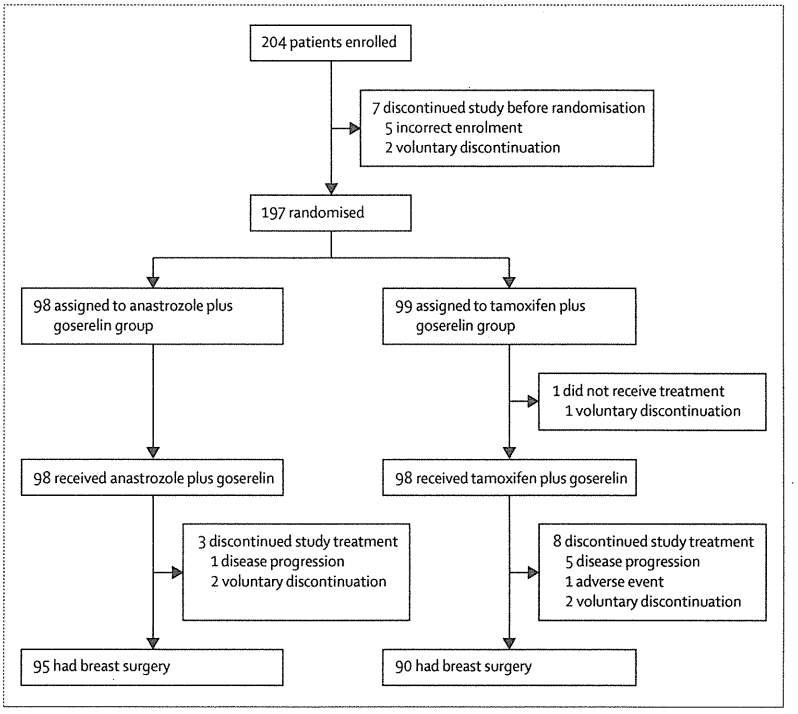

Figure: **Trial profile**

Role of the funding source

AstraZeneca employees participated in the conception and design of the study, collection and assembly of data, data analysis and interpretation, and drafting of the manuscript. All authors had full access to the study data and the corresponding author had the final responsibility to submit for publication.

Results

Between Oct 2, 2007, and May 29, 2009, at 27 centres in Japan, 197 patients were randomly assigned to receive anastrozole plus goserelin (anastrozole group, n=98) or tamoxifen plus goserelin (tamoxifen group, n=99; figure). 185 patients completed the 24-week neoadjuvant treatment period and received breast surgery (figure).

Patient demographics and baseline characteristics were generally well balanced between the treatment groups (table 1). The number of patients with tumour grade 3 was higher in the tamoxifen group than in the anastrozole group (table 1). More patients had a negative PgR status in the tamoxifen group (12 of 98 [12%]) than in the anastrozole group (5 of 98 [5%]; table 1).

Significantly more women in the anastrozole group achieved a complete or partial response (measured with callipers) than did those in the tamoxifen group from baseline to week 24 (table 2). More patients in the anastrozole group had an overall tumour response than in the tamoxifen group when response was measured by ultrasound, MRI or CT (table 2).

	Anastrozole plus goserelin (n=98)	Tamoxifen plus goserelin (n=99)
Age group at baseline (years)		
20–29	2 (2%)	0
30–39	21 (21%)	20 (20%)
40–49	65 (66%)	68 (69%)
50–59	10 (10%)	11 (11%)
Body-mass index (kg/m²)		
Mean (SD)	22·2 (3·5)	22·1 (3·3)
Body-mass index >25 kg/m²	21 (21%)	13 (13%)
Histology type		
Infiltrating ductal carcinoma	87 (89%)	91 (92%)
Infiltrating lobular carcinoma	3 (3%)	3 (3%)
Other*	8 (8%)	5 (5%)
Tumour grade		
1	42 (43%)	48 (48%)
2	36 (37%)	26 (26%)
3	4 (4%)	14 (14%)
Not assessable	1 (1%)	0
Not done	15 (15%)	11 (11%)
Longest breast tumour diameter at baseline (calliper measurement; cm)		
Mean (SD)	3·21 (0·85)	3·24 (0·97)
Median	3·00	3·00
Hormone-receptor status		
ER-positive	98 (100%)	99 (100%)
PgR-positive	93 (95%)	87 (88%)
HER2 status		
Negative	98 (100%)	99 (100%)

Data are n (%) unless otherwise stated. ER=oestrogen receptor. PgR=progesterone receptor. HER2=human epidermal growth factor receptor 2. *Including adenocarcinoma (n=3), mucinous carcinoma (n=9), and scirrhous carcinoma (n=1).

Table 1: **Patient demographics and baseline tumour characteristics**

	Anastrozole plus goserelin (n=98)	Tamoxifen plus goserelin (n=99)
Best overall tumour response		
Calliper*		
CR	12 (12·2%)	7 (7·1%)
PR	57 (58·2%)	43 (43·4%)
CR+PR	69 (70·4%)	50 (50·5%)
Ultrasound†		
CR	1 (1·0%)	0
PR	56 (57·1%)	42 (42·4%)
CR+PR	57 (58·2%)	42 (42·4%)
MRI or CT‡		
CR	2 (2·0%)	0
PR	61 (62·2%)	37 (37·4%)
CR+PR	63 (64·3%)	37 (37·4%)
Histopathological response§		
Grade 0 (no response)	12 (12·2%)	19 (19·2%)
Grade 1a (mild response)	42 (42·9%)	44 (44·4%)
Grade 1b (moderate response)	28 (28·6%)	18 (18·2%)
Grade 2 (marked response)	12 (12·2%)	9 (9·1%)
Grade 3 (complete response)	1 (1·0%)	0
Missing	3 (3·1%)	9 (9·1%)
Grade ≥1b	41 (41·8%)	27 (27·3%)

Data are n (%). CR=complete response. PR=partial response. *Estimate of difference between treatment groups 19·9% (95% CI 6·5–33·3); p=0·004. †Estimate of difference between treatment groups 15·7% (95% CI 1·9–29·5); p=0·027. ‡Estimate of difference between treatment groups 26·9% (95% CI 13·5–40·4); p=0·0002. §Estimate of difference between treatment groups 14·6% (95% CI 1·4–27·7); p=0·032. p values calculated by χ^2 test.

Table 2: **Summary of best overall tumour response and histopathological response from baseline to week 24 (intention-to-treat population)**

These differences were still apparent after adjustment for PgR status, tumour grade, and longest length of tumour measurement, irrespective of means of measurement: calliper odds ratio [OR] 2·23, 95% CI 1·22–4·06, p=0·009; ultrasound OR 1·71, 0·96–3·06, p=0·071; and MRI or CT OR 2·76, 1·52–5·03, p=0·0009.

Tumour responses increased gradually throughout the 24-week treatment period for both treatment groups (table 3). At every visit, tumour responses were higher for anastrozole versus tamoxifen with calliper measurement (table 3).

See Online for appendix

One patient (1%) showed no tumour shrinkage in the anastrozole group compared with eight (8%) in the tamoxifen group. All patients received breast surgery except those who withdrew prematurely. 84 (86%) of 98 patients in the anastrozole group had breast-conserving surgery, compared with 67 (68%) of 99 patients in the tamoxifen group.

A significantly higher proportion of patients in the anastrozole group had a histopathological response (tumours of grade 1b or higher at week 24) than in the tamoxifen group (table 2).

Mean Ki67 index at baseline was 21·9% in the anastrozole group (n=92) and 21·6% in the tamoxifen group (n=96). At week 24, Ki67 index was reduced in both treatment groups (2·9% in the anastrozole group [n=91] and 8·0% in the tamoxifen treatment group [n=87]). Reduction in Ki67 index from baseline to week 24 was significantly greater with anastrozole versus tamoxifen (estimated ratio of reduction between groups 0·35, 95% CI 0·24–0·51; p<0·0001).

Geometric mean serum concentrations of oestrone and oestradiol decreased from baseline in both treatment groups, with maximum decrease of both oestrone and oestradiol achieved in both groups by week 4; this was maintained throughout the 24-week treatment period for both oestrone and oestradiol (appendix). Reductions in concentrations of oestrone and oestradiol were significantly greater with anastrozole than with tamoxifen at week 24 (p<0·0001 for both oestrone and oestradiol). In an exploratory analysis of histopathological samples (n=13 for anastrozole and n=21 for tamoxifen), concentrations of oestrone and oestradiol in the breast tumour tissue were reduced in both treatment groups from baseline to week 24 (appendix). Oestrone suppression was greater in the anastrozole group than in the tamoxifen group (estimated ratio 0·14, 95% CI 0·06–0·31; p<0·0001), whereas

	Anastrozole plus goserelin (n=98)		Tamoxifen plus goserelin (n=99)	
	n (%)	95% CI	n (%)	95% CI
Week 4	10 (10·2%)	5·0–18·0	6 (6·1%)	2·3–12·7
Week 8	35 (35·7%)	26·3–46·0	20 (20·2%)	12·8–29·5
Week 12	49 (50·0%)	39·7–60·3	34 (34·3%)	25·1–44·6
Week 16	61 (62·2%)	51·9–71·8	47 (47·5%)	37·3–57·8
Week 20	69 (70·4%)	60·3–79·2	50 (50·5%)	40·3–60·7
Week 24	74 (75·5%)	65·8–83·6	56 (56·6%)	46·2–66·5

Where patients discontinued treatment, tumour response was considered non-response at each timepoint following discontinuation. CR=complete response. PR=partial response.

Table 3: **Tumour response rates by visit (CR+PR; intention-to-treat population)**

	Anastrozole plus goserelin (n=98)	Tamoxifen plus goserelin (n=98)
Vascular disorders	52 (53%)	53 (54%)
Hot flush	51 (52%)	51 (52%)
Musculoskeletal and connective tissue disorders	49 (50%)	29 (30%)
Arthralgia	35 (36%)	19 (19%)
Musculoskeletal stiffness	19 (19%)	9 (9%)
Joint stiffness	5 (5%)	1 (1%)
Myalgia	5 (5%)	1 (1%)
Nervous system disorders	22 (22%)	13 (13%)
Headache	10 (10%)	10 (10%)
Reproductive system and breast disorders	20 (20%)	13 (13%)
Menopausal symptoms	6 (6%)	4 (4%)
Metrorrhagia	5 (5%)	2 (2%)
Gastrointestinal disorders	9 (9%)	14 (14%)
Constipation	3 (3%)	10 (10%)
General disorders and administration site conditions	9 (9%)	14 (14%)
Fatigue	3 (3%)	5 (5%)
Psychiatric disorders	9 (9%)	10 (10%)
Insomnia	6 (6%)	6 (6%)
Skin and subcutaneous tissue disorders	8 (8%)	11 (11%)
Hyperhidrosis	4 (4%)	8 (8%)

Data are n (%). System organ class or preferred term.

Table 4: **Treatment-related adverse events occurring in at least 5% of patients (safety-analysis-set population)**

oestradiol suppression did not differ between groups (estimated ratio 0·63, 95% CI 0·26–1·54; p=0·301).

In both treatment groups, the ES and FACT-B TOI scores decreased slightly from baseline at week 12 and week 24. Mean ES score decreased from 64·7 at baseline to 55·5 at week 24 in the anastrozole group and from 63·4 at baseline to 57·1 at week 24 in the tamoxifen group. The FACT-B TOI mean score decreased from 69·6 at baseline to 64·9 at week 24 in the anastrozole group and from 68·8 at baseline to 66·2 at week 24 in the tamoxifen group. Although the study was not specifically powered to detect a difference in the quality-of-life outcome measures, groups did not differ significantly (estimated difference for anastrozole–tamoxifen; ES subscale −2·14, 95% CI −4·58 to 0·29, p=0·084; FACT-B TOI −1·52, −4·02 to 0·98, p=0·231). No significant changes from baseline to week 24 were observed for the subscales of emotional wellbeing and social and family wellbeing in either treatment group.

Adverse events were reported by 87 (89%) of 98 anastrozole-treated patients and 84 (86%) of 98 tamoxifen-treated patients. Treatment-related adverse events were reported by 82 (84%) patients in the anastrozole group and 75 (77%) patients in the tamoxifen group. Table 4 shows the most common treatment-related adverse events.

Most adverse events were mild or moderate (grade 1 or 2). Treatment-related grade 3 adverse events were reported in two patients in the anastrozole group (arthralgia and syncope) and one patient in the tamoxifen group (depression). No events at grade 4 were recorded. One serious adverse event was reported in the anastrozole group (grade 3 incidence of benign neoplasm), which was not considered related to treatment. No serious adverse events were reported in the tamoxifen group. One patient in the tamoxifen group discontinued treatment because of a grade 1 adverse event (liver disorder), which was considered related to treatment.

Mean bone mineral density at lumbar spine decreased by 5·8% in the anastrozole group and by 2·9% in the tamoxifen group, and mean bone mineral density at cervical thighbone decreased by 2·5% in the anastrozole group and by 0·8% in the tamoxifen group. The reduction in bone mineral density was significantly greater in the anastrozole group at lumbar spine (p<0·0001) and cervical thighbone (p=0·0045) than in the tamoxifen group. Bone turnover marker BAP increased slightly in the anastrozole group (EIA method [n=66], mean 20·97 to 28·11 U/L; CLEIA method [n=32], 10·98 to 16·58 μg/L), whereas no change was recorded in the tamoxifen group. Bone turnover marker NTX increased numerically in both treatment groups (anastrozole mean 13·22 to 22·43 nmol BCE/L [bone collagen equivalents per L of serum]; tamoxifen 12·66 to 14·99 nmol BCE/L).

No clinically important changes in laboratory parameters or vital signs were recorded. Treatment compliance for the tablet medication, measured by confirmed tablet counting, was 98·9% for the anastrozole group and 99·3% for the tamoxifen group.

Discussion

During 24 weeks of neoadjuvant treatment, a greater proportion of premenopausal women with ER-positive, HER2-negative breast cancer who received anastrozole plus goserelin showed a tumour response benefit than did those who received tamoxifen plus goserelin. Further, a higher proportion of patients in the anastrozole group

Panel: Research in context

Systematic review

We searched PubMed and ClinicalTrials.gov with the search terms "aromatase inhibitor", "goserelin", "premenopausal", and "neoadjuvant", to identify all studies and publications to July, 2007. We did not find any randomised trials and, therefore, we identified the need for a new study comparing an aromatase inhibitor with tamoxifen in the neoadjuvant treatment setting for premenopausal breast cancer.

Subsequently, we have identified studies investigating the use of aromatase inhibitors in premenopausal breast cancer, including a single-arm, phase 2 study of anastrozole plus goserelin in premenopausal advanced breast cancer,[31] which reported a clinical benefit rate (partial response plus complete response plus stable disease ≥6 months) of 71·9%. Additionally, we identified a non-randomised study[32] that suggested that concomitant goserelin plus letrozole together with presurgical chemotherapy was effective in premenopausal women with locally advanced breast cancer in terms of improved disease-free survival. Results from a phase 3 study (ABCSG-12),[11] comparing anastrozole plus goserelin with tamoxifen plus goserelin in the adjuvant setting in premenopausal women, showed disease-free survival rates to be similar between the treatment groups. A recent analysis of ABCSG-12[24] suggests that body-mass index significantly affects the efficacy of anastrozole plus goserelin in premenopausal patients with breast cancer. Given the available evidence at the time, we decided to undertake this randomised phase 3 trial to compare an aromatase inhibitor with tamoxifen in the neoadjuvant treatment setting for premenopausal breast cancer.

Interpretation

To our knowledge, our results have shown for the first time that neoadjuvant treatment with anastrozole plus goserelin has a better risk–benefit profile than does tamoxifen plus goserelin as neoadjuvant treatment for premenopausal women with early-stage breast cancer. As such, this combination could represent an alternative neoadjuvant treatment option for premenopausal women with early-stage breast cancer.

than in the tamoxifen group received breast-conserving surgery. These data suggest that anastrozole plus goserelin is an effective neoadjuvant treatment option in this patient population, and might enable tumour downstaging to allow for breast-conserving surgery.

A favourable response to neoadjuvant therapy usually translates into a better clinical prognosis.[20] In the ABCSG-12 study,[11] which compared anastrozole plus goserelin with tamoxifen plus goserelin in the adjuvant setting in premenopausal women, disease-free survival rates were similar between the treatment groups. It might be expected that the greater efficacy in the anastrozole group in the neoadjuvant setting noted in this present study would translate to improved disease-free survival compared with the tamoxifen group with continued treatment in the adjuvant setting.

This study recruited only patients with ER-positive and HER2-negative tumours. Our own experience, together with data from other studies, has shown ER-positive and HER2-negative tumours to be more hormone dependent and therefore more responsive to endocrine therapy than ER-positive and HER2-positive tumours.[21]

Although similar disease-free survival rates were reported between the groups in the ABCSG-12 study,[11] a strong trend was noted for improved overall survival in the tamoxifen group compared with the anastrozole group. Although the precise reason for improved overall survival in favour of tamoxifen is unclear, it was speculated that the absence of palliative treatment with aromatase inhibitors in the anastrozole group after relapse could affect overall survival.[22,23]

Interestingly, a retrospective analysis of the ABCSG-12 data[24] reported that the better overall survival for tamoxifen plus goserelin than for anastrozole plus goserelin was only noted in a subset of patients with body-mass index (BMI) higher than 25 kg/m², but not in those patients with BMI lower than 25 kg/m².[24] Similarly, obese women (BMI >30 kg/m²) treated with anastrozole in the Arimidex, Tamoxifen, Alone or in Combination (ATAC) trial[25] were associated with poorer overall prognosis than were women with BMI lower than 23 kg/m². The proportion of women with BMI higher than 25 kg/m² was lower in the STAGE study (34 [17·3%] of 197 women) than in the ABCSG-12 study (573 [33·0%] of 1736 women),[24] which might also partly explain the better efficacy for anastrozole than for tamoxifen in STAGE.

The optimum duration of neoadjuvant hormone therapy has yet to be fully elucidated. We report an increase in tumour responses from week 16 to week 24 of 13·3% in the anastrozole group and 9·1% in the tamoxifen groups. As a result, although we have shown that treatment duration of 24 weeks was preferable over 16 weeks, it is possible that the optimum treatment duration may even be greater than 24 weeks. These results correspond to those reported by Dixon and colleagues,[26] in which clinical response was greater with extended neoadjuvant letrozole treatment beyond 3 months, than with a shorter treatment duration.

The clinical response during the 24-week treatment period of 70% achieved by the anastrozole group in our study seems similar to the clinical response rate of 66% achieved with chemotherapy in a similar patient population in a previous study,[27] but a definitive randomised trial that compares neoadjuvant endocrine therapy with chemotherapy has yet to be reported.[28] Although clinical response might not be consistent with the pathological response,[29] and it is possible that pathological responses might ultimately be higher with chemotherapy, anastrozole plus goserelin might offer a treatment option for patients with large ER-positive and HER2-negative tumours for which downstaging could allow breast-conserving surgery.

A possible limitation of this study is that, although a higher proportion of patients in the anastrozole group received breast-conserving surgery, a prediction of the expected method of surgery was not done at baseline, which would be necessary for a meaningful comparison between best overall tumour response and the actual surgical method used. With only two treatment groups, the effect of the individual treatments (anastrozole, tamoxifen, or goserelin) used in the study could not be

determined. Definitive results are also unlikely to be shown for long-term outcomes because of the small sample size.

Reduction in Ki67 index was significantly greater with anastrozole than with tamoxifen treatment, consistent with results observed in the IMmediate Preoperative Anastrozole, Tamoxifen, or Combined with Tamoxifen (IMPACT) trial.[21] The relation between reduction in Ki67 index in the IMPACT trial correlated with the long-term outcome of improved disease-free survival for anastrozole versus tamoxifen in the adjuvant ATAC trial.[25] However, the tumour response rates under neoadjuvant treatment did not seem to predict for long-term outcome with adjuvant therapy.[23]

Both treatment regimens were well tolerated during the 24-week neoadjuvant treatment period, consistent with the known safety profile of the individual treatments. The incidence of hot flushes reported here was higher than that reported for any of the drugs as monotherapy.[23] However, as hot flushes are a known side-effect of all three drugs, an additive effect of combination therapy cannot be discounted. An exploratory analysis showed that no significant relation existed between those patients who responded to treatment and those patients who had hot flushes in both treatment groups (data not shown). Consistent with the known safety profiles of each treatment, musculoskeletal disorders seemed higher with anastrozole than with tamoxifen treatment.[30] Although this was a short-term study, results of bone mineral density and bone turnover markers BAP and NTX seem consistent with the known safety profile of anastrozole.

In conclusion, results from this study have, to the best of our knowledge (panel), shown for the first time that neoadjuvant treatment with anastrozole plus goserelin has a better risk–benefit profile than tamoxifen plus goserelin as neoadjuvant treatment for premenopausal women with early-stage breast cancer.

Contributors

NM, YS, TK, HIwat, SNa, YY, RN, HIwas, SK, and HT contributed to provision of study patients, data collection, data interpretation, and writing. SNo contributed to study design, data interpretation, and writing. All authors critically reviewed the draft manuscript and approved the final report.

Conflicts of interest

HIwas has received honoraria from AstraZeneca and Pfizer. SNo has received honoraria, consultancy fees, and research funding from AstraZeneca. The other authors declare that they have no conflicts of interest.

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