Ⅲ. 学会等発表実績

学 会 等 発 表 実 績

委託業務題目「乳癌に対する術前薬物療法における治療戦略研究」
機関名　国立がん研究センター

１．学会等における口頭・ポスター発表

発表した成果（発表題目、口頭・ポスター発表の別）	発表者氏名	発表した場所（学会等名）	発表した時期	国内・外の別
Safety, pharmacokinetics (PK) and efficacy of cyclin-dependentkinase (CDK) 4 and 6 inhibitor, paldociclib (PD-0332991) Developmental therapeutics	Mukai H, Yoshino T, Osera S, Sasaki M, Shimizu C, Yonemori K, Koudaira M, Tanabe Y, Matsuda N, Mizutani N, Mori Y, Hashigaki S, Nagasawa T, Umeyama Y, Randolph S, Tamura K.	ESMO2014	2014.9	国外
Transition of recurrence-free survival for early-stage breast cancer at National Cancer Center Hospital East (Poster Presentation (Display) Brest cancer early stage)	Kaneko M, Hosono A, Sasaki M, Matsubara N, Naito Y, Saito S, Yamanaka T, Wada N, Mukai H.	ESMO2014	2014.9	国外
転移・再発乳癌に対するタキサン系薬剤とTS-1のランダム化比較試験（SELECT-BC）（優秀演題講演 PS-1-001-03）	相良吉昭 高島勉 原文堅 渡辺隆紀 穂積康夫 鶴谷純司 井本滋 西村令喜 大橋靖雄 向井博文	第22回日本乳癌学術学会	2014.7	国内
Luminalタイプ乳癌における予後因子としての組織グレードの意義（ポスター掲示 GP-2-059-11）	佐々木政興 内藤陽一 藤井誠志 細野亜古 松原伸晃 和田徳昭 米山公康 向井博文	第22回日本乳癌学術学会	2014.7	国内

発表した成果（発表題目、口頭・ポスター発表の別）	発表者氏名	発表した場所（学会等名）	発表した時期	国内・外の別
CpG islands and genomic regions with basal low-level methylation to aberrant DNA methylation induction （ポスター）	Yamashita S, Nanjo S, Rehnberg E, Ando T, Maekita T, Ichinose M, Sugiyama T, Ushijima T	第8回日本エピジェネティクス研究会年会	2014.5	国内
遺伝子点突然変異のターゲットシークエンシングによる高感度解析法の開発とGeneticな発がんの素地の解析（口頭）	山下聡, 岸野貴賢, 永野玲子, 牛島俊和	第29回発癌病理研究会	2014.9	国内
High-Sensitivity Analysis of Aberrant DNA Methylation by Targeted Deep Sequencing using a Benchtop Sequencer（ポスター）	Yamashita S, Kishino T, Nagano R, Ushijima T	第73回日本癌学会学術総会	2014.9	国内

２．学会誌・雑誌等における論文掲載

掲載した論文（発表題目）	発表者氏名	発表した場所（学会誌・雑誌	発表した時期	国内・外の別
Comprehensive DNA Methylation and Extensive Mutation Analyses of HER2-Positive Breast Cancer.	Yamaguchi T, Mukai H, Yamashita S, Fujii S, Ushijima T.	Oncology	2015 Jan 14	国外
Prospective cohort study: whether or not patients benefit from participation itself in randomized-controlled trials (SELECT BC ECO)	Mukai H, Ohno S, Ohashi Y	Jpn J Clin Oncol	2014	国外
Phase 1 combination study of eribulin mesylate with trastuzumab for advanced or recurrent human epidermal growth factor receptor 2 positive breast cancer.	Mukai H, Saeki T, Shimada K, Naito Y, Matsubara N, Nakanishi T, Obaishi H, Namiki M, Sasaki Y	Invest New Drugs	2015 Feb	国外
Pathologic complete response after neoadjuvant chemotherapy in HER2-overexpressing breast cancer according to hormonal receptor status.	Tanioka M, Sasaki M, Shimomura A, Fujishima M, Doi M, Matsuura K, Sakuma T, Yoshimura K, Saeki T, Ohara M, Tsurutani J, Watatani M, Takano T, Kawabata H, Mukai H, Naito Y, Hirokaga K, Takao S, Minami H	Breast	2014	国外
Survival outcome and reduction rate of Ki-67 between pre- and post-neoadjuvant chemotherapy in breast cancer patients with non-pCR.	Matsubara N, Mukai H, Masumoto M, Sasaki M, Naito Y, Fujii S, Wada N	Breast Cancer Res Treat	2014	国外

掲載した論文（発表題目）	発表者氏名	発表した場所（学会誌・雑誌	発表した時期	国内・外の別
Factors affecting enrollment in a randomized controlled trial for Japanese metastatic breast cancer patients (SELECT BC-FEEL) a prospective study.	Ohsumi S, Mukai H, Ohashi Y	Jpn J Clin Oncol	2014	国外
Anastrozole versus tamoxifen as adjuvant therapy for Japanese postmenopausal patients with hormone-responsive breast cancer: efficacy results of long-term follow-up data from the N-SAS BC 03 trial.	Aihara T, Yokota I, Hozumi Y, Aogi K, Iwata H, Tamura M, Fukuuchi A, Makino H, Kim R, Andoh M, Tsugawa K, Ohno S, Yamaguchi T, Ohashi Y, Watanabe T, Takatsuka Y, Mukai H	Breast Cancer Res Treat	2014	国外
Evaluation of HER2-based biology in 1,006 cases of gastric cancer in a Japanese population	Aizawa M, Nagatsuma AK, Kitada K, Kuwata T, Fujii S, Kinoshita T, Ochiai A.	Gastric Cancer	2014	国外
ZNF695 methylation predicts a response of esophageal squamous cell carcinoma to definitive chemoradiotherapy.	Takahashi T, Yamahsita S, Matsuda Y, Kishino T, Nakajima T, Kushima R, Kato K, Igaki H, Tachimori Y, Osugi H, Nagino M, Ushijima T.	J Cancer Res Clin Oncol.	2015	国外

掲載した論文（発表題目）	発表者氏名	発表した場所（学会誌・雑誌	発表した時期	国内・外の別
Establishment of a DNA Methylation Marker to Evaluate Cancer Cell Fraction in Gastric Cancer.	Zong L, Hattori N, Yoda Y, Yamashita S, Takeshima H, Takahashi T, Maeda M, Katai H, Nanjo S, Ando T, Seto Y, Ushijima T.	Gastric Cancer	online	国外
High-sensitivity troponin T as a marker to predict cardiotoxicity in breast cancer patients with adjuvant trastuzumab therapy.	Katsurada K, Ichida M, Sakuragi M, Takehara M, Hozumi Y, Kario K	Springerplus	2014	国外
RhoC upregulation is correlated with reduced E-cadherin in human breast cancer specimens after chemotherapy and in human breast cancer MCF-7 cells.	Kawata H, Kamiakito T, Omoto Y, Miyazaki C, Hozumi Y, Tanaka A.	Horm Cancer.	2014	国外
Treatment outcomes and prognostic factors for patients with brain metastases from breast cancer of each subtype: a multicenter retrospective analysis.	Niikura N, Hayashi N, Masuda N, Takashima S, Nakamura R, Watanabe K, Kanbayashi C, Ishida M, Hozumi Y, Tsuneizumi M, Kondo N, Naito Y, Honda Y, Matsui A, Fujisawa T, Oshitanai R, Yasojima H, Tokuda Y, Saji S, Iwata H.	Breast Cancer Res Treat.	2014	国外

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Survival of HER2-positive primary breast cancer patients treated by neoadjuvant chemotherapy plus trastuzumab: a multicenter retrospective observational study (JBCRG-C03 study).	Takada M, Ishiguro H, Nagai S, Ohtani S, Kawabata H, Yanagita Y, Hozumi Y, Shimizu C, Takao S, Sato N, Kosaka Y, Sagara Y, Iwata H, Ohno S, Kuroi K, Masuda N, Yamashiro H, Sugimoto M, Kondo M, Naito Y, Sasano H, Inamoto T, Morita S, Toi M.	Breast Cancer Res Treat.	2014	国外
Incidence of contralateral breast cancer in Japanese patients with unilateral minimum-risk primary breast cancer, and the benefits of endocrine therapy and radiotherapy.	Aihara T, Tanaka S, Sagara Y, Iwata H, Hozumi Y, Takei H, Yamaguchi H, Ishitobi M, Egawa C.	Breast Cancer	2014	国内
Pathological responses and survival of patients with human epidermal growth factor receptor 2-positive breast cancer who received neoadjuvant chemotherapy including trastuzumab.	Ohzawa H, Sakatani T, Niki T, Yasuda Y, Hozumi Y.	Breast Cancer	2014	国内
Magnetic resonance lymphography of sentinel lymph nodes in patients with breast cancer using superparamagnetic iron oxide: a feasibility study.	Shiozawa M, Kobayashi S, Sato Y, Maeshima H, Hozumi Y, Lefor AT, Kurihara K, Sata N, Yasuda Y	Breast Cancer	2014	国内

Ⅳ．研究成果の刊行物・別刷

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Comprehensive DNA Methylation and Extensive Mutation Analyses of HER2-Positive Breast Cancer

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Key Words
Breast cancer · Epigenome · Gene mutation · HER2 · Ki-67 · Neoadjuvant chemotherapy · Next-generation sequencer · Trastuzumab

Abstract
Objective: Resistance to trastuzumab is a problem that remains to be solved in HER2-positive breast cancer. We aimed to characterize profiles of genetic and epigenetic alterations in cancer-related pathways in HER2-positive breast cancers, using biopsy tissue samples obtained from patients enrolled in a prospective neoadjuvant clinical trial. ***Methods:*** HER2-positive breast cancer tissue samples were collected and processed with the PAXgene Tissue System. A total of 24 breast cancers were analyzed. Genetic alterations of 409 cancer-related genes were analyzed by a bench-top next-generation sequencer. DNA methylation statuses were analyzed by a bead array with 485,512 probes. ***Results:*** The WNT pathway was potentially activated by aberrant methylation of its negative regulators, such as *DKK3* and *SFRP1*, in 9 breast cancers. The AKT/mTOR pathway was activated by mutations of *PIK3CA* in 5 breast cancers. The Notch pathway was potentially activated by mutations of *NOTCH1* and *NOTCH2* in 4 breast cancers. The p53 pathway was inactivated by mutations of *TP53* in 13 breast cancers and potentially by aberrant methylation of its downstream genes in 10 breast cancers. Cell adhesion was affected by mutations of *CDH1* in 1 breast cancer. ***Conclusion:*** Genes involved in cancer-related pathways were frequently affected not only by genetic but also by epigenetic alterations in HER2-positive breast cancer.

Introduction

Overexpression of human epidermal growth factor receptor 2 (HER2) or amplification of the *HER2* gene is observed in 15–25% of breast cancers (BCs), and, independently, both are factors discriminating for poor prognosis [1]. Adding trastuzumab, which is a humanized monoclonal antibody that targets HER2, to chemotherapy improves survival in patients with HER2-positive metastatic BC [2]. Therefore, HER2 was recognized as an important therapeutic target in BC. Trastuzumab has also shown efficacy against early-stage BC as an adjuvant and neoadjuvant therapy [3]. Although trastuzumab has revolutionized the treatment of HER2-positive BC, resis-

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tance to this substance is a problem that remains to be solved. Response rates to trastuzumab monotherapy as first- and second-line treatments for metastatic BC have been reported to be 15 and 26%, respectively [4, 5]; most patients do not respond to this agent alone (de novo resistance). The combination of chemotherapy and trastuzumab shows a high response rate in metastatic BC; however, all patients eventually show resistance (acquired resistance). Therefore, the discovery of new biomarkers and an elucidation of the mechanisms of resistance to trastuzumab are needed for the development of new treatments in the future.

Genetic and epigenetic alterations are involved in cancer development and progression by activating growth-promoting pathways and inactivating tumor-suppressive pathways. Various studies have been conducted in vivo and in vitro, and several mechanisms of resistance to HER2-targeted therapy have been proposed. However, many of these studies are retrospective [6–9]. Until recently, these genetic and epigenetic alterations have been assessed individually, because technologies for their comprehensive analysis have not been available at a reasonable cost. Now, point mutations and gene amplifications of a large number of target genes can be analyzed by bench-top next-generation sequencers [10]. A comprehensive DNA methylation profile can be analyzed using a bead array [11]. The process of the combination of comprehensive DNA methylation and mutation analyses as well as pathway analysis using these data was established in our previous studies of gastric cancer [12, 13].

In this study, we aimed to establish an integrated profile of genetic and epigenetic alterations in HER2-positive BC by using tissue samples obtained from a neoadjuvant clinical trial.

Methods

Study Design of the Neoadjuvant Clinical Trial

We have conducted a trial to determine if neoadjuvant chemotherapy can be optimized by using the Ki-67 index changes during neoadjuvant chemotherapy in HER2-positive BC. This trial was registered in the UMIN Clinical Trials Registry (registration No. UMIN000007074). The details of the trial have been described in a previous paper [14]. In brief, this was a randomized phase II trial in which women aged 20–75 years with histologically confirmed HER2-positive BC stage II–III were eligible patients. HER2 positivity was defined as overexpression by immunohistochemistry (3+) or gene amplification by fluorescence in situ hybridization.

The patients were required to have a good performance status, sufficient organ functions, a normal left ventricular ejection fraction as well as no prior endocrine therapy or chemotherapy for BC. They were randomly assigned to weekly paclitaxel (80 mg/m^2 a week) and trastuzumab (a loading dose of 4 mg/kg followed by 2 mg/kg a week) for a total of 12 doses or to Ki-67 index-guided treatment as a preoperative treatment. In Ki-67 index-guided treatment, the patients initially received weekly paclitaxel and trastuzumab as in the reference arm, and a primary tumor was biopsied during days 15–21 for estimation of the Ki-67 index. The subsequent chemotherapy regimen was adjusted according to changes in Ki-67 index from baseline.

Between December 2011 and December 2013, 133 patients were registered, with the aim of reaching 200 patients; registration is currently still in progress (as of April 2014). The study protocol was approved by the National Cancer Center Ethics Committee (approval No. 2010-250). The trial met the Ethical Guidelines for Clinical Studies of the Japanese Ministry of Health, Labor and Welfare and was conducted in compliance with the Declaration of Helsinki. The patients provided written informed consent.

Samples

From among the patients enrolled in the neoadjuvant clinical trial so far, samples obtained from 24 patients were used. One or two core needle biopsy samples of the primary tumors were collected at baseline and days 15–21. An experienced pathologist (S.F.) evaluated whether the components of the tumor cells in the samples were sufficient enough for molecular analysis. In the present study, 24 HER2-positive BC tissue samples obtained at baseline were analyzed (22 invasive ductal carcinoma samples, 1 invasive lobular carcinoma sample and 1 invasive micropapillary carcinoma sample); 8 BCs were estrogen receptor positive, and 8 were progesterone receptor positive.

Tissue samples were fixed and stored with the PAXgene Tissue System according to the manufacturer's instructions (Qiagen, Germany). Genomic DNA was extracted with a PaxGene DNA Kit (Qiagen), and extracted DNA was quantified using a Quant-iT PicoGreen dsDNA Assay Kit (Life Technologies, USA). Total RNA was extracted with a PaxGene RNA Kit (Qiagen). The PAXgene Tissue System is a recently developed tissue stabilization technology. So far, researchers have reported that RNA and DNA purified from PAXgene-fixed tissues are of high integrity and that they perform as well as those from fresh frozen tissue [15–17].

Analysis of Somatic Mutations

Four independent multiplex PCRs amplifying a total of 15,991 regions in 409 cancer-related genes were performed on genomic DNA, using the Ion AmpliSeq Library Kit 2.0 with the Comprehensive Cancer Panel (Life Technologies). The amplified products were mixed equally and then uniquely barcoded with an Ion Xpress Barcode Adapters 1-96 Kit (Life Technologies); 4–8 barcoded libraries were pooled, and an emulsion PCR was performed with an Ion OneTouch 2 device with an Ion PI Template OT2 200 Kit v2 (Life Technologies). Template-positive Ion Sphere particles were concentrated with an Ion OneTouch ES (Life Technologies) and loaded onto an Ion PI Chip v2 (Life Technologies). Sequencing was performed with a bench-top next-generation sequencer, an Ion Proton sequencer with an Ion PI Sequencing 200 Kit v2 (Life Technologies). Primer sequences were removed from the obtained sequences, and the trimmed sequences were aligned onto the human reference genome hg19 with Torrent Suite (Life Technologies). By using CLC Genomics Workbench 6.0 (CLC bio, Denmark), a variation was identified as a functional mutation only

if (1) its frequency was >10%, (2) its read count was >40, (3) it was found in both at least 5 forward and 5 reverse reads, (4) it was not present in >10% of the reads in any normal samples and (5) it caused amino acid changes or splicing defects. All the mutations identified by computational analysis were validated by manual inspection of the alignment data.

Analysis of Gene Amplifications

Gene amplifications of 409 genes were analyzed using the alignment data; the average reading depth of each target region (amplicon) in each sample was obtained by CLC Genomics Workbench. To evaluate copy number variations in a gene, the relative reading depth to the reference (RRDR) of an individual gene was calculated as following: RRDR = (average of the reading depths of the target regions in an individual gene/average of the reading depths of all regions in the panel)$_{cancer}$/(average of the reading depths of the target regions in an individual gene/average of the reading depths of all regions in the panel)$_{normal}$, where 'cancer' and 'normal' mean a cancerous sample and its matched normal tissue (blood), respectively. Regions in which the average reading depth of all the samples was >50 were employed for the analysis. Genes whose RRDRs were larger (two-fold or more) than those of the other genes were defined as amplified genes.

Selection of Genes of Cancer-Related Pathways

A total of 64 genes involved in 9 cancer-related pathways (MAPK, WNT, AKT/mTOR, Notch, Hedgehog, cell cycle regulation, mismatch repair, p53 and cell adhesion) were selected according to our previous study [13]. Regarding the signaling pathways activated in BCs, their negative regulators were selected. Regarding the pathways inactivated in BCs, their positive regulators and downstream effectors were selected.

Analysis of DNA Methylation

DNA methylation levels of 485,512 probes (482,421 probes for CpG sites and 3,091 probes for non-CpG sites) were obtained using an Infinium HumanMethylation450 BeadChip array, as previously described [18]. To adjust for probe design biases, intra-array normalization was performed using a peak-based correction method, BMIQ (Beta Mixture Quantile dilation) [19]. The methylation level of each CpG site was represented by a β-value that ranged from 0 (unmethylated) to 1 (fully methylated).

DNA methylation of a CpG island (CGI) in a promoter region, especially in the 200-bp upstream region from a transcription start site (TSS200), is known to consistently silence its downstream gene, while that of downstream exons is weakly associated with increased expression [20–23]. Therefore, we paid as much attention as possible to analyze the DNA methylation of a CGI in a TSS200. To achieve this, probes for CpG sites were assembled into 296,494 genomic blocks <500 bp. Of these blocks, 59,757 were located in CGIs, and 11,307 of them were located in TSS200s. The selection of genomic blocks to analyze genes of cancer-related pathways was performed according to our previous study [13]. The DNA methylation level of a genomic block was evaluated using the mean β-value of all the probes within the genomic block. The methylation levels were corrected using cancer cell contents pathologically analyzed by an experienced pathologist (S.F.). The methylation status of the genomic blocks was classified into unmethylated (corrected β-value: 0–0.2), partially methylated (corrected β-value: 0.2–0.8) and heavily methylated (corrected β-value: 0.8–1.0).

Analysis of Gene Expression

Gene expression in normal human mammary epithelial cells (HMECs) was analyzed with the GeneChip Human Genome U133 Plus 2.0 microarray (Affymetrix, USA). Genes with signal intensities of ≥250 were defined as expressed genes [24].

Results

Methylation Silenced Genes in HER2-Positive BC

Comprehensive DNA methylation analysis was performed on 24 HER2-positive BCs. The analysis was conducted using 7,103 TSS200 CGIs unmethylated in normal mammary epithelial cells (genes unmethylated in HMECs), because a TSS200 CGI is known to play a critical role in methylation silencing [25]. The number of aberrantly methylated genes ranged from 9 to 629. Next, we focused on TSS200 CGIs of genes with positive expression in normal cells but aberrantly methylated in cancer cells, because this group of genes is known to frequently contain driver genes in carcinogenesis [26]. Using 292 TSS200 CGIs whose downstream genes were expressed in normal mammary epithelial cells and aberrantly methylated in ≥1 HER2-positive BCs (methylation-silenced genes; online suppl. table S1; for all online suppl. material, see www.karger.com/doi/10.1159/000369904), the number ranged from 2 to 132. These results showed that the number of aberrantly methylated genes was highly variable even among individual HER2-positive BCs.

Point Mutations and Gene Amplifications in HER2-Positive BC

Of the 24 HER2-positive BCs analyzed for mutations of the 409 cancer-related genes, 23 had 80 somatic mutations (online suppl. table S2). The *PIK3CA* oncogene was mutated in 20.8% of the HER2-positive BCs. *TP53* was most frequently mutated (54.2% of the HER2-positive BCs), and *PIK3CA*, *NOTCH2*, *RNF213*, *ADAMTS20*, *PKHD1*, *ROS1*, *SETD2*, *ZNF521* and *TAF1L* were mutated in ≥2 HER2-positive BCs (fig. 1).

Gene amplification was analyzed as well for the 409 cancer-related genes in the 24 HER2-positive BCs, and all BCs had 169 amplifications in 80 genes (online suppl. table S3). *ERBB2* amplification was detected in 22 of the 24 HER2-positive BCs. The remaining 2 samples (12D, 25D) showed 1.2- and 1.3-fold RRDRs, respectively. *MAF*, *PLAG1*, *EXT*, *CSMD2* and *MYC* were also amplified in ≥3 HER2-positive BCs, except for genes on chromosome 17q (close to HER2).

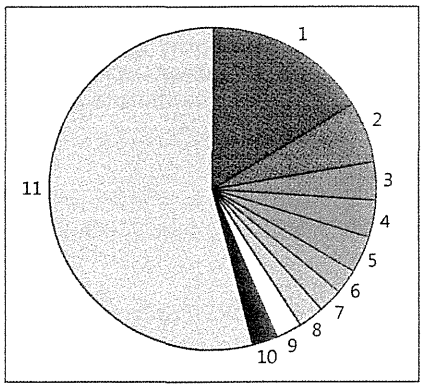

Fig. 1. Gene mutations in 24 HER2-positive BCs. A total of 80 somatic mutations found in these BCs are shown in the circle graph on the left side. Genes mutated in ≥2 HER2-positive BCs are listed on the right side.

Growth-Promoting Pathways Affected by Epigenetic and Genetic Alterations

Aberrant DNA methylation of the 64 genes involved in the 9 cancer-related pathways was combined with genetic alterations in the 24 HER2-positive BCs. First, the potential activation of growth-promoting pathways by aberrant methylation of their negative regulators, in addition to activating genetic alterations (point mutations and gene amplifications), were analyzed (fig. 2).

Regarding the MAPK pathway, in addition to amplification of *ERBB2*, 15 of the 24 BCs had heavy aberrant methylation of its 1 negative regulator: *RASSF1* (fig. 2a). As for the AKT/mTOR pathway, only 1 of the 24 BCs had heavy aberrant methylation of its 4 negative regulators, and 5 BCs had point mutations of *PIK3CA* or *PTPN11* (fig. 2b).

Regarding the WNT pathway, all the BCs had heavy or partially aberrant methylation of ≥1 of its 16 negative regulators, such as *DKK3* and *SFRP1* (fig. 2c). To exclude concerns that we analyzed the methylation of genes that had little expression in normal epithelial cells and thus were susceptible to methylation [24], we confirmed that 7 of the 16 negative regulators were moderately or abundantly expressed (signal intensity >250) in normal epithelial cells. Even when limited to these 7 genes, *DKK3* or *SFRP1* were heavily methylated in 9 BCs. In contrast, no BCs had point mutations of *CTNNB1*.

With regard to the Notch pathway, no BCs had heavy aberrant methylation of its negative regulators, and 4 BCs had a mutation of *NOTCH1* or *NOTCH2* (fig. 2d). Regarding the Hedgehog pathway, only 1 BC had heavy aberrant methylation of its 1 negative regulator, and no BCs had a mutation of *PTCH1* (fig. 2e).

Tumor-Suppressive Pathways Affected by Epigenetic and Genetic Alterations

Tumor-suppressive pathways inactivated in BCs were also analyzed. Regarding cell cycle regulation, none of the 24 BCs had heavy aberrant methylation of *CDKN2A* and/or *CHFR*, and none of the 24 BCs had point mutations of *CDKN2A* (fig. 3a). Concerning mismatch repair, none of the 24 BCs had heavy aberrant methylation of *MLH1*, and none of the BCs had a point mutation (fig. 3b).

As for the p53 pathway, 13 BCs had point mutations of *TP53*. Twenty-four downstream genes had promoter CGIs, and 10 BCs had heavy aberrant methylation of ≥1 of the 24 genes (fig. 3c). Among the 24 genes, *IGFBP7* was abundantly expressed in normal mammary epithelial cells, and 6 BCs had heavy aberrant methylation.

Regarding cell adhesion, 13 BCs had partial aberrant methylation of *CDH1*, but none of the 24 BCs had heavy aberrant methylation. At the same time, 1 BC had its point mutations (fig. 3d). Taken together, these results demonstrate that genes in cancer-related pathways were frequently affected by epigenetic alterations even in HER2-positive BCs.

Discussion

In this study, we performed genetic and epigenetic profiling of 24 HER2-positive BCs and showed that genes in cancer-related pathways were frequently affected by not only genetic but also epigenetic alterations in HER2-positive BCs. When genetic and epigenetic alterations were combined, almost all of the 24 HER2-positive BCs had alterations in cancer-related pathways. The alterations in these pathways are potential targets for therapy, and they possibly have a role in the mechanism of resistance to trastuzumab.

In addition to amplification of HER2, 15 of the 24 HER2-positive BCs had heavy aberrant methylation of *RASSF1*. Since *RASSF1* is a negative regulator of the MAPK pathway, silencing of *RASSF1* by DNA methylation is equivalent to releasing the brakes restraining the activation of the MAPK pathway by HER2 amplification. Methylation of *RASSF1* may play an important role in the activity of the MAPK pathway in HER2-positive BC and might also be involved in the therapeutic response.

Among the 24 HER2-positive BCs, some had mutations and amplifications of target genes for molecular tar-

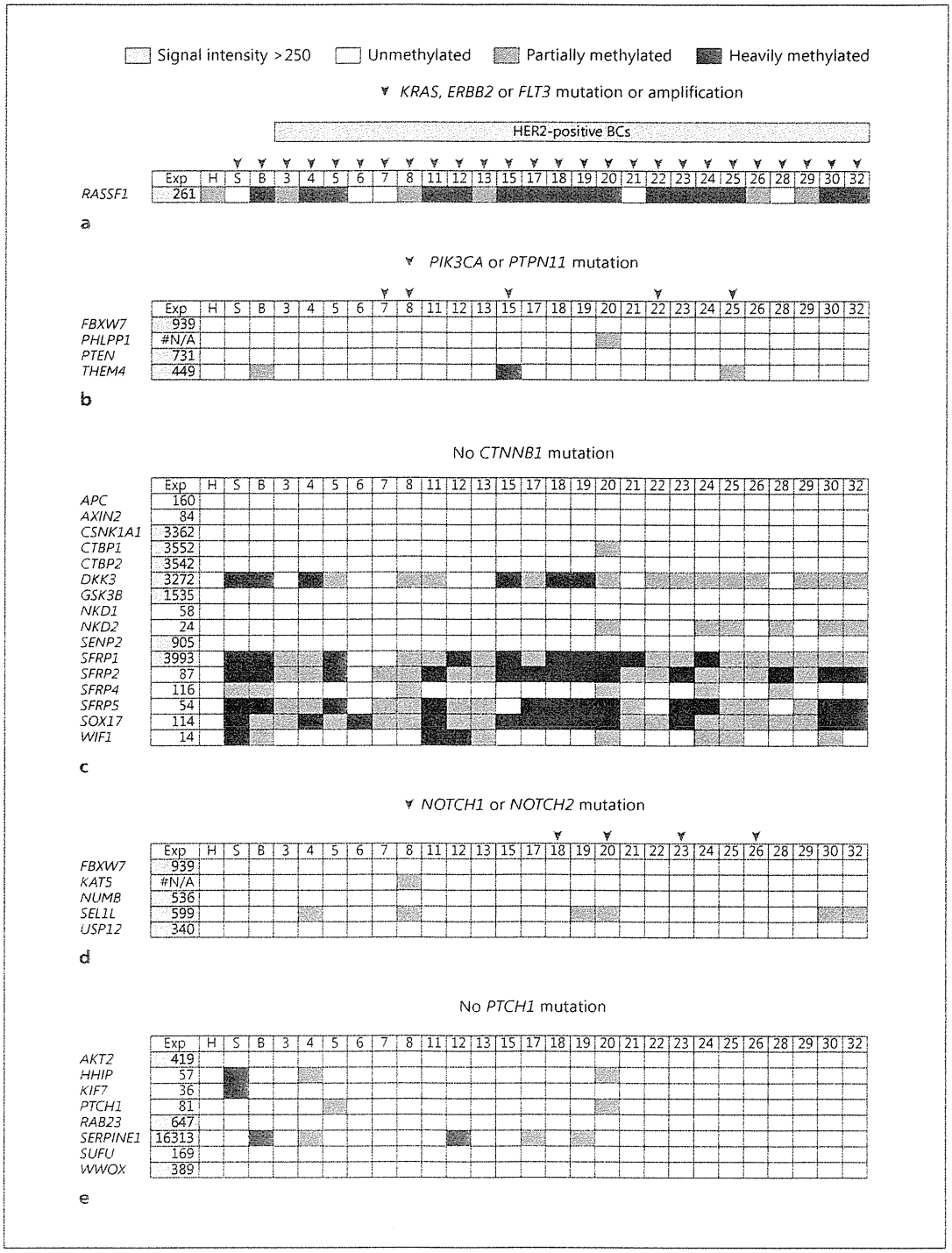

Fig. 2. Genetic and epigenetic alterations in 5 growth-promoting pathways. **a** In the MAPK pathway, 15 of the 24 HER2-positive BCs had heavy aberrant methylation of *RASSF1*. **b** In the AKT/mTOR pathway, 4 BCs had point mutations of *PIK3CA* or *PTPN11* (shown by arrowheads). In contrast, none of the 24 BCs had heavy aberrant methylation of negative regulators of the AKT/mTOR pathway. **c** WNT pathway. **d** Notch pathway. **e** Hedgehog pathway. Exp = Expression levels in HMECs; H = HMECs (normal cell lines); S = SK-BR-3 (HER2-positive BC cell lines); B = BT474 (HER2-positive BC cell lines).

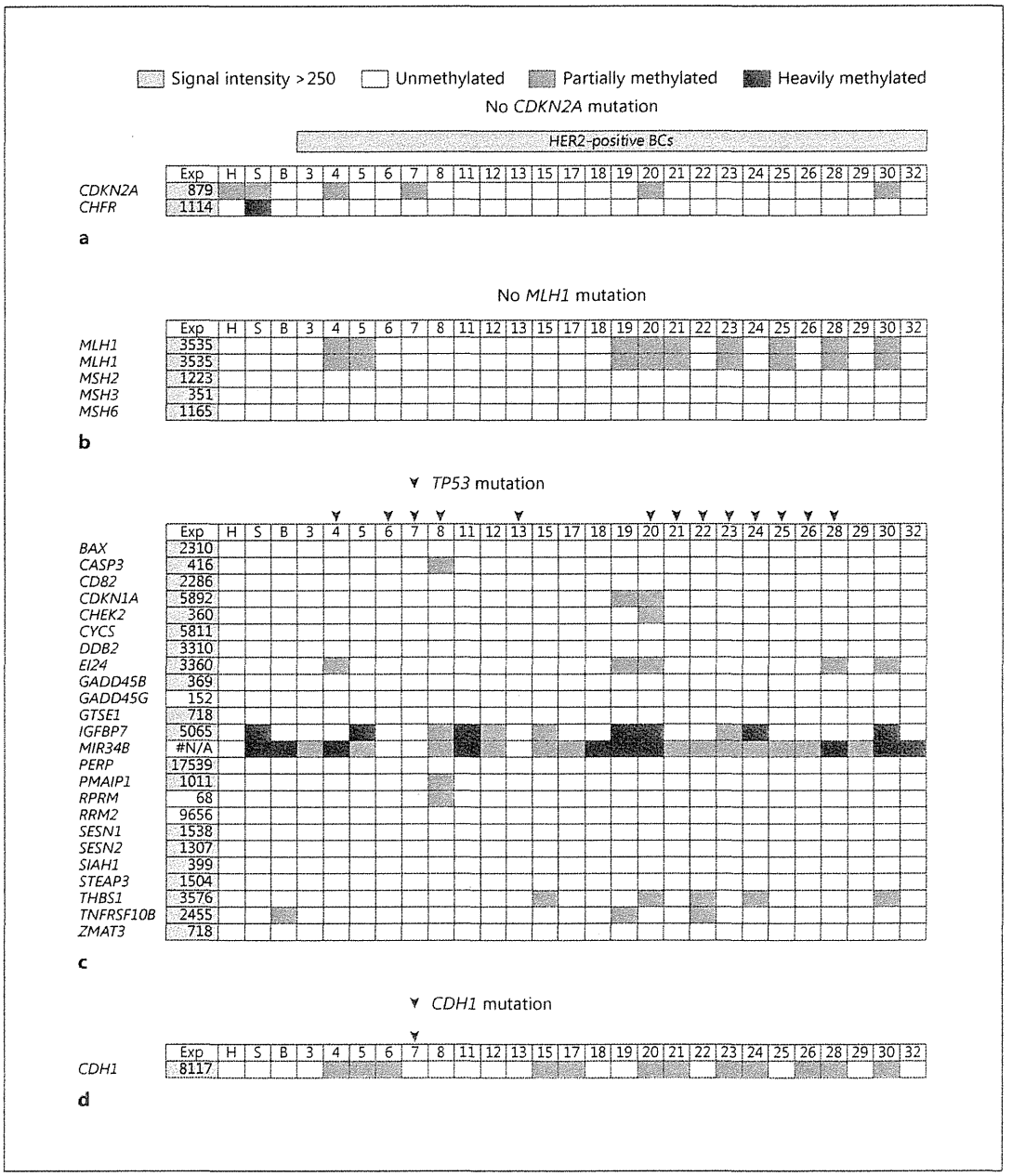

Fig. 3. Genetic and epigenetic alterations in 4 tumor-suppressor pathways. **a** Cell cycle regulation. None of the BCs had point mutations of *CDKN2A* or heavy aberrant methylation of *CDKN2A* and/or *CHFR*. **b** Mismatch repair. For *MLH1*, 2 genomic blocks in its 2 TSS200s were analyzed. **c** p53 pathway. Thirteen BCs had point mutations of *TP53* (shown by arrowheads), and 6 BCs had heavy aberrant methylation of *IGFBP7*. **d** Cell adhesion. One BC had a mutation of *CDH1* (shown by an arrowhead), but none of the 24 BCs had heavy aberrant methylation of *CDH1*.

geted therapy additionally to ERBB2 targeting. In this study, 21% of the BCs had point mutations of the *PIK3CA* gene, which is involved in the AKT/mTOR pathway. This is concordant with a study reporting that 20% of HER2-positive BCs have point mutations of this gene [11]. Activation of this pathway has been reported to be involved in therapeutic efficacy in HER2-positive BC. However, this study suggests that the possibility of activation by aberrant DNA methylation is low.

Aberrant DNA methylation of the negative regulator of the WNT pathway was observed in almost all HER2-positive BCs. Mutations of the Notch pathway-related genes were observed in 4 of the 24 BCs. As for the p53 pathway, mutations of *TP53* and aberrant methylation of the genes of the p53 pathway were observed in a complementary manner. When aberrant methylation and genetic abnormalities were combined, alterations to the p53 pathway were present in almost all HER2-positive BCs. On the other hand, the frequency of alterations in the Hedgehog pathway, cell cycle regulation, mismatch repair and cell adhesion was low both genetically and epigenetically. These results suggest the importance of the WNT and p53 pathways in HER2-positive BC. *DKK3* and *SFRP1* (involved in the WNT pathway) and *IGFBP7* (involved in the p53 pathway) were expressed in normal mammary epithelial cells and frequently methylated in HER2-positive BCs. It is known that downregulation of *DKK3* is correlated with tumor progression [27] and that *IGFBP7* can inhibit cell growth and induce apoptosis [28]. These results support that aberrant methylation of *DKK3*, *SFRP1* and *IGFBP7* was involved in the carcinogenesis of HER2-positive BCs.

ERBB2 amplification was detected with a bench-top next-generation sequencer in 22 of the 24 HER2-positive BCs. This discrepancy can be explained by the low cancer cell contents and low-level amplification of *ERBB2*. In this case, the relative copy number of *ERBB2* is low in DNA samples including both cancer and non-cancer cells.

In conclusion, an integrated profile of genetic and epigenetic alterations to cancer-related pathways in HER2-positive BCs was obtained using a bench-top next-generation sequencer and a bead array. The profile is expected to play an important role in the development of new treatments and the identification of biomarkers.

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Disclosure Statement

The authors declare that no competing interests exist.

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Clinical Trial Note

Prospective Cohort Study: Whether or Not Patients Benefit from Participation Itself in Randomized-controlled Trials (SELECT BC ECO)

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The randomized-controlled trial is widely accepted as a clinical trial to decide a standard therapy. It remains to be concluded whether or not patients benefit from participation itself in a randomized-controlled trial. This study was aimed at comparing prognoses between trial participants and participation-refusers. Concerned randomized trials are 'selection of effective chemotherapy for breast cancer' (SELECT BC) and its successor trial, SELECT BC-CONFIRM. Study subjects are all of metastatic breast cancer patients who are requested by their doctors to participate in these two trials. This trial is a hitherto exceptional prospective study and is suitable to clarify the effects of participation *per se* in such a trial on prognosis when compared with previous two studies.

Key words: metastatic breast cancer – prospective cohort study – SELECT BC – SELECT BC-CONFIRM

INTRODUCTION

At the present time, the randomized-controlled trial is a widely accepted procedure to establish a standard therapy.

In 1985, Davis et al. (1) compared participants and non-participants in a randomized-controlled trial in postoperative non-small cell lung cancer patients, reporting a significantly better survival in the trial participants. Similarly, as reported in 2000 by Gnant (2), participants in nine randomized-controlled trials showed a longer overall survival than non-participants among patients with Stage I or II early breast cancer.

Therefore, participation itself has the potential to improve patients' prognoses in the randomized-controlled trial. On the other hand, Peppercorn et al. (3) reviewed 26 reports on comparison of clinical trial participants and non-participants, concluding that there was no evidence enough to definitely favor the prognosis of the participants. Furthermore, in 2009, a similar comparative study was performed by Tanai et al. in Japanese advanced lung cancer patients. These investigators found no significant difference in prognosis between participants and non-participants in a randomized-controlled trial (4). As mentioned above, it has not been concluded yet whether or not participation *per se* in a randomized-controlled trial provides a better prognosis to patients. If non-participants include patients who are in such a bad condition that they do not meet entry criteria of a trial, non-participants have logically worse prognoses. Additionally, a trial may have the following effects and biases: effects of protocol therapy *per se*, care effects, Horson effects and placebo effects; and patient selection biases, clinical doctor selection biases and movement biases (5). Unless these biases are well controlled and minimized, it cannot be clarified whether or not participation *per se* in a randomized-controlled trial benefits participants.

This is an accompanying study of 'selection of effective chemotherapy for breast cancer' (SELECT BC) and its successor, SELECT BC-CONFIRM (6). The SELECT BC and

SELECT BC-CONFIRM trials are one of the largest scale randomized-controlled trials that are now ongoing in Japan in metastatic or recurrent breast cancer patients (Fig. 1).

This study has been designed so that study subjects are all patients who meet the eligibility criteria of SELECT BC as well as SELECT BC-CONFIRM trials and are requested for participation by attending doctors to compare prognoses between participants and participation-refusers. Since therapeutic agents that are to be administered to participants in both trials have been already approved, it is expected that participants and participation-refusers receive almost identical therapy except for orders of agent administration.

Treatment methods, examinations to be performed during therapy and observation methods of participants of both trials take a practical approach, and so care seems to be scarcely different in its effects between both trials. It is also expected that backgrounds of the participants and participation-refusers are eventually similar, because study subjects have to meet the eligibility criteria of both trials and are free to participate or refuse. Accordingly, this study is more suitable for clarifying effects of participation itself in randomized-controlled trials on prognosis than previous studies. Moreover, this study is a rare prospective study of great significance.

DIGEST OF THE STUDY PROTOCOL

Purpose

To prospectively perform a prognostic study of participants and participation-refusers of SELECT BC and SELECT BC-CONFIRM trials to compare their life prognoses.

Resources

This study was funded by Comprehensive Support Project for Oncology Research (CSPOR) of Public Health Research Foundation. The research fund was provided to CSPOR by Taiho Pharmaceutical Co., Ltd. Taiho Pharmaceutical took no part in this study other than providing information relevant to proper use of the study drug. All decisions concerning the planning, implementation and publication of this study were made by the executive committee of this study.

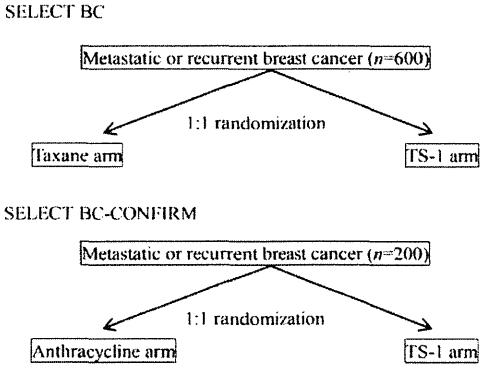

Figure 1. Study design of SELECT BC and SELECT BC-CONFIRM.

Endpoint

The endpoint is overall survival.

ELIGIBILITY CRITERIA

All patients that were proposed to participate in SELECT BC and SELECT BC-CONFIRM trials are study subjects of SELECT BC ECO. A participation-refuser is defined as 'a person who meets the eligibility criteria of SELECT BC and SELECT BC-CONFIRM but refuses to participate on their own will'. The inclusion and exclusion criteria of both trials are described below.

Inclusion Criteria

(i) Women with a histologically confirmed diagnosis of breast cancer.
(ii) One of the following conditions has to be met for a diagnosis of metastatic breast cancer:
  (a) At presentation, the patient has distant metastasis.
  (b) The patient has breast cancer that has worsened or recurred in association with distant metastasis after treatment (after surgery and preoperative and postoperative treatment); however, local recurrence is excluded.
(iii) The presence of at least one assessable lesion. However, sites treated by radiotherapy are not considered assessable lesions.
(iv) No chemotherapy with anticancer drugs since the diagnosis of metastatic breast cancer.
(v) An age of 20–75 years.
(vi) A performance status of 0–1 according to the Eastern Cooperative Oncology Group scale.
(vii) Either of the following conditions has to be met concerning previous treatment with taxane derivatives (paclitaxel or docetaxel):
  (a) Not administered previously.
  (b) If such drugs have been administered as preoperative or postoperative adjuvant chemotherapy, at least 6 months (168 days, 24 weeks) should have elapsed since the final day of treatment.
(viii) Either of the following conditions has to be met concerning a history of treatment with oral 5-fluorouracil (5-FU) derivatives:
  (a) Not administered previously.
  (b) If such drugs have been administered as preoperative or postoperative adjuvant chemotherapy, at least 6 months (168 days, 24 weeks) have elapsed since the final day of treatment.
(ix) Both of the following conditions have to be met concerning preceding treatment:

(a) Hormone therapy: At least 7 days have elapsed since the final day of drug treatment (irrespective of the details of treatment).
(b) Radiotherapy: At least 14 days have elapsed since the final dose of radiation.

(x) Resistance to hormone therapy is defined as any of the following:
(a) Estrogen receptors or progesterone receptors are negative on examination of the primary lesion or recurrent lesion(s). However, if both the primary lesion and recurrent lesion(s) are examined and the results differ, the results for the recurrent lesion(s) will apply.
(b) Hormone therapy is ineffective after recurrence.
(c) Recurrence occurs during postoperative adjuvant hormone therapy or within 6 months after the final dose.

(xi) All of the following conditions have to be met regarding organ function (within 21 days before registration):
(a) A neutrocyte count (stab cells + segmented cells) of 1500/mm^3 or higher, or a white cell count of 3000/mm^3 or higher.
(b) A platelet count of 100 000/mm^3 or higher.
(c) A total bilirubin concentration of not more than 2.5 times the upper limit of normal at the laboratory where the test was performed.
(d) Aspartate aminotransferase (AST, GOT) and alanine aminotransferase concentrations (ALT, GPT) of not more than 2.5 times the upper limit of normal at the laboratory where the test was performed.
(e) A serum creatinine concentration of not more than the upper limit of normal at the laboratory where the test was performed.

(xii) At least one of the following conditions has to be met for cardiac function:
(a) No cardiac disease: absence of fatigue, palpitations, shortness of breath and anginal pain during daily activities as confirmed by interview.
(b) Cardiac disease is present, but exercise restriction is not required, and the absence of fatigue, palpitations, shortness of breath and anginal pain during daily activities can be confirmed, and is expected to be maintained during treatment.

(xiii) Written informed consent has been obtained directly from the subject.

The requirement of informed consent as in the above inclusion criteria is not applied to participation-refusers.

Exclusion Criteria

(i) Women who are pregnant, nursing infants or intend to become pregnant.
(ii) Overexpression of HER2 (Her2/neu, Erb B2), or the results of fluorescence *in situ* hybridization are positive.
(iii) A past history of hypersensitivity to the protocol-treatment drugs or their solvents.
(iv) The presence of other active cancers (synchronous double cancers or metachronous double cancers with a disease-free interval of 5 years or less).
(v) The presence of brain metastasis requiring treatment because of increased intracranial pressure or emergency brain irradiation.
(vi) The presence of extensive liver metastasis or lymphatic pulmonary metastasis associated with dyspnea.
(vii) The presence of only one assessable lesion located at a previously irradiated site.
(viii) The presence of pleural effusion, ascites or pericardial effusion requiring emergency treatment.
(ix) Concurrent active infections.
(x) The presence of interstitial pneumonia or pulmonary fibrosis.
(xi) Positive test results for HBs antigen.
(xii) Patients with diabetes mellitus that is poorly controlled or being treated with insulin.
(xiii) Participation in the study is precluded by mental disease or psychological symptoms.
(xiv) Other reasons that preclude participation in the study as judged by the investigator.

TREATMENT

Taxane Arm

For the taxane arm, one of the three regimens described below will be selected. Before the start of treatment, the treatment regimen will be selected at the discretion of the investigator. The same regimen will be used for the duration of first-line treatment. The reason for selecting the regimen will be reported in the 'Follow-up Report'.

(i) Docetaxel 60–75 mg/m^2 administered at 3- or 4-week intervals. Treatment will be repeated until tumor progression or for at least six courses (18 or 24 weeks).
(ii) Paclitaxel 175 mg/m^2 administered at 3- or 4-week intervals. Treatment will be repeated until tumor progression or for at least six courses (18 or 24 weeks).
(iii) Paclitaxel 80–100 mg/m^2 administered every week. Weekly treatment for three consecutive weeks, followed by a 1-week rest period will comprise one course. Treatment will be repeated until tumor progression or for at least six courses (24 weeks).

Anthracycline Arm

One of the following regimens will be selected at the discretion of the attending physician, and treatment will be repeated until disease progression or for at least six courses.

(i) Doxorubicin 40–60 mg/m^2 + Cyclophosphamide 400–600 mg/m^2 given at 3- or 4-week intervals.
(ii) Epirubicin 60–90 mg/m^2 + Cyclophosphamide 400–600 mg/m^2 given at 3- or 4-week intervals.

(iii) Fluorouracil 500 mg/m^2 + Doxorubicin 40–50 mg/m^2 + Cyclophosphamide 500 mg/m^2 given at 3- or 4-week intervals.
(iv) Fluorouracil 500 mg/m^2 + Doxorubicin 60–100 mg/m^2.

Cyclophosphamide 500 mg/m^2 + given at 3- or 4-week intervals.

TS-1 Arm

TS-1 will be administered orally in doses of 40–60 mg twice daily for 28 consecutive days. The dose will be assigned according to body weight. Treatment will be followed by a 14-day rest period to complete one course. Treatment will be repeated until tumor progression or for at least four courses (24 weeks).

Participating doctors must send each patient's information to the CSPOR Data Center for registering patients to this study. Detailed information, for example, why patients refused to participate in the original clinical trial 'SELECT-BC, SELECT-BC CONFIRM', will be gathered afterward with a submitted Case Report Form. This study has been ongoing completely in parallel with the original prospective study.

Treatment methods for participation-refusers are not defined.

PERIOD OF THIS STUDY

This study ends when both SELECT BC and SELECT BC-CONFIRM trials end.

Statistical Analysis

Ratios of participants to participation-refusers in this study are assumed to fall between 1 to 2 and 2 to 1. Two groups of participants and refusers are examined by the Kaplan–Meier method for cumulative survival rates. In addition, the log–log plot is applied to Kaplan–Meier curves of both groups to confirm inter-group proportionality of hazards.

In both SELECT BC and SELECT BC-CONFIRM trials, the following items are used as allocation adjustment factors: (1) institution, (2) the presence or absence of liver metastasis, (3) the presence or absence of hormone sensitivity, (4) administration or non-administration of taxanes, (5) oral administration or non-administration of 5-FU agents and (6) period from surgery to recurrence. If there is a significant difference in these allocation adjustment factors in comparison of the two groups, adjustment is made before the analysis.

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Conflict of interest statement

None declared.

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