Abstract. Endocrine therapy (ET) is one of a number of targeted therapies for estrogen receptor-positive breast cancer (BRCA); however, resistance to ET has become the primary issue affecting treatment outcome. In the present study, a predictive classifier was created using a DNA methylation dataset to identify patients susceptible to endocrine resistance. DNA methylation and RNA sequencing data, and the clinicopathological features of BRCA, were obtained from The Cancer Genome Atlas. Stringent criteria were set to select and classify patients into two groups, namely those resistant to ET (n=11) and sensitive to ET (n=21) groups. Bump hunting analysis revealed that 502 out of 135,418 genomic regions were differentially methylated between these two groups; these regions were differentially methylated regions (DMRs). The majority of the CpG sites contained in the DMRs mapped to the promoter region. Functional enrichment analyses indicated that a total of 562 specific genes encompassing these DMRs were primarily associated with ‘biological progress of organ morphogenesis and development’ and ‘cell-cell adhesion’ gene ontologies. Logistic regression and Pearson’s correlation analysis were conducted to construct a predictive classifier for distinguishing patients resistant or sensitive to ET. The highest areas under the curve and relatively low Akaike information criterion values were associated with a total of 60 DMRs; a risk score retained from this classifier was revealed to be an unfavorable predictor of survival in two additional independent datasets. Furthermore, the majority of genes (55 /63) exhibited a statistically significant association between DNA methylation and mRNA expression (P<0.05). The association between the mRNA expression of a number of genes (namely calcium release activated channel regulator 2A, Schlafen family member 12, chromosome 3 open reading frame 18, zinc finger protein 880, dual oxidase 1, major histocompatibility complex, class II, DP β1, C-terminal binding protein 1, ALG13 UDP-N-acetylglucosaminyltransferase subunit and RAS protein activator like 2) and the prognosis of patients with estrogen receptor-positive BRCA and ET resistance was determined using Kaplan-Meier Plotter. In summary, the predictive classifier proposed in the present study may aid the identification of patients sensitive or resistant to ET, and numerous genes maybe potential therapeutic targets to delay the development of resistance to ET.

Introduction

Breast cancer (BRCA) is the most common type of cancer in women globally (1) and is characterized by notable heterogeneity (2). The expression levels of estrogen and progesterone receptors (ER/PR) and human epidermal growth factor receptor (HER2) have been investigated to further classify BRCA into numerous subtypes: Luminal (ER+/PR+), HER2-positive (ER-/PR-/HER2+), basal-like or triple negative (ER-/PR-/HER2-), claudin-low and normal-like BRCA (3). Based on these guidelines (4), ~70% of patients with BRCA may be classified as the luminal subtype (5). Endocrine therapy (ET), one of the crucial adjuvant treatments for luminal BRCA, suppresses tumor growth by targeting the ER signaling pathways. Unfortunately, >30% of ER-positive tumor types are intrinsically endocrine-resistant at diagnosis; ~40% of breast tumor types that initially respond to ET eventually acquire resistance (6). Additionally, the clinical characteristics of BRCA may be notably heterogeneous even when similar expression levels of ER are observed (7).

ET resistance in ER-positive tumor cells may be ascribed to a variety of factors, including the post-transcriptional modifications of ERs (8,9) or activation of the ER-independent growth factor signaling pathways (10); however, these results have not been further investigated for the effective clinical treatment of BRCA (11,12). Upon metastasis, surgical intervention and present second-line therapeutic strategies have

Dysregulation of DNA methylation patterns may identify patients with breast cancer resistant to endocrine therapy: A predictive classifier based on differentially methylated regions

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limited effectiveness. Thus, precisely predicting the prognosis of patients with BRCA and ET resistance is vital for generating the most appropriate individualized treatment.

As ER status alone is inadequate for identifying patients responsive to ET, multi-gene signatures from gene transcripts have been obtained via analyses with Oncotype DX (13) and MammaPrint tests (14). The expression profiles of these biomarkers may substantially aid the prediction of therapeutic outcomes and the selection of adjuvant therapy (3); however, transcriptional expression may be regulated by a variety of factors and appears to be unstable. Additionally, gene transcripts may not reflect marked changes in regulatory mechanisms, including epigenetic alterations, which may result in disease susceptibility. This represents a limitation of current molecular diagnostic tools based on gene expression assays (15).

DNA methylation is a chemical modification of DNA that does not result in alterations in its sequence and may be inherited during cell division. It is well established that notable alterations to the genome-wide DNA methylation landscape may occur in the early stages of cancer initiation and during cancer progression, and throughout the acquisition of drug resistance (16,17). The hypermethylation of tumor suppressor genes or the hypomethylation of oncogenes maybe associated with the development of BRCA (18,19). DNA methylation is an enzymatic process and may be reversed by epigenetic inhibitors (17). Compared with genetic transcription (mRNA), DNA is inherently stable and may be obtained from numerous sources, including tissue, plasma, saliva and urine (20). Therefore, the DNA methylation profile is promising for identifying patients susceptible to ET. In addition, specific epigenotypes have been identified for the characterization and molecular subtyping of BRCA (21-23); however, few studies focusing on the DNA methylome associated with endocrine-resistant BRCA have been conducted. Furthermore, previous studies have revealed that remodeling of the epigenome is associated with the endocrine-resistant cell phenotype (24,25). Thus, DNA methylation signatures may serve as predictive biomarkers to identify ET-responsive patients with BRCA.

DNA methylation levels maybe simultaneously determined via microarray analyses of numerous CpGs. An increasing number of genome-wide DNA methylation profiles of various types of cancer are available from public databases (26). Previous studies have revealed the importance of methylation in genomic regions compared with that at a single CpG island (27,28). A genome-wide bump hunting approach, introduced by Jaffe et al. (29), was originally designed to identify differentially methylated regions (DMRs) detected on numerous microarray platforms, including the Infinium HumanMethylation450 BeadArray (HM450 array). This approach was demonstrated to effectively model expression profiles without measurement errors, remove batch effects and detect regions of interest. The present study aimed to identify a novel predictive classifier of BRCA by applying the bump hunting method and logistic regression to The Cancer Genome Atlas (TCGA) BRCA datasets on the basis of the DNA methylation profile of BRCA. The results of the present study may aid the identification of patients susceptible to endocrine resistance.

Materials and methods

Data downloading and processing. The DNA methylation profiles associated with BRCA were determined using an HM450 array; the corresponding RNA sequencing data (IlluminaHiSeq_RNASeqV2 arrays; measured using RSEM software; version 1.2.31) (30) and detailed clinicopathological features, including ET information, were downloaded from TCGA (accessed: January 2016; known as the Genomic Data Common Data Portal; https://portal.gdc.cancer.gov/); there were a total of 885 and 1,213 tumor/adjacent tissues with DNA methylation and RNA sequencing data, respectively. Among them, 787 tumor/adjacent tissues possessed both DNA methylation and RNA sequencing data. These samples were used to examine the association between DNA methylation and mRNA expression for the DMRs included in the predictive classifier. Additionally, two DNA methylation datasets based on HM450 array analysis, namely GSE75067 (31) and GSE72251 (32) from the Gene Expression Omnibus database (33) (http://www.ncbi.nlm.nih.gov/geo/), were used as independent datasets to assess the predictive potential of DNA methylation as a classifier of endocrine resistance.

Patient enrollment. In order to investigate the DNA methylation patterns associated with sensitivity to ET, inclusion criteria for patient enrollment were set. In the present study, all patients were: i) Diagnosed with BRCA; ii) female and ≤75-year-old; iii) positive for tumor types of tumor, node and metastasis (TNM) stage <4 (34,35) and ERα expression; and iv) treated with ET. Consequently, of the 1,097 patients with BRCA, 404 patients were selected. Of these patients, those with disease free survival (DFS) ≤30 months were regarded to resist ET and were defined as the resistant to ET (RTE) group. Those with DFS >100 months were classified as the sensitive to ET (STE) group; patients without DNA methylation data were excluded. Furthermore, there were 11 and 21 patients in the RTE and STE groups, respectively; the data of these patients were included for the predictive classifier building. Either a Fisher’s exact test or a Student’s t-test were conducted to determine differences of clinicopathological features between these two groups.

Model construction and selection. The level 3 DNA methylation β-value of BRCA from TCGA was defined as the percentage of DNA methylation in the tissue samples at each CpG probe; the methylation ranged from 0.0 (unmethylated) to 1.0 (fully methylated). In the present study, the M-value \[\text{logit}(\beta)\] was used instead of the β-value to calculate the test statistics; however, for ease of interpretation, the β-value was employed to report differences in methylation levels between the groups. The association between M- and β-values were determined as follows: M-value = \[\logit(\beta) + \logit(1 - \beta)\].

For the identification of DMRs, CpG sites that did not target specific genes and sites without data in any patients were excluded. Additionally, differential expression analysis was conducted to reveal the DMRs between the RTE and SET groups with the R-package ‘bumphunter’ (version 1.10.0) (29). This package was used to determine regions of methylation that deviated from the baseline values. DMRs were defined as genomic regions of differential methylation between
two populations with a P-value <0.001 and covering ≥3 CpG sites. The diagnostic potential of these DMRs was further investigated by producing receiver operating characteristic curves (ROCs) and calculating the area under the curve (AUC). The median β-value across the CpG sites in each DMR (median) was calculated and the difference of median between the two groups (d) was determined. Finally, DMRs with an AUC ≥0.6 and |d|>0.2 were included to build the predictive classifier, and were ranked in an ascending order of P-values obtained from the bumphunter analysis.

In the first step of the classifier building, 3 DMRs per analysis were added into the predictive classifier. The mean \( m_r \) of each DMR across all patients in the STE group, representing relatively normal DNA methylation, was calculated as \( m_r \). In the second step, a Pearson's correlation coefficient was calculated between the \( m_r \) and \( m_e \) of each patient, labeled as the \( r_P \) value (-1 to 1). A positive \( r_P \) value represented the expression profile, indicating an association with patients in the STE group; otherwise, the expression profile of patients was considered to be less associated with the STE group. In step three, the effect of \( r_P \) values on the prediction of patients with ER-positive BRCA resistant to ET was evaluated via the logistic regression analysis; the predictive classifier was then generated. Simultaneously, ROCs in addition to the AUC were used to assess the diagnostic potential of the predictive classifier. A 95% confidence interval (CI) of the AUC was calculated according to the order of the observed AUC values among 1,000 permutations. Finally, for the model selection, the Akaike information criterion (AIC) was used to determine the goodness of fit and the simplicity of the classifier. As aforementioned, 3 DMRs were included each time and the process (steps 1-3) was repeated until all 80 DMRs were included. A risk score (RS) was then able to be calculated based on the final classifier.

**Model validation.** As public datasets with both DNA methylation (determined via an HM450 array) and treatment information were unavailable, patients with ER-positive BRCA without explicit information regarding ET were used. The independent datasets, GSE75067 and GSE72251, which determined DNA methylation in 87 and 70 patients with ER-positive BRCA, respectively, were used as external validations of the predictive classifier. An RS was assigned to each patient; Kaplan-Meier (KM) survival analysis was then performed to investigate the association between RS and cumulative rates of overall survival (OS) and invasive disease-free survival (IDFS). As for GSE75067, univariate and multivariate analyses using Cox regressions were also performed to screen out the independent factors affecting OS.

**Identification of the function of genes included in DMRs.** Functional enrichment analysis was performed using the R-package ‘clusterProfiler’ (version 3.6.0) (36) to investigate the well-known database, Gene Ontology (GO; http://geneontology.org/) (37,38). The specific genes, which were mapped by DMR analysis, were annotated with GO ‘biological process’ (BP), ‘molecular function’ (MF) and ‘cellular component’ (CC) terms. GO terms of \( P<0.01 \) and \( P_s<0.05 \) obtained via the Benjamini and Hochberg method (39) were considered to be statistically enriched.

**Correlation between DNA methylation and mRNA transcripts.** Pearson's correlation coefficients were further calculated to reveal the correlation between DNA methylation and mRNA expression. Since the sample sizes of the groups were limited, the data of 787 tumor/adjacent tissues in the TCGA dataset were employed for the correlation analysis. Since one DMR could contain several CpG sites mapped to one specific gene, several Pearson's correlation analyses were conducted separately to examine the associations between these CpG sites and the mRNA level of the gene. The CpG site in a specific DMR with the lowest P-value was demonstrated to exhibit the strongest correlation with the mRNA expression, and presented.

**Effects of numerous specific genes on relapse-free survival (RFS).** KM Plotter (http://kmplot.com/analysis/) (40,41), a tool containing the gene expression and survival data of >4,000 patients with BRCA, was used to perform KM survival analyses to further assess the association between mRNA expression and RFS. Patients with ER-positive BRCA and ET were selected, and divided into the high and low expression groups based on the median expression levels of each specific gene. Subsequently, survival curves were created and log-rank tests were conducted.

**Results**

**Identification of DMRs associated with the response to ET in patients with ER-positive BRCA.** As presented in Fig. 1A, patients with BRCA meeting the inclusion criteria were divided into two groups according to their DFS, namely the RTE and STE groups. Detailed clinicopathological and treatment information of these 32 patients were presented in Table I. Of them, five patients had received tamoxifen, while 14 patients had been treated with aromatase inhibitors (anastrozole, letrozole and aromasin). The remaining patients were treated with one type of these drugs for a period of time, and subsequently treated with another type. A Fisher's exact test and a Student's t-test were conducted to determine differences between these two groups. No statistically significant differences in TNM stage and receptors status were observed between the two groups. In the RTE group, the tumor types of eight patients (8/11, 72.7%) were TNM stage II and three (3/11, 27.3%) were TNM stage III; however, in the STE group, five (5/21, 23.8%), 12 (12/21, 57.1%) and four (4/21, 19.0%) tumor types were classified as TNM I, II and III stages (P=0.205), respectively. As for receptor status, nine tumor types (9/11, 81.8%) from the RTE group and 16 (16/21, 76.2%) tumor types from the STE group were PR-positive (P=1.000). In addition, only two tumor types (2/11, 18.2%) from the RTE group and two tumor types (2/21, 9.5%) from the STE group expressed HER-2 (P=0.738). The mean age of the patients in the RTE groups was slightly higher compared with that of the patients in the STE group; no statistical significance was observed (P=0.235). Based on the aforementioned results, the clinicopathological data of these two groups were comparable.

Aberrant methylation profiles of DMRs were identified between the RTE and STE groups (Fig. 1B). Genomic locations were grouped into clusters (regions) based on a maximum distance of 500 base pairs (bp); of the 135,418 genomic
regions, 502 regions, including 5,252 CpG sites were significantly differently methylated (P<0.001) and had ≥3 individual CpG sites (Fig. 2A). Due to the limited sample sizes of these two groups, multiple testing correction was not conducted. Of the 502 DMRs, the median size was 803 bp, with a range of 41-3,509 bp; the median number of CpGs in each DMR was 10, with a range of 3 -32. According to the annotation files, the majority of the 5,252 CpG sites were mapped to the promoter region (TSS1500 and TSS200), the gene body and the 5'-untranslated region (UTR) and 3'-UTR, in addition to the first exon (Fig. 2B). Of note, each CpG site may simultaneously be detected in the promoter and other regions due to the various transcripts of a specific gene. These 502 DMRs encompassed 562 specific genes, and genes in the top ranked 20 DMRs were calcium release activated channel regulator 2A (EFCAB4B), paraoxonase 3, homeobox C4 (HOXC4), podoplanin (PDPN), major histocompatibility complex, class II, DQ β2, haptoglobin-like protein (HPHL), transcription factor, major histocompatibility complex, class I, J (proliferation/terminal differentiation) containing 1 antisense, pseudogene, proline rich transmembrane protein 1/palmitoyl-protein thioesterase 2, family with sequence similarity 24 member B/chromosome 10 open reading frame 88 pseudogene, achaete-scute family bHLH transcription factor 2, histone cluster 1 H4 family member L/histone cluster 1 H3 family member I, tenascin XB, EYA transcriptional coactivator and phosphatase 4, lymphoid enhancer factor 1, epithelial stromal interaction 1 (EPSTI1), dimethylarginine dimethylaminohydrolase 2, GATA-binding protein 5, heparan sulfate-glucosamine 3-sulfotransferase 1, major histocompatibility complex, class II, DOα and homeobox C8 (HOXC8) (genes in the top ranked 50 DMRs were listed in Table II). Functional enrichment analysis was performed on these genes. Consequently, 48 BP, 8 MF and only one CC GO terms were significantly enriched with a P-value <0.01 and a false discovery rate q-value <0.05 (Fig. 2C-E). The top-ranked representative 10 BP GO terms were ‘skeletal system development’ (P=6.24x10^{-7}), ‘cell-cell adhesion via plasma-membrane adhesion molecules’ (P=1.86x10^{-5}), ‘homophilic cell adhesion via plasma membrane adhesion molecules’ (P=9.51x10^{-2}), ‘pattern specification process’ (P=7.88x10^{-7}), ‘embryonic organ morphogenesis’ (P=1.34x10^{-4}), ‘transcription factor activity, RNA polymerase II core promoter proximal region sequence-specific binding’ (P=3.85x10^{-5}), ‘glucuronosyltransferase activity’ (P=1.38x10^{-7}), ‘retinoid binding’ (P=1.21x10^{-4}), ‘retinoic acid binding’ (P=2.77x10^{-4}) and ‘oxidoreductase activity and acting on NAD(P)H, oxygen

Figure 1. Flow chart of patient selection and model construction. (A) Publicly available DNA methylation, RNA sequence and clinicopathological features of BRCA were downloaded from The Cancer Genome Atlas. Stringent criteria were set to select and classify patients into two groups, namely the RTE (n=11) group and the STE (n=21) group. (B) Bump hunting was used to identify DMRs, which was used to construct a predictive classifier. The final classifier had 60 DMRs and was validated in an independent dataset (GSE75067). BRCA, breast cancer; TNM, Tumor Node Metastasis; ERs, estrogen receptor α; RTE, resistant to endocrine therapy; STE, sensitive to endocrine therapy; ROC, receiver operating characteristic; AUC, area under the curve; DMR, differentially methylated regions; ET, endocrine therapy.
Table I. Clinicopathological features of patients with breast cancer from The Cancer Genome Atlas included in the present study.

| Patient ID     | Age (year) | TNM stage | DFS (month) | Relapse/death | ER | PR | HER2 | Group | Drug name                                 | Regimen indication | Radiation therapy | History of neoadjuvant treatment |
|----------------|------------|-----------|-------------|---------------|----|----|------|-------|------------------------------------------|--------------------|-------------------|---------------------|
| TCGA-A2-A0YCY  | 59         | 2         | 26.2        | Yes           | Pos | Pos | Neg  | RTE   | Arimidex                                | Adjuvant           | -                 | No                  |
| TCGA-A7-A13E   | 62         | 2         | 18.3        | Yes           | Pos | Neg | Neg  | RTE   | Arimidex, Anastrozole                    | Adjuvant           | -                 | No                  |
| TCGA-A7-A13H   | 61         | 2         | 26.1        | Yes           | Pos | Pos | Neg  | RTE   | Anastrozole                              | Adjuvant           | -                 | No                  |
| TCGA-A7-A26H   | 72         | 2         | 10.2        | Yes           | Pos | Neg | Pos  | RTE   | Anastrozole                              | Adjuvant           | -                 | No                  |
| TCGA-A7-A425   | 70         | 3         | 14.6        | Yes           | Pos | Pos | Neg  | RTE   | Arimidex                                | -                   | Yes               | No                  |
| TCGA-A0-A0JA   | 36         | 3         | 4.7         | Yes           | Pos | Pos | Neg  | RTE   | Tamoxifen                               | Recurrence         | -                 | No                  |
| TCGA-A0-A126   | 39         | 2         | 8.8         | Yes           | Pos | Pos | Neg  | RTE   | Tamoxifen                               | Recurrence         | -                 | No                  |
| TCGA-E2-A10B   | 67         | 2         | 6.4         | Yes           | Pos | Pos | Neg  | RTE   | Arimidex                                | Adjuvant           | -                 | No                  |
| TCGA-E9-A1N6   | 52         | 2         | 22.1        | Yes           | Pos | Pos | Pos  | RTE   | Tamoxifen, Letrozole                     | -                   | -                 | No                  |
| TCGA-E9-A1NF   | 60         | 2         | 3.0         | Yes           | Pos | Pos | Neg  | RTE   | Tamoxifen                               | -                   | -                 | No                  |
| TCGA-LQ-A4E4   | 73         | 3         | 22.4        | Yes           | Pos | Pos | Neg  | RTE   | Anastrozole                              | -                   | Yes               | No                  |
| TCGA-A2-A04R   | 36         | 1         | 121.9       | No             | Pos | Pos | Pos  | STE   | Tamoxifen, Anastrozole                   | Adjuvant           | -                 | No                  |
| TCGA-A2-A0CR   | 54         | 2         | 107.9       | No             | Pos | Pos | Neg  | STE   | Tamoxifen, Anastrozole                   | Adjuvant           | -                 | No                  |
| TCGA-A2-A26A   | 70         | 2         | 134.3       | No             | Pos | Pos | Neg  | STE   | Anastrozole, Tamoxifen                   | Adjuvant           | -                 | No                  |
| TCGA-A2-A0EP   | 56         | 1         | 118.4       | No             | Pos | Neg | Neg  | STE   | Tamoxifen                               | Adjuvant           | -                 | No                  |
| TCGA-A2-A25A   | 44         | 2         | 107.6       | No             | Pos | Pos | Neg  | STE   | Tamoxifen, Aromasin                     | Adjuvant           | -                 | No                  |
| TCGA-AO-A125   | 72         | 2         | 113.5       | No             | Pos | Pos | Neg  | STE   | Tamoxifen, Aromasin                     | Adjuvant           | -                 | No                  |
| TCGA-AO-A04L   | 48         | 2         | 130.0       | No             | Pos | Neg | Pos  | STE   | Tamoxifen                               | Adjuvant           | -                 | No                  |
| TCGA-AR-A0TT   | 53         | 3         | 108.9       | No             | Pos | Neg | Neg  | STE   | Tamoxifen, Anastrozole                   | Adjuvant           | -                 | No                  |
| TCGA-AR-A0U3   | 59         | 2         | 134.0       | No             | Pos | Pos | Neg  | STE   | Anastrozole                             | Adjuvant           | -                 | No                  |
| TCGA-AR-A1AK   | 70         | 1         | 103.8       | No             | Pos | Pos | Neg  | STE   | Anastrozole                             | Adjuvant           | -                 | No                  |
| TCGA-AR-A24H   | 65         | 2         | 160.8       | No             | Pos | Pos | Neg  | STE   | Anastrozole, Tamoxifen                   | Adjuvant           | -                 | No                  |
| TCGA-AR-A24M   | 38         | 3         | 120.2       | No             | Pos | Neg | Pos  | STE   | Letrozole, Tamoxifen                     | Adjuvant           | -                 | No                  |
| TCGA-AR-A24Q   | 49         | 2         | 104.2       | No             | Pos | Neg | Neg  | STE   | Anastrozole                             | Adjuvant           | -                 | No                  |
| TCGA-AR-A24R   | 45         | 3         | 112.7       | No             | Pos | Pos | Neg  | STE   | Letrozole, Tamoxifen                     | Adjuvant           | -                 | No                  |
| TCGA-AR-A24T   | 46         | 3         | 105.2       | No             | Pos | Pos | Neg  | STE   | Letrozole, Tamoxifen                     | Adjuvant           | -                 | No                  |
| TCGA-AR-A24V   | 52         | 2         | 105.2       | No             | Pos | Neg | Pos  | STE   | Tamoxifen, Anastrozole                   | -                   | -                 | No                  |
| TCGA-AR-A2LE   | 69         | 1         | 138.1       | Yes            | Pos | Neg | -    | STE   | Tamoxifen                               | -                   | -                 | No                  |
| TCGA-GM-A2DA   | 46         | 2         | 214.7       | Yes            | Pos | Pos | Pos  | STE   | Tamoxifen, Aromasin, Letrozole           | -                   | -                 | No                  |
| TCGA-GM-A2DL   | 50         | 1         | 115.6       | No             | Pos | Pos | Neg  | STE   | Tamoxifen, Arimidex                      | -                   | -                 | No                  |
| TCGA-GM-A2DM   | 57         | 2         | 106.0       | No             | Pos | Pos | Neg  | STE   | Arimidex                                | -                   | -                 | No                  |
| TCGA-GM-A2DN   | 58         | 2         | 101.5       | No             | Pos | Pos | Neg  | STE   | Arimidex                                | -                   | -                 | No                  |

TNM stage was determined according to the staging system of American Joint Committee on Cancer, either version 5th, 6th or 7th (34,35). Pos, positive; neg, negative; RTE, resistant to endocrine therapy; STE, sensitive to endocrine therapy; DFS, disease free survival; ER, estrogen receptor status determined by immunohistochemistry; PR, progesterone receptor status determined by immunohistochemistry; HER2, human epidermal growth factor receptor 2 status determined by immunohistochemistry.
as acceptor’ (P=3.85x10^{-5}). The only significant CC GO term was ‘transcription factor complex’ (P=1.31x10^{-5}).

Classifier building for predicting the response to ET in patients with ER-positive BRCA based on DMR patterns. Following the process presented in Fig. 1B, 502 DMRs were identified, of which 457 DMRs had an AUC ≥0.6. Finally, 80 DMRs remained to build the predictive classifier set (with a difference of mS between the RTE and STE groups >0.2).

As presented in Fig. 3A, the logistic regression, including 60 DMRs, had the highest AUC to distinguish the RTE group from the STE group, and a relatively low AIC to indicate the goodness of fit and the simplicity of the classifier. Therefore, these 60 DMRs listed in Table IV were included. Among them, 31 DMRs, including EFCAB4B, secretoglobin family 3A member 1 (SCGB3A1) and dual oxidase maturation factor 1 (DUOXA1), were hypermethylated in the RTE group, and the remaining DMRs, including tripartite motif containing 58 and ELF5, were hypomethylated in the RTE group.

Unsupervised hierarchical clustering analysis of these patients in the RTE and STE groups was performed based on these 60 DMRs. As presented in Fig. 3B, patients were divided into two classes, as follows: Class 1 was mainly enriched for patients in the STE group (20/21), while class 2 comprised 10 patients in the RTE group and one in the STE group. In total, only two samples (2/32, 6.25%) were incorrectly sorted into the wrong group.

Based on the aforementioned 60 DMRs, an equation based on the aforementioned logistic regression, ln(p/1-p)=7.207-12.610x, was generated to evaluate the probability of resistance to ET in patients with ER-positive BRCA. The denoting of letters in the equation is as follows: p, the probability of resistance to ET, and x, rP values between mR and mS across 60 DMRs in each patient. Thus, the RS was generated and described as follows: RS=e^{7.207-12.610x}/(1+e^{7.207-12.610x}).

Application of RS on the prognosis of patients with ER-positive BRCA in two additional independent test datasets. The GSE75067 dataset containing the data of 87 patients with ER-positive BRCA was used instead to externally analyze the prognostic power of the RS on BRCA. The RS of each ER-positive patient was calculated; the median RS among these patients was 0.161, with a range of 0.051-0.982. As observed in Fig. 3C and D, whether the cutoff value of RS was 0.2 or 0.5, patients with higher RS values tended to exhibit significantly shorter OS times compared with those with lower RS values (log-rank test, P=0.042 and P<0.001, respectively). Furthermore, Cox regression analysis...
Table II. DMRs between patients resistant and sensitive to endocrine therapy.

| Bumps (no.) | Start coordinate of DMR | End coordinate of DMR | Chr of DMR | Gene(s) in DMR | P-value |
|-------------|-------------------------|-----------------------|------------|----------------|---------|
| 23474       | 3862221                 | 3862597               | 12         | EFCAB4B        | 1.28x10^-5 |
| 95719       | 95025194                | 95026937              | 7          | PON3           | 1.46x10^-5 |
| 25363       | 54446100                | 54448913              | 12         | HOXC4          | 2.21x10^-5 |
| 2013        | 13909161                | 13910796              | 1          | PDPN           | 2.35x10^-5 |
| 88062       | 32729174                | 32730299              | 6          | HLA-DQB2       | 3.60x10^-5 |
| 81042       | 185938933               | 185941625             | 4          | HELT           | 4.27x10^-5 |
| 87538       | 29974022                | 29975078              | 6          | HLA-A:NCRNA00171 | 4.52x10^-5 |
| 87958       | 32119616                | 32121249              | 6          | PRRT1;PPT2     | 4.96x10^-5 |
| 15619       | 124638756               | 124639892             | 10         | FAM24B;LOC399815 | 5.27x10^-5 |
| 16983       | 2291347                 | 2292905               | 11         | ASCL2          | 6.87x10^-5 |
| 87359       | 27840957                | 27842098              | 6          | HIST1H4L;HIST1H3I | 8.00x10^-5 |
| 87946       | 32063774                | 32064749              | 6          | TNXB           | 8.77x10^-5 |
| 90707       | 133561614               | 133562196             | 6          | EYA4           | 9.02x10^-5 |
| 87773       | 31539601                | 31540750              | 6          | LTA            | 9.29x10^-5 |
| 29316       | 43565901                | 43566902              | 13         | EPST1          | 9.85x10^-5 |
| 87830       | 31695903                | 31697276              | 6          | DDAH2          | 9.90x10^-5 |
| 66889       | 61050560                | 61051561              | 20         | GATA5          | 1.00x10^-4 |
| 77952       | 11430022                | 11431359              | 4          | HS3ST1         | 1.03x10^-4 |
| 88100       | 32975875                | 32978129              | 6          | HLA-DOA        | 1.27x10^-4 |
| 25345       | 54402431                | 54403314              | 12         | HOXC8          | 1.24x10^-4 |
| 12810       | 50969997                | 50970591              | 10         | OGDHL          | 1.71x10^-4 |
| 17099       | 2890019                 | 2891118               | 11         | KCNQ1DN        | 1.74x10^-4 |
| 84428       | 140305713               | 140306458             | 5          | PCDHAC1; PCDHA7; PCDHAC1; PCDHA12; PCDHA6; PCDHA10; PCDHA4; PCDHA11; PCDHA8; PCDHA1; PCDHA2; PCDHA9; PCDHA1; PCDHAC1; PCDHA13; PCDHA5; PCDHA3; PCDHA10 | 1.80x10^-4 |
| 16989       | 2397201                 | 2397977               | 11         | CD81           | 1.80x10^-4 |
| 36576       | 72667883                | 72669149              | 15         | HEXA; C15orf34 | 1.99x10^-4 |
| 84434       | 140345966               | 140346403             | 5          | PCDHAC2; PCDHA7; PCDHA12; PCDHA6; PCDHA10; PCDHA4; PCDHA11; PCDHA8; PCDHA1; PCDHA2; PCDHA9; PCDHA1; PCDHAC1; PCDHA13; PCDHA5; PCDHA3; PCDHAC2; PCDHA10 | 2.01x10^-4 |
| 23388       | 2800055                 | 2801584               | 12         | CACNA1C        | 2.03x10^-4 |
| 16985       | 2321770                 | 2323059               | 11         | C11orf21; TSPAN32 | 2.09x10^-4 |
| 46839       | 46655164                | 46656543              | 17         | HOXB4          | 2.52x10^-4 |
| 5778        | 92951355                | 92952268              | 1          | GFI1           | 2.56x10^-4 |
| 83500       | 112073348               | 112073769             | 5          | APC            | 2.70x10^-4 |
| 26639       | 103351180               | 103352454             | 12         | ASCL1          | 2.76x10^-4 |
| 109271      | 153236083               | 153238579             | X          | HCFC1; TMEM187 | 2.81x10^-4 |
| 78985       | 76555547                | 76556042              | 4          | CDKL2          | 2.94x10^-4 |
| 42578       | 88717134                | 88717989              | 16         | CYBA           | 3.13x10^-4 |
| 107070      | 16729564                | 16731095              | X          | CTPS2          | 3.16x10^-4 |
| 80825       | 174449827               | 174451468             | 4          | HAND2; NBLA00301 | 3.34x10^-4 |
indicated that, following the adjustment for age, tumor type, lymph node status and PR expression, RS was an independent predictor for OS in patients with ER-positive BRCA (hazard ratio: 2.551; 95% confidence interval, 1.048-6.206; P=0.039; Table V).

Additionally, the GSE72251 dataset containing the data of 70 patients with tumor types expressing ER was used for further validation; the median RS among these patients was 0.227, with a range of 0.053-0.957. As presented in Fig. 3E, patients with RS≤0.2 tended to have a better OS times, but statistical significance was not observed (log-rank test, P=0.717); however, the OS times of patients with RS ≤0.5 were significantly longer compared with that of their counterparts with RS >0.5 (log-rank test, P=0.006; Fig. 3F). IDFS data were available in this dataset and were also analyzed. As presented in Fig. 3G, no significant differences in IDFS were observed between the two groups (patients with BRCA and RS >0.2 compared with those with RS ≤0.2); however, patients assigned a higher RS exhibited a significantly longer IDFS compared with those with a lower RS when the cutoff of RS was set as 0.5 (P=0.009; Fig. 3H).

Correlation between DNA methylation and mRNA expression in DMRs included in the predictive classifier. Of the 60 DMRs, 63 specific genes were encompassed, and Pearson's correlation coefficients were determined to reveal the effects of epigenetic regulation. The CpG site in one specific DMR exhibiting the strongest correlation with mRNA expression was identified. The majority of the genes (55/63) had a statistically significant correlation between DNA methylation and mRNA expression with a P-value <0.05; 17 genes had an r-value ≤−0.3 (Table IV). Numerous representative DMRs and their correlation with mRNA expression in specific genes were presented in Fig. 4. Of these genes, EFCAB4B (r=1.28x10^-1), Schlafen family member 12 (SLFN12; r=5.33x10^-3), chromosome 3 open reading frame 18 (C3orf18; r=5.53x10^-3), zinc finger protein 880 (ZNF880; P=9.37x10^-4), dual oxidase 1 (DUOX1; P=1.08x10^-41) and major histocompatibility complex, class II, DPβ1 (HLA-DPB1; P=2.27x10^-3) were hypermethylated in patients in the RTE group, while ELF5 (P=5.32x10^-4), phospholipase A2 group III (PLA2G3; P=1.63x10^-3), metallothionein 1G (MT1G; P=3.70x10^-3), C-terminal binding protein 1 (CTBP1; P=4.15x10^-3), ALG13 UDP-N-acetylglucosaminyltransferase subunit (ALG13; P=2.92x10^-3) and RAS protein activator like 1 (RASAL1; P=5.93x10^-3) were hypomethylated in patients in the RTE group compared with that in the STE group. Among them, ELF5 (r=-0.594, P=2.53x10^-76) exhibited the strongest negative correlation with mRNA expression, followed by PLA2G3 (r=-0.581, P=3.43x10^-72), C3orf18 (r=-0.567, P=3.93x10^-68), MT1G (r=-0.536, P=7.32x10^-66), SLFN12 (r=-0.533, P=3.98x10^-66), ZNF880 (r=-0.464, P=3.35x10^-45), DUOX1 (r=-0.456, P=1.08x10^-41), EFCAB4B (r=-0.358, P=3.20x10^-25), HLA-DPB1 (r=-0.300, P=8.83x10^-18), RASAL2 (r=-0.279, P=1.67x10^-15), CTBP1 (r=-0.175, P=7.47x10^-7) and ALG13 (r=-0.122, P=6.13x10^-4).

Effects of the mRNA expression levels of numerous genes on the prognosis of patients with ER-positive BRCA and ET. Considering the association between DNA methylation and mRNA expression levels in the majority of the DMRs included in the prediction model, the effects of the mRNA expression levels of numerous genes on the prognosis of patients with ER-positive BRCA and ET were investigated using independent datasets in the KM Plotter tool. The cutoff value of the mRNA expression level for each gene was set as the median. A number of representative genes are presented in Fig. 5. Patients with higher mRNA expression levels of C3orf18 (log-rank P=0.003), ZNF880 (P=0.035), DUOX1 (P=0.013) and HLA-DPB1 (P=0.033) exhibited significantly longer RFS times compared with those with lower mRNA expression levels. Conversely, patients with lower expression levels of RASAL2...
exhibited a significantly longer RFS (P=0.006) compared with those with increased expression levels. Additionally, separate survival curves associated with EFCAB4B (P=0.170), SLFN12 (P=0.063), CTBPI (P=0.170) and ALGI3 (P=0.092) expression were generated; however, statistical significance was not observed.

### Table III. Top-ranked 10 biological process terms enriched by genes included in differentially methylated regions determined through the functional enrichment analysis.

| Description                                      | Gene ratio | Gene ID                                      | P-value          |
|--------------------------------------------------|------------|----------------------------------------------|------------------|
| Homophilic cell adhesion via plasma membrane adhesion molecules | 33/446     | PCDHA7/PCDHA12/PCDHA6/PCDHA10/PCDHA4/PCDHA11/PCDHA8/PCDHA1/PCDHA2/PCDHA9/PCDHA13/PCDHA5/PCDHA3/PCDHA2/GYPC/FAT1/SDK1/IGSF9B/PCDH8/CDH7/PCDHA4/PCDHA6/PCDHA1/A/PCDHA5/PCDHB1/PCDHB4/PCDHA3/PCDHA8/PCDHA2/PCDHA7/PCDHB2/PCDHB3 | 9.51x10^{-21}   |
| Cell-cell adhesion via plasma-membrane adhesion molecules | 33/446     | PCDHA7/PCDHA12/PCDHA6/PCDHA10/PCDHA4/PCDHA11/PCDHA8/PCDHA1/PCDHA2/PCDHA9/PCDHA13/PCDHA5/PCDHA3/PCDHA2/GYPC/FAT1/SDK1/IGSF9B/PCDH8/CDH7/PCDHA4/PCDHA6/PCDHA1/A/PCDHA5/PCDHB1/PCDHB4/PCDHA3/PCDHA8/PCDHA2/PCDHA7/PCDHB2/PCDHB3 | 1.64x10^{-15}   |
| Cell fate commitment                              | 28/446     | ASCL1/EVX1/OLIG1/TBX2/ELF5/TRIM15/SOX2/NKX2-2/NOTCH4/FGFR1/GDF7/FGF10/PROX1/WT1/SOX8/BCL11B/EBF2/PTX1/GS1/GLI3/FGF13/PAX7/NKX2-5/LBX1/GATA3/NR2F2/TGFBI11/GATA2 | 4.35x10^{-11}   |
| Forebrain development                             | 29/446     | ASCL1/CXCL12/SOX2/KCNA1/FGFR1/GDF7/FGF10/NPY/SRD5A2/PROX1/ALK/BCL11B/PTX1/GS1/GLI3/FGF13/DLX5/4/AQP1/DAB2/EP/EGFR/NR2F2/RARB/TACC2/DUOX2/TRAPPC9/GATA2/PTX2/HTR5A/INHBA | 9.95x10^{-8}    |
| Skeletal system development                       | 33/446     | HOXC4/HOXC8/HOBX4/HAND2/HOXD1/HOBX5/HOBX9/HAPLN3/HOXD4/FGFR1/TBX15/SRD5A2/COL11A2/PTX1/HOXC6/HOX5/GLI3/DLX5/COL1A2/PAX7/GNAS/CX1/TLL1/ALPL/RUNX3/RARB/CDKN1C/PTX2/SHOX2/BARX2/BMP8/B/COL2A1/MEIS1 | 6.24x10^{-7}    |
| Gland development                                 | 30/446     | ASCL1/HAND2/TBX2/ELF5/GPX1/HOXD9/SOX2/KALRN/TNF/NOTCH4/FGFR1/GDF7/FGF10/PROX1/WT1/BCL11B/PTX1/GS1/GLI3/BXS/LIM5/SOX2-5/GATA3/EGFR/CDKN1C/DUOX2/GATA2/PTX2/UGT1A1/IRS2 | 6.45x10^{-7}    |
| Embryonic organ development                       | 30/446     | HOXC4/ASCL2/HOBX4/HAND2/TBX2/HOBX5/HOBX9/TNF/HOXD4/FGFR1/CITED1/TBX15/FGF10/VANGL2/PROX1/EN2/GLI3/DLX5/GNAS/NKX2-5/LBX1/GATA3/EGFR/NR2F2/RARB/CDKN1C/GATA2/PTX2/SHOX2/COL2A1 | 7.50x10^{-7}    |
| Pattern specification process                     | 30/446     | HOXC4/HOXC8/HOBX4/ASCL1/HAND2/EVX1/TBX2/HOBX5/HOXD9/NKX2-2/HOXD4/FGFR1/CITED1/IRX4/FGF10/VANGL2/GDNF/WT1/HOXC6/HOX5/GLI3/PAX7/CX1/SYNGAP1/NKX2-5/LBX1/PCHD8/NR2F2/PTX2/BCOR | 7.88x10^{-7}    |
| Embryonic organ morphogenesis                     | 23/446     | HOXC4/HOBX4/HAND2/TBX2/HOBX5/HOXD9/HOXD4/FGFR1/TBX15/FGF10/VANGL2/PROX1/EN2/GLI3/DLX5/GNAS/NKX2-5/LBX1/GATA3/RARB/GATA2/PTX2/SHOX2/COL2A1 | 1.86x10^{-6}    |
| Regulation of hormone levels                      | 28/446     | CACNA1C/TACR1/OPRK1/TRH/KALRN/TNF/FGFR1/DUOX1/NDUOX1/SRD5A2/SOX8/GALR1/P2RY1/GNAS/KCN5S3/CDM3/EGFR/DUOX2/DUOX2/UCN/RAB11/FIP3/UGTI1A1/UGTI1A8/UGTI1A3/UGTI1A9/UGTI1A7/IRS2/INHBA | 5.88x10^{-5}    |
Figure 3. Construction and validation of the predictive classifier. (A) When modeling, 3 DMRs were included into the prediction classifier each time, followed by an assessment of the rs in the STE group and the calculation of the r-value. The effect of the r-values was evaluated using the logistic regression, and AUC and AIC were simultaneously created to assess its diagnostic capacity, the goodness of fit and the simplicity of the classifier. The 95% CI of the AUC were calculated following 1,000 permutations. The left y-axis represented the AIC values, and the right y-axis represented the AUC values. The x-axis indicated the number of DMRs included in the logistic regression. (B) Hierarchical clustering was conducted on the DMRs. Each row of the heat map represented one of the DMRs with each column representing a different sample belonging to the RTE or STE group. RS was retained from each patient in the independent dataset GSE75067 on the basis of the predictive classifier. Kaplan-Meier analyses with log-rank tests were used to assess the effect of RS on OS time. The cutoff value of RS was set to be either (C) 0.2 or (D) 0.5. Similarly, for GSE72251, RS was assigned to each patient and Kaplan-Meier analyses were also used to assess the association between RS and OS, when the cutoff value was set to be (E) 0.2 or (F) 0.5. In addition, survival curves were also created by Kaplan-Meier analyses for invasive disease-free survival time, and the cutoff of RS was set to be (G) 0.2 or (H) 0.5. AIC, Akaike information criterion; AUC, area under the curve; CI, confidence interval; RS, risk score; DMR, differentially methylated regions; RTE, resistant to endocrine therapy; STE, sensitive to endocrine therapy; OS, overall survival.
Table IV. Differentially methylated regions for predicting patients who are resistant to endocrine therapy.

| Bumps (no.) | Gene symbol | r-value | P-value | DNA methylation (resistant/sensitive to endocrine therapy) | Chr | No. of CpG sites | P-values |
|-------------|-------------|---------|---------|-------------------------------------------------------------|-----|-----------------|---------|
| 23474       | EFCAB4B     | -0.358  | 3.20x10^-25 | Up                                                                    | 12  | 12              | 1.28x10^-5 |
| 2013        | PDPN        | -0.210  | 2.94x10^-9  | Up                                                                     | 1   | 17              | 2.35x10^-5 |
| 87359       | HIST1H4L,   | -0.233  | 3.41x10^-11 | Up                                                                     | 6   | 16              | 8.00x10^-5 |
|             | HIST1H3I    | -0.270  | 1.66x10^-14 |                                                                        |
| 29316       | EPSTI1      | -0.581  | 2.89x10^-72 | Up                                                                     | 13  | 14              | 9.85x10^-5 |
| 23388       | CACNA1C     | 0.305   | 1.26x10^-18 |                                                                        |
| 83500       | APC         | 0.075   | 3.57x10^-2  |                                                                        |
| 109271      | HCFC1,      | 0.072   | 4.37x10^-2  | Down                                                                  | X   | 19              | 2.81x10^-1 |
| 87226       | SCGN        | -0.194  | 3.83x10^-8  |                                                                        |
| 11057       | TRIM58      | -0.619  | 1.39x10^-84 |                                                                        |
| 93160       | MIR589,     | -0.255  | 4.23x10^-13 | Down                                                                  | 7   | 12              | 5.07x10^-4 |
|             | FBXL18      |         |          |                                                                        |
| 18397       | ELF5        | -0.594  | 2.53x10^-76 | Down                                                                  | 11  | 13              | 5.32x10^-4 |
| 45490       | SLFN12      | -0.553  | 3.98x10^-64 |                                                                        |
| 72514       | C3orf18     | -0.567  | 3.93x10^-68 |                                                                        |
| 86221       | SCGB3A1     | -0.309  | 7.02x10^-19 |                                                                        |
| 19965       | SIPA1       | -0.260  | 1.13x10^-13 |                                                                        |
| 53071       | ACP5        | -0.222  | 3.18x10^-10 |                                                                        |
| 67311       | MIR155HG    | -0.424  | 1.01x10^-35 |                                                                        |
| 16291       | NNX6-2      | -0.048  | 1.76x10^-1  | Down                                                                  | 10  | 12              | 8.42x10^-4 |
| 56468       | ZNF880      | -0.464  | 3.35x10^-43 |                                                                        |
| 78770       | IGF2BP7     | 0.084   | 1.82x10^-2  | Down                                                                  | 4   | 8               | 1.01x10^-3 |
| 12633       | ALOX5       | -0.189  | 8.55x10^-8  |                                                                        |
| 20753       | PHOX2A      | -0.225  | 1.79x10^-10 | Down                                                                  | 11  | 7               | 1.09x10^-3 |
| 33797       | PPP2R5C     | 0.068   | 5.51x10^-2  |                                                                        |
| 51880       | NFIC        | 0.140   | 8.11x10^-5  |                                                                        |
| 87280       | HIST1H4F    | -0.228  | 1.14x10^-10 |                                                                        |
| 94632       | IKZF1       | -0.265  | 4.47x10^-14 | Down                                                                  | 7   | 6               | 1.25x10^-3 |
| 67598       | CBR1        | -0.621  | 3.20x10^-45 |                                                                        |
| 102219      | ZNF572      | -0.645  | 1.22x10^-93 | Down                                                                  | 8   | 8               | 1.30x10^-3 |
| 35402       | DUOX1,      | -0.456  | 1.08x10^-21 |                                                                        |
|             | DUOXA1      | -0.485  | 1.03x10^-47 |                                                                        |
| 108507      | CHRD1       | 0.120   | 7.29x10^-4  | Down                                                                  | X   | 9               | 1.61x10^-3 |
| 69212       | PLA2G3      | -0.581  | 3.43x10^-72 | Down                                                                  | 22  | 6               | 1.63x10^-3 |
| 23113       | GLB1L3      | -0.175  | 8.25x10^-7  | Down                                                                  | 11  | 7               | 1.89x10^-3 |
| 98905       | VIPR2       | -0.433  | 2.76x10^-37 | Down                                                                  | 7   | 6               | 1.92x10^-3 |
| 28896       | GSX1        | -0.094  | 8.09x10^-5  | Down                                                                  | 13  | 9               | 1.98x10^-3 |
| 88104       | HLA-DPB1    | -0.300  | 8.83x10^-18 | Up                                                                    | 6   | 9               | 2.26x10^-3 |
| 86545       | CDYL        | -0.073  | 4.05x10^-2  |                                                                        |
| 11209       | DIP2C       | -0.194  | 3.95x10^-8  | Down                                                                  | 10  | 15              | 2.50x10^-3 |
| 97788       | FAM115A     | 0.061   | 8.98x10^-2  |                                                                        |
| 84265       | LOC589333   | -0.165  | 3.14x10^-6  |                                                                        |
| 17233       | TRIM68      | -0.573  | 5.39x10^-70 |                                                                        |
| 57826       | KCNS3       | -0.227  | 1.13x10^-10 |                                                                        |
| 30133       | DOCK9       | -0.119  | 7.89x10^-4  | Down                                                                  | 13  | 6               | 2.74x10^-3 |
| 108522      | ALG13       | -0.122  | 6.13x10^-4  | Down                                                                  | X   | 5               | 2.92x10^-3 |
| 7843        | SLAMF1      | -0.270  | 1.33x10^-14 |                                                                        |
| 99079       | ARHGEF10    | -0.030  | 4.00x10^-1  | Down                                                                  | 8   | 4               | 3.47x10^-3 |
| 40848       | MT1G        | -0.536  | 7.32x10^-90 | Down                                                                  | 16  | 6               | 3.70x10^-3 |

*a: r-value for DNA methylation
*b: P-value for DNA methylation
*c: P-values for DNA methylation
Previous studies have indicated that dysregulated DNA methylation is associated with carcinogenesis and therapeutic effectiveness (16,17,42). In the present study, DMRs were identified via the bumphunting analysis, followed by the building of a predictive classifier to identify ET-responsive patients with ER-positive BRCA. The RS was then calculated, which served as an indicator to classify patients with ER-positive BRCA into two groups with distinct survival outcomes in two additional independent datasets.

Functional enrichment analyses demonstrated that genes with DMRs associated with ET sensitivity were associated with organ morphogenesis and development and cell-cell adhesion. The present study mainly reported on two groups of genes, namely the PCDH family and homeobox genes. PCDHs, as part of the cadherin superfamily, were originally identified in the rat brain via polymerase chain reaction analysis and were associated with certain types of neurological disease (43,44). Previously, aberrant PCDH expression was observed in a variety of human malignant tumor types, potentially due to post-translational regulatory mechanisms, including DNA methylation (45). In the present study, it was reported that the methylation status of numerous PCDHs, including PCDHA4, PCDHA7, PCDHA10, PCDH8 and PCDHGA1 may be associated with the resistance to ET. In addition, the tumor suppressor and oncogenic functions of PCDHs have been reported in BRCA (46,47). The effects of certain PCDHs, including PCDH10, have been associated with fulvestrant resistance in BRCA (48). The function of PCDHs is associated with numerous signaling pathways, including the Wnt/β-catenin (49) and receptor tyrosine kinase (50) pathways, which have been proposed to be associated with tamoxifen resistance. The results of

| Bumps (no.) | Gene symbol | r-value | P-value | DNA methylation (resistant/sensitive to endocrine therapy) | Chr | No. of CpG sites | P-values |
|-------------|-------------|---------|---------|----------------------------------------------------------|-----|-----------------|---------|
| 26270       | LIN7A       | -0.291  | 7.61x10^{-17} | Up | 12 | 4 | 4.08x10^{-3} |
| 57453       | TTC15       | 0.195   | 3.45x10^{-8} | Down | 2 | 5 | 4.13x10^{-3} |
| 77073       | CTBP1       | -0.175  | 7.47x10^{-7} | Down | 4 | 6 | 4.15x10^{-3} |
| 42328       | FBXO31      | -0.075  | 3.59x10^{-2} | Up | 16 | 3 | 4.28x10^{-3} |
| 85239       | EBF1        | 0.035   | 3.28x10^{-1} | Down | 5 | 4 | 4.31x10^{-3} |
| 19658       | FERMT3      | 0.102   | 4.24x10^{-3} | Down | 11 | 4 | 4.32x10^{-3} |
| 7847        | CD48        | -0.143  | 5.38x10^{-5} | Up | 1 | 4 | 4.56x10^{-3} |
| 102571      | TRAPPc9     | 0.060   | 9.15x10^{-2} | Down | 8 | 3 | 5.43x10^{-3} |
| 49506       | HEXDC       | 0.078   | 2.90x10^{-2} | Down | 17 | 3 | 5.80x10^{-3} |
| 81036       | ACSL1       | 0.060   | 9.37x10^{-2} | Down | 4 | 3 | 5.89x10^{-3} |
| 8503        | RASAL2      | -0.279  | 1.67x10^{-15} | Down | 1 | 3 | 5.93x10^{-3} |
| 40647       | ZNF423      | 0.384   | 4.02x10^{-20} | Up | 16 | 4 | 6.19x10^{-3} |
| 393         | PRKcz       | -0.065  | 6.77x10^{-2} | Down | 1 | 3 | 6.31x10^{-3} |
| 11176       | DIP2C       | -0.194  | 3.95x10^{-8} | Down | 10 | 4 | 6.67x10^{-3} |

*rPearson’s correlation coefficient between mRNA expression and DNA methylation; *P-values were obtained from Pearson’s correlation analyses; *P-values represented the statistical significance of differentially methylated regions on identifying patients with estrogen receptor-positive breast cancer sensitive to endocrine therapy; Chr, chromosome.

### Table V. Univariate and multivariate Cox regressions in the dataset GSE75067.

| Clinical features                                | Univariate | Multivariate |
|--------------------------------------------------|------------|--------------|
|                                                  | HR (95%CI) | P-value      | HR (95%CI) | P-value |
| Age (years, >50/≤50)                             | 1.286 (0.677, 2.442) | 0.442 | 1.688 (0.800, 3.563) | 0.170 |
| Tumor types (non-ductal/ductal)                  | 0.613 (0.290, 1.294) | 0.199 | 0.528 (0.224, 1.245) | 0.145 |
| Lymph node status (positive/negative)            | 4.199 (2.096, 8.411) | <0.001 | 5.319 (2.440, 11.596) | <0.001 |
| Progesterone receptor expression (positive/negative) | 0.472 (0.215, 1.036) | 0.061 | 1.282 (0.429, 3.835) | 0.656 |
| Risk Score (>0.5/≤0.5)                           | 3.463 (1.742, 6.887) | <0.001 | 2.551 (1.048, 6.206) | 0.039 |

HR, hazard ratio; CI, confidence interval.

### Discussion

Previous studies have indicated that dysregulated DNA methylation is associated with carcinogenesis and therapeutic effectiveness (16,17,42). In the present study, DMRs were identified via the bumphunting analysis, followed by the building of a predictive classifier to identify ET-responsive patients with ER-positive BRCA. The RS was then calculated, which served as an indicator to classify patients with ER-positive BRCA into two groups with distinct survival outcomes in two additional independent datasets.

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the present study suggested a potential association between PCDHs and resistance to ET; however, further investigation is required.

Additionally, the HOX genes encode a family of highly conserved homeodomain-containing transcription factors that serve crucial functions during embryogenesis (51). In BRCA,
the expression of numerous HOX genes has been reported to be up- or downregulated, which may be associated with carcinogenesis, metastasis and tamoxifen resistance (52,53). It was proposed that HOX genes contribute to a major part of DNA methylation profiles in BRCA subtypes (54). In the present study, the methylation patterns of numerous HOX genes, including HOXB4, HOXB5, HOXC4, HOXC8, HOXD1 and HOXD4, were observed to be associated with resistance to ET.

DNA methylation of gene promoters may downregulate transcriptional expression, affecting tumorigenesis or the progression of tumor types (52). Therefore, gene promoters have become the focus of research to investigate DNA methylation. Li et al (55) reported that 3.3% inter-tumor gene expression may be attributed to DNA methylation in gene promoters. The present study revealed that the locations of DMRs were primarily in the gene promoter, namely TSS200 and TSS1500; however, other regions, including the gene body, additionally exhibited a large proportion of methylation, which indicated that other regions containing CpG sites may regulate gene expression, contributing to ET resistance. Li et al (55) revealed that in addition to the gene promoter, other regions may substantially affect inter-tumor gene

Figure 5. Prognostic significance of mRNA expression of a number of specific genes in estrogen receptor-positive patients with BRCA receiving ET. Effect of mRNA expression on the relapse-free survival time of patients with BRCA receiving ET was assessed using the Kaplan Meier plotter. P-values were obtained from Kaplan Meier analysis with a log-rank test. ET, endocrine therapy; BRCA, breast cancer; EFCAB4B, calcium release activated channel regulator 2A; SLFN12, Schlafen family member 12; C3orf18, chromosome 3 open reading frame 18; ZNF880, zinc finger protein 880; DUOX1, dual oxidase 1; HLA-DPB1, major histocompatibility complex, class II, DPβ1; CTBP1, C-terminal binding protein 1; ALG13, ALG13 UDP-N-acetylgalactosaminyltransferase subunit; RASAL2, RAS protein activator like 2.
expression. For instance, enhancer methylation was associated with 4.0% of inter-tumor gene expression variation (56). Compared with a single CpG site, the varied methylation of genomic regions containing a number of CpG sites was more stable. Integrating the bumphunting method and logistic regression, 60 DMRs were reported to have the potential to identify patients with ER-positive BRCA and ET resistance. Due to the inadequate treatment information of the data from TCGA and the stringent criteria set, sample sizes in the present study were limited. Therefore, 11 patients with BRCA possessing a DFS ≤30 months were regarded as exhibiting ET resistance, while 21 patients exhibiting a DFS >100 months were regarded as sensitive to ET (57,58). The limited sample sizes may reduce the comparative power of the identification of DMRs. Therefore, multiple test adjustment was not applied to retain potentially genuine biomarkers. Furthermore, the GSE75067 and GSE72251 datasets lacking treatment information were included to externally validate the model proposed in the present study. The survival curves, particularly when the cutoff value of RS was set as 0.5, demonstrated notable curve separation in patients with ER-positive BRCA. These survival analyses indicated the potential application of RS in the prediction of the prognosis of patients with ER-positive BRCA and suggested its potential for identifying patients resistant to ET; however, cohorts with a large sample size are required to further support this predictive classifier.

Pearson's correlation analyses revealed that the majority of DMRs (46/60) in the predictive model of the present study exhibited a negative correlation with the transcript expression. Numerous genes in this model, including ELF5 (59), CTBP1 (60) and zinc finger protein 423 (ZNF423) (61), have previously been reported to be involved in anti-estrogen resistance. Elevated expression levels of ELF5 were detected in luminal BRCA cells that had acquired resistance to tamoxifen (59). In addition, ELF5 may be a key transcriptional determinant of BRCA molecular subtypes by suppressing estrogen sensitivity in luminal BRCA cells (59). Furthermore, as a corepressor, CTBP1 was reported to be associated with the silencing of ubiquitin-conjugating enzyme E2 D1 and simultaneously elevated cyclin D1 expression levels, which may underlie the mechanism of acquired resistance to 4-hydroxytamoxifen (60). It was demonstrated that ZNF423 may be an estrogen-inducible BRCA1 transcription factor, and may contribute to variations in selective ER modulators in the prevention of BRCA (61). Additionally, a number of other genes, namely APC regulator of WNT signaling pathway, PDPN, EPSTI1, SCGB3A1, signal-induced proliferation-associated 1, acid phosphatase 5, tartrate resistant, MIR155 host gene, insulin like growth factor binding protein 7, arachidonate 5-lipoxygenase, protein phosphatase 2 regulatory subunit Bγ, nuclear factor I C, IKAROS family zinc finger 1, carbonyl reductase 1, DUOXA1, chordin like 1, disco interacting protein 2 homolog C, signaling lymphocytic activation molecule family member 1, Rho guanine nucleotide exchange factor 10, MT1G, lin-7 homolog A, crumb cell polarity complex component, F-box protein 31, EBF transcription factor 1, trafficking protein particle complex 9, acyl-CoA synthetase long chain family member 1, RASAL2, ZNF423, protein kinase CZ, PLA2G3 and ALG13 have been reported to be involved in the development of cancer (62-65), including BRCA; however, their association with ET resistance remains unknown. For instance, previous studies have identified the increased methylation of SCGB3A1 in metastases compared with that in primary breast tumor types (62,63). In non-invasive MCF7 cells, DUOXA1 expression was upregulated compared with that in highly metastatic cells; DUOXA1 overexpression sensitized cells to doxorubicin (64). In the present study, the increased DNA methylation of SCGB3A1 and DUOXA1 were observed in the RTE group, indicating their potential function in resistance to anti-estrogenic treatment. Additionally, a significant difference in the methylation frequencies and expression levels of MT1G was reported between BRCA subtypes (65). MT1G hypomethylation in patients who were resistant to ET was detected in the present study, indicating its association with antiestrogen therapy. The present study also reported numerous genes that have been rarely investigated in cancer research, including EFCAB4B. EFCAB4B is a Ca²⁺-binding protein that serves a key function in store-operated calcium entry in T-cells (66). A previous study demonstrated EFCAB4B hypermethylation in a twin with rheumatoid arthritis compared with their healthy co-twin (67). The results of the present study indicate a putative function of EFCAB4B in ET or potential immune/inflammatory alterations in the tumor microenvironment; however, further investigation is required.

Generally, the targeting of numerous genes has been reported to be superior to targeting an individual target, and DNA methylation patterns have become a promising diagnostic tool in addition to gene transcripts in BRCA. In the present study, a number of DMRs were detected between patients with ET resistance and those sensitive to ET; DMRs were used to build a predictive classifier. Furthermore, an RS was generated based on the classifier, which may determine the distinct outcomes of patients with ER-positive BRCA, suggesting a beneficial function in the identification of patients who are resistant to ET. Additionally, a potential function underlying the development of BRCA and resistance to ET was indicated for a number of genes (EFCAB4B and SLFN12); however, further investigation using a larger cohort is required. The present study primarily proposed a useful tool for assessing patient responses to ET and a number of potential therapeutic targets to promote the sensitivity of patients to ET with ER-positive BRCA.

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Availability of data and materials

The datasets analyzed during the present study are available from The Cancer Genome Atlas (https://portal.gdc.cancer.gov/) and the Gene Expression Omnibus (https://www.ncbi.nlm.nih.gov/geo/) databases.

Authors' contributions

FZ and YC participated in the conception and design of the study. FZ downloaded, analyzed the data and drafted the manuscript. YC revised the manuscript prior to submission. Both authors have read and approved the final version of the manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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