Quantitative DNA methylation and recurrence of breast cancer: A study of 30 candidate genes

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Abstract. Background: The need for new prognostic factors in breast cancer is ever increasing as breast cancer management evolves. Aberrant DNA methylation plays a pivotal role in cancer development and progression; DNA methylation-based biomarkers may provide independent prognostic information. We used pyrosequencing to investigate the prognostic potential of quantitative DNA methylation of a large set of candidate genes in a Korean single-institution series of operable breast cancer. Methods: Absolute DNA methylation in 20 candidate genes from an initial set of 30 genes was measured by pyrosequencing of bisulfite converted DNA in 121 fresh frozen breast cancer cases. Survival analyses used continuous and categorized (quintile-based) gene methylation data with time to recurrence (TTR) as an endpoint. Prognostic abilities of gene-only and risk-score models were explored. Results: Median follow-up was 5.1 years; 25 recurrences (21\%) were observed. Nodal status, methylation of TWIST1, SLIT2 (both as continuous and categorized variables) and APC, HLA-A, NKX2-5, SERPINB5, SFN (as categorized variables) were significantly prognostic; grade showed a prognostic trend. A multivariate model containing nodal status, grade and TWIST1 was a best fit (p < 0.001) in stepwise regression; risk-score based on this model separated patients into 3 distinct risk-groups (p < 0.001). A gene-only model based on TWIST1 and SFN also classified patients into distinct risk-groups (p = 0.009).

Conclusions: This study shows that accurate quantitative measurement of DNA methylation by pyrosequencing identifies a small set of genes with independent prognostic potential in breast cancer. These genes complement the current clinico-pathological prognostic factors and appear to be potential biomarkers that warrant further validation.

Keywords: Breast cancer, prognosis, DNA Methylation, pyrosequencing, biomarker

1. Background

Breast cancer (BC) is the most common cancer in women worldwide with an estimated 1.38 million new cancer cases diagnosed every year, accounting for 23\% of all cancers in women and 10.9\% of all cancers (second most common cancer) globally [1]. Overall incidence of BC is steadily increasing in most parts of the world, USA being one of the recent exceptions [2]. Trends in mortality from BC, however, show a mixed picture, with mortality declining in developed countries for the past two decades [3]. Evolved management of breast cancer and availability of various treatment options has played a major role in the welcome de-
cline in mortality [4]. Adjuvant systemic treatment in BC includes various chemotherapy regimens, a choice of endocrine therapy drugs and targeted agents like Trastuzumab. Each therapeutic option has its own characteristic benefit and adverse effect profile and therefore selecting treatment options requires precise estimation of risk of recurrence or death due to BC, particularly in view of the heterogeneous nature of the disease. This underscores the continuing need for novel prognostic biomarkers.

A few new prognostic factors like uPA [5], Oncotype Dx and Mammaprint are being evaluated [6,7] and/or used [8] in clinical practice. The use of gene-signature based prognostic tools is still quite uncommon and some consider it premature [9] due in part to limitations like the need for centralized processing and high costs, which in the developing world often exceed the cost of the entire generic drug-based chemotherapy regimen [10]. Moreover, Oncotype Dx in particular is applicable only in the subset of breast cancer patients with ER positive node negative disease. uPA was recently recommended [5] as a prognostic factor in breast cancer, its use is also limited due to a requirement for frozen tumour tissue. Therefore, there is an urgent need for robust cost-effective prognostic biomarkers that can be used in different subtypes of breast cancers; employing assays performed locally on formalin-fixed paraffin embedded (FFPE) tissues.

Epigenetic alterations are one of the most common molecular changes in human cancer [11–13]. These changes differ from genetic mutations in that they occur at higher frequency and may be reversible upon treatment with pharmacological agents. DNA methylation is one well known epigenetic-mechanism and it has become clear in recent years that there is a synergy between genetic and epigenetic changes [13]. Recognizing that aberrant DNA methylation plays a pivotal role in carcinogenesis and disease progression we assumed that accurate measurement of differential gene methylation patterns among many genes is likely to reveal biomarkers for improved risk assessment. Since most new prognostic markers in breast cancer are based on RNA expression; DNA methylation based biomarkers may provide independent prognostic information and may even complement RNA expression based prognostic factors. DNA methylation status has the ability to reflect multiple aspects of disease; types of genes methylated reflecting the biology [14,15] and the number of genes methylated and quantitative level of methylation in these genes reflecting the chronology. Therefore, DNA methylation biomarkers can prove to be useful in both diagnosis and prognosis of breast cancer.

Various approaches including high-throughput approaches could be used for identification of candidate biomarkers. Alternatively numerous (hundreds) of candidate genes (Web-appendix) have been reported in literature as differentially methylated in cancer but have not yet been validated as a combined prognostic set. Thus, selecting from among the myriad of candidates initially provides an enriched population from which to discover a key set, which may have a higher chance of successfully completing validation [16]. Indeed such an approach has been productive in the past for RNA expression biomarkers, e.g. Oncotype Dx assay was developed using a similar approach [17]. For biomarkers to be useful and widely used, the method/assay should be accurate, reproducible, relatively simple to perform, not prone to biases, feasible on very small amounts of FFPE material and affordable. PCR followed by pyrosequencing (PSQ) is one such sensitive, highly reproducible and cost-effective method [18] for DNA methylation analyses. It provides absolute quantitative information on methylated bases at each interrogated CpG site (CG dinucleotides in DNA where methyl group can be enzymatically added to the 5' position of cytosine), which is not possible with most other methods.

We investigated the prognostic potential of DNA methylation of 20 preferred genes chosen from an initial set of 30 candidate genes using a PCR-PSQ method on frozen tissues in a single-institution series of Korean breast cancer patients with median follow-up of 5.1 years.

2. Materials and methods

2.1. Patients, pathology specimens and handling

The study included 121 breast cancer patients presenting at Dong-A University Medical Centre, Busan, Republic of Korea from January 2004 to December 2006. All consecutive operable breast cancer patients who consented to provide fresh breast tumour and adjoining normal tissue sample (measuring approximately 0.5 × 0.5 cm each) at the time of surgery and in whom such sample collection (and immediate freezing of samples) was possible without jeopardizing clinical diagnosis and management were included in the study. Patients receiving neo-adjuvant chemotherapy (NACT) were included. Patients with missing clinical-pathological data for 2 or more variables were excluded (n = 3), resulting in 121 eligible participants.
All specimens were centrally reviewed to confirm diagnosis by the breast pathologist (DCK). Histopathological evaluation and immunohistochemistry for ER (DAKO, Clone 1D5, 1:50), PgR (DAKO, PgR 636, 1:100) and HER2 (Neomarkers, Clone e2-4001/3B5, Fremont, CA, USA:1:200) were done as per manufacturer recommended protocols. Histological grade was determined by the Nottingham Modification of Richardson Bloom Score (RBS). HER2 expression was assessed by immunohistochemistry (IHC). HER2 FISH was not performed in all cases of 2+ by IHC, therefore, only those 3+ on IHC were considered to be HER2 positive. Clinico-pathological and treatment variables including age, tumour size, type, histological grade, lymph node status, ER, PgR, HER2, type of surgery, neo-adjuvant/adjuvant treatment details (e.g. type of chemotherapy, no. of cycles, no. of fractions of radiotherapy, tumour bed boost etc.) were recorded in the study database for all eligible patients. Data on tumour size were a mixture of clinical and pathological tumour size, and some patients also received NACT. In view of the heterogeneous data on the tumour size variable, all analyses were done excluding this variable. Patients were regularly followed up every 6 months.

This study was approved by the Institutional Review Board of Dong-A University Medical Centre (IRB-2519 /SangYun-07-04 Ho) and all patients gave written informed consent.

Laboratory methods were as previously described [19]; detailed description is included in the Web-appendix.1

2.2. DNA extraction and bisulfite conversion

A simple macrodissection of sections from frozen tissue was performed before DNA extraction to enrich for areas of cancer. Five consecutive sections per specimen (10 µm thickness) were obtained by cryosectioning the cancer tissues and staining the first and fifth sections by H&E for histopathology review to confirm the areas of cancer and to guide the dissections of the three central sections. Genomic DNA was extracted and bisulfite converted as previously described [19].

2.3. Primer design

Primer sets with one biotin-labelled primer were used to amplify bisulfite converted DNA. Thirty genes were identified from the literature as candidate genes for this study. These genes were differentially methylated in either breast or prostate cancer, a combined candidate gene pool was based on a rationale that both of these are endocrine sensitive cancers, therefore likely to have major overlap of important biological pathways [19]. New primers for each of the 30 genes (Web-Table W2) were designed as previously described [19]. Most amplicons were kept short with lengths between 90 to 140 base pairs (bp) to facilitate later studies on FFPE specimens. Maximum permissible size of the amplicons was 210 bp.

2.4. PCR and pyrosequencing

PCRs followed by pyrosequencing were performed using 1.2 to 2.4 ng bisulfite converted DNA as previously described [19]. Optimized primer-specific annealing temperatures are given in Web-Table W2. All runs contained standard curves, which comprised a range of control methylated DNA (0%, 25%, 50%, 75%, and 100%) to allow standardized direct comparisons between different experiments.

A further selection of preferred genes from the initial 30 candidate genes was performed after the first 30 samples were processed. Genes (n = 20) correlating (p < 0.1) to any of Age, Nodal status, Histological grade, ER, PgR, HER2 were selected as preferred genes for further investigation. The remaining 10 genes (Web-Table W2) also had very low methylation frequency and levels and therefore were even more unlikely to succeed as biomarkers. These genes were therefore not investigated further in this study; we report findings on 20 selected preferred genes.

We have previously established reproducibility of the PCR-PSQ method [19]; therefore, all samples were assayed once except for the samples which did not yield pass results on first assay.

2.5. Statistical methods and data analyses

2.5.1. Data normalization

The main analyses used average of methylation values across all CG analyzed in a particular gene segment. The number of CGs analysed varied between two to six in each gene (Web-Table W2). Methylation data were adjusted for primer bias through re-scaling each gene’s methylation measurements by the median standard curve obtained using control mixtures for each primer set as previously described [19].
2.5.2. Correlations
Correlation analyses were performed using Spearman’s test to investigate associations between the categorical clinico-histopathological variables: age, nodal status, grade, ER, PgR, HER2 and continuous percentage methylation (%MeC) of genes.

2.5.3. Univariate analyses and determination of gene cut-off values
Univariate analyses using log-rank test were performed with time to recurrence (TTR) as an endpoint to probe relationship between nodal status, grade, ER, PgR, and HER2.

2.5.3.1. Genes as continuous variables
Univariate analyses with time to recurrence (TTR) as an endpoint and gene methylation levels as continuous variables were performed using the likelihood-ratio test. Only genes with p-value at or below 0.05 were considered for further analyses.

2.5.3.2. Genes as categorized variables
Relationship between DNA methylation and gene function may be non-linear in some cases and therefore analyses as continuous variables may not identify significant relationships between DNA methylation and survival. Secondly, if a biomarker emerges as successful candidate, categorized values often simplify its clinical application. Therefore, we also analysed genes as categorized variables using cut-offs determined as described below.

Various approaches are used for biomarker assessment like fixed percentage or median-based cut-off. These may not yield an optimum cut-off. Outcome-based cut-point optimization, X-Tile [20] is another approach. Cut-off determination, however, is subjective in the X-Tile approach and may also result in overfitting.

We employed an outcome-based approach (log-rank test) with 4 cut-offs based on quintiles (at 20, 40, 60 and 80 percentile methylation values) to determine optimal cut-off. The cut-offs yielding a lowest p-value were chosen (optimal cut-off by p-value method). Only genes with p-value at or below 0.05 were considered for further analyses using gene methylation categorized variable. 10000-fold permutation analyses were performed to provide exact p values.

Multiple comparisons in a small sample can result in over-fitting and yield false positive results. However, correction for multiple testing in the early phase of biomarker discovery-credentialing can also be an excessively conservative approach. We, therefore, adopted a middle path approach of accepting those genes which showed a p value < 0.05 in a relevant significance test and a p value < 0.2 in 10000-fold permutation analyses.

2.5.4. Multivariate analyses
2.5.4.1. Genes as continuous variables
Clinico-pathological variables with p-value < 0.1 in univariate analysis and genes with p-value at or below 0.05 in univariate analyses using gene methylation as continuous variable were considered for multivariate analyses. Stepwise regression using the likelihood ratio test was performed to identify the best fitting multivariate model. Multivariate analyses were performed by Cox’s Proportional Hazards method.

2.5.4.2. Genes as categorized variables
Genes with p-values at or below 0.05 in univariate analyses using gene methylation as categorized variable were considered for gene-only multivariate analyses. Stepwise regression using likelihood ratio test was performed to identify the best fitting gene-only model. Multivariate analyses were performed by the Cox’s Proportional Hazards method.

2.5.5. Risk score stratification
A risk-score was calculated for each case based on a multivariate model (using β coefficients) and the cases were stratified into 3 groups, low risk-score (cases within 0–25 percentiles), intermediate risk-score (cases within 26–75 percentiles) and high risk-score (cases within 76–100 percentiles).

All statistical analyses were conducted using software R version 2.11.2 [21] and STATA version 11.2 (StataCorp LP, College Station, TX, USA). All tests were two-sided and p-values of <= 0.05 were accepted as statistically significant.

3. Results

3.1. Patient treatment details
One hundred and twenty-one patients met the inclusion criteria for this study. All patients underwent either a breast conservation surgery or a mastectomy. Axillary management was either a complete axillary clearance or a sentinel node biopsy (SNB); with complete axillary clearance in whom SNB showed metastatic node/s. Sixteen patients underwent breast conservation surgery,
ally received Doxil or Docetaxel. Twenty-three patients addition-
of anthracyclines and taxanes (AC/EC followed by Pa-
FEC) and 32 patients received a sequential combination
received Anthracycline-based chemotherapy (FAC or
received methotrexate-based chemotherapy (CMF), 19
Nine patients did not receive any chemotherapy, 46
patients received chemotherapy only post-operatively.
Thirteen patients received some NACT, while 99 pa-
went SNB as the initial axillary staging procedure.
metabolism was performed in 1 patient. Forty nine patients un-
derwent SNB as the initial axillary staging procedure.

table 1. Methylation distribution of 20 genes is also displayed in Supplementary Fig. 1; pa-
patients with and without recurrence are separated in two groups.

3.3. Correlations among histopathological and gene
variables

ER showed a strong positive correlation with PR
(Spearman’s Rho = 0.79, \( p < 0.001 \)), and negative
relation with HER2 (Rho = −0.172, \( p < 0.042 \)), and
grade (less ER positivity with increasing grade; Rho
= −0.361, \( p < 0.001 \)). PgR expectedly showed similar
correlations; HER2 (Rho = −0.252, \( p = 0.004 \)), grade
(Rho = −0.366, \( p < 0.001 \)). HER2 and grade did not
 correlate with each other and nodal status did not show
 correlation with any other variable.

Correlations between \%MeC of genes and clinicopathological factors are displayed in Supplementary Ta-
ble 2. None of the genes correlated with age. Only SER-
PINB5 and PDLIM4 correlated positively with grade and
odal status respectively. HER2 correlated posi-
tively with PDLIM4, RARB and RASSF1A. A majority of
correlations were between genes and ER or PgR. ER
and PgR correlated positively with CDH13, EDNRB,
EGFR5, HIN1, RASSF1A and negatively with RARB
and SERPINB5. ER alone correlated positively with
SLIT2 (Supplementary Table 2).

Correlations among the 20 genes are displayed as a
heatmap in Supplementary Fig. 2. While most genes
showed weak to moderate positive correlations, cer-
tain negative correlations were also observed, partic-
ularly of SERPINB5, SFN and TWIST1. SERPINB5
correlated negatively with EGFR5, MAL, SLIT2, HIN1,
CDH13, EDNRB and RASSF1A.

3.4. Univariate survival analyses

Univariate survival analysis explored relationships
between age, histological grade, nodal status, ER, PgR, HER2 and TTR. Of the clinical variables, only
nodal status (\( p = 0.004 \)) was significantly associated
with TTR (Fig. 1A). Histological grade showed a non-
significant trend of association with TTR (\( p = 0.086 \)).
Both variables were considered for multivariate analy-

Univariate survival analysis of genes as continuous
variables and as categorized variables is displayed in
Table 2. Resulting quintile-based cut-offs, number of
cases and events in two groups created by dichotomiza-
tion at these cut-offs are also displayed. In analyses

Table 1

<table>
<thead>
<tr>
<th>Variable</th>
<th>Groups</th>
<th>Number</th>
<th>Events*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>≤50</td>
<td>59</td>
<td>13</td>
</tr>
<tr>
<td>&gt;50</td>
<td>62</td>
<td>12</td>
<td></td>
</tr>
<tr>
<td>Grade</td>
<td>I</td>
<td>18</td>
<td>2</td>
</tr>
<tr>
<td>II</td>
<td>38</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>III</td>
<td>61</td>
<td>17</td>
<td></td>
</tr>
<tr>
<td>Not Avbl</td>
<td>4</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Nodal status</td>
<td>Negative</td>
<td>60</td>
<td>6</td>
</tr>
<tr>
<td>1–3 Positive</td>
<td>27</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>&gt;4 Positive</td>
<td>34</td>
<td>13</td>
<td></td>
</tr>
<tr>
<td>ER</td>
<td>Positive</td>
<td>63</td>
<td>13</td>
</tr>
<tr>
<td>Negative</td>
<td>58</td>
<td>12</td>
<td></td>
</tr>
<tr>
<td>PgR</td>
<td>Positive</td>
<td>60</td>
<td>12</td>
</tr>
<tr>
<td>Negative</td>
<td>61</td>
<td>13</td>
<td></td>
</tr>
<tr>
<td>HER2</td>
<td>Positive</td>
<td>46</td>
<td>12</td>
</tr>
<tr>
<td>Negative</td>
<td>72</td>
<td>11</td>
<td></td>
</tr>
<tr>
<td>Not Avbl</td>
<td>3</td>
<td>2</td>
<td></td>
</tr>
</tbody>
</table>

*The recurrence of breast cancer.

104 underwent total mastectomy and radical mastecto-
my was performed in 1 patient. Forty nine patients un-
derwent SNB as the initial axillary staging procedure.
Thirteen patients received some NACT, while 99 pa-
ents received chemotherapy only post-operatively.
Nine patients did not receive any chemotherapy, 46
received methotrexate-based chemotherapy (CMF), 19
received Anthracycline-based chemotherapy (FAC or
FEC) and 32 patients received a sequential combination
of anthracyclines and taxanes (AC/EC followed by Pa-

citaxel or Docetaxel). Twenty-three patients addition-
ally received Doxiluridine after completion of standard
adjuvant chemotherapy. Sixty patients received radio-
therapy (all conservative surgery patients included), 16
of them received tumour bed boost. All premenopausal
hormone receptor positive patients were prescribed Ta-
moxifen for 5 years, while postmenopausal patients
were prescribed aromatase inhibitors. After a median
follow-up of 5.1 years (4.87–5.4) 25 patients experi-
enced recurrences, and there were 3 deaths; one patient
died due to chemotherapy related complications after
first cycle of CMF chemotherapy, cause of death was
not known in the other two patients.

3.2. Patient characteristics and gene methylation

Patient characteristics are displayed in Table 1. Most
(\( n = 112 \)) patients had Invasive Ductal Carcinoma
(IDC), 10 of these showed medullary features; 4 pa-
ients had Invasive Lobular Carcinoma (ILC), and 5 tu-
mours had other morphologies like mucinous carci-
oma or tubular carcinoma. Number of samples anal-
ised and methylation values for 20 genes are displayed
in Supplementary Table 1. Methylation distribution of 20 genes is also displayed in Supplementary Fig. 1; pa-
patients with and without recurrence are separated in two groups.
Fig. 1. Kaplan-Meier survival estimates of nodal status (A), APC (B), HLA-A (C), NKX2-5 (D), SERPINB5 (E), SFN (F), SLIT2 (G) and TWIST1 (H) in univariate analysis. + sign refers to cases with methylation values above cut-off and – sign refers to those below cut-off.
Table 2

Univariate survival analysis of genes as categorized variables, their derived cut-offs (percentile and corresponding level of methylation), resulting groups, p-values and as continuous variables (p-values in the last column)

<table>
<thead>
<tr>
<th>Gene</th>
<th>Categorized</th>
<th>Continuous</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cut-off percentile</td>
<td>Cut-off %MeC</td>
</tr>
<tr>
<td>APC*</td>
<td>20</td>
<td>1.1</td>
</tr>
<tr>
<td>CDH13</td>
<td>20</td>
<td>3.8</td>
</tr>
<tr>
<td>DAPK1</td>
<td>40</td>
<td>2.0</td>
</tr>
<tr>
<td>DPYS</td>
<td>20</td>
<td>12.6</td>
</tr>
<tr>
<td>EDNRB</td>
<td>80</td>
<td>42.5</td>
</tr>
<tr>
<td>EGFR5</td>
<td>20</td>
<td>33.8</td>
</tr>
<tr>
<td>GSTP1</td>
<td>80</td>
<td>19.8</td>
</tr>
<tr>
<td>HLA-A*</td>
<td>80</td>
<td>26.6</td>
</tr>
<tr>
<td>MAL</td>
<td>20</td>
<td>10.5</td>
</tr>
<tr>
<td>MDR1</td>
<td>40</td>
<td>4.8</td>
</tr>
<tr>
<td>NKX2-5*</td>
<td>20</td>
<td>5.1</td>
</tr>
<tr>
<td>PDLIM4</td>
<td>60</td>
<td>23.3</td>
</tr>
<tr>
<td>PTGS2</td>
<td>20</td>
<td>2.1</td>
</tr>
<tr>
<td>RARB</td>
<td>80</td>
<td>15.5</td>
</tr>
<tr>
<td>RASSF1A</td>
<td>20</td>
<td>2.4</td>
</tr>
<tr>
<td>SERPINB5*</td>
<td>60</td>
<td>67.3</td>
</tr>
<tr>
<td>SFN*</td>
<td>80</td>
<td>86.2</td>
</tr>
<tr>
<td>SLIT2*</td>
<td>60</td>
<td>31.3</td>
</tr>
<tr>
<td>TWIST1*</td>
<td>40</td>
<td>7.0</td>
</tr>
</tbody>
</table>

Low methylation: ≤ Cut-off; High methylation: > Cut-off; *p < = 0.05.

of genes as continuous variables, TWIST1 (p = 0.034) and SLIT2 (p = 0.015) were significantly associated with TTR, both genes were considered for subsequent multivariate analysis.

Seven genes (APC, HLA-A, NKX2-5, SERPINB5, SFN, SLIT2 and TWIST1) were significantly associated with TTR (as categorized variables). Cut-offs derived by the quintile-based method were tested through permutations (B = 10000) and 5 of 7 genes showed p-values less than 0.1 while SERPINB5 and SLIT2 had p between 0.1 and 0.16 (Data not shown). All these genes except NKX2-5, showed p-values less than 0.2 in survival analyses as continuous variables as well (Table 2). Kaplan-Meier survival plots are displayed for APC, HLA-A, NKX2-5, SFN, SLIT2 and TWIST1 in Figs 1B, C, D, E, F, G, and H respectively.

3.5. Multivariate analyses

3.5.1. Genes as continuous variables

Stepwise regression using the likelihood ratio test and nodal status, grade, SLIT2 and TWIST1 as variables was performed on 109 cases (24 events, data available on all 4 variables); the best fitting multivariate model comprised of 3 variables, nodal status [HR (95%CI) = 1.81(1.12–2.95); p = 0.016], grade [HR (95%CI) = 1.77(0.88–3.55); p = 0.108], and TWIST1 [HR of 1% change in methylation (95%CI) = 0.94(0.88–1.01); p = 0.092].

3.5.2. Genes as categorized variables: Gene-only model

Stepwise regression using the likelihood ratio test and 7 genes as categorised variables was performed on 72 cases (17 events, data available on all 7 variables); the best fitting gene-only model comprised SFN, and TWIST1 (Fig. 2). Cases with both genes showing methylation either below or above their respective cut-offs belonged to an intermediate risk group, whereas those with SFN methylated below cut-off and TWIST1 methylated above cut-off belonged to a low-risk group. A small group of cases with SFN methylated above cut-off and TWIST1 methylated below cut-off showed very poor survival.

3.6. Risk score stratification

A risk-score was calculated for each case based on a multivariate model (using β coefficients). The information was entered in the model as follows: node negative = 0, 1-3 nodes positive = 1, 4 or more nodes positive = 2; grade 1 = 0, grade 2 = 1, grade 3 = 2, and TWIST1 methylation values as follows:
Risk-Score = (0.60 × Node) + (0.57 Grade) − (0.06 × TWIST1)

A risk-score based stratification of the cases in 3 groups, low risk-score (cases within 0–25 percentiles), intermediate risk-score (cases within 26–75 percentiles) and high risk-score (cases within 76–100 percentiles) resulted in a very clear separation of patients with different risk profiles, with low-risk group patients being virtually recurrence free (having received systemic treatment) and high-risk group patients having less than 50% 5-year survival despite receiving systemic treatment (Fig. 3).

4. Discussion

Alterations in DNA methylation contribute to development and progression of invasive breast cancer [22], and studying the methylation of genes is likely to reveal useful novel biomarkers. Several methods of determination of promoter methylation have been described including the use of restriction enzymes [23], genomic bisulfite sequencing [24] and microarray-based methylation analysis [25]. The overwhelming majority of published data use methylation-specific PCR following bisulfite treatment (MSP) [26,27]. MSP is highly sensitive and has been used widely in this context. However, as there is no in-built measure of adequacy of bisulfite treatment, the possibility of false positives due to inadequate conversion of non-methylated cytosine to uracil exists. Another potential source of false positives is mis-priming, and this may be a greater problem when high numbers of PCR cycles or if nested primers are used. Previously described methods of controlling for mis-priming include re-analysis by methylation-sensitive restriction enzymes [28] or subsequent bisulfite DNA sequencing [29]. Accurate quantitative analysis is very important for DNA methylation analysis and MSP has many weaknesses in this regard. One of the major issues for MSP is the use of relative quantification by means of an external control with a different PCR of a control gene. The accuracy and reproducibility of this approach is still not adequately answered for a large diversity of genes. Development of DNA methylation risk prediction panels based on diverse genes will however require an accurate and reproducible method. High reliability and flexibility have made pyrosequencing an analysis platform that has been widely used for various diagnostic applications such as routine (multiplex) genotyping [30], sensitive detection of mutations [31] and microbacterial identification [32]. Pyrosequencing combines the ability of direct quantitative sequencing, reproducibility, speed and ease-of-use and is becoming more widely used [33].

Our aim was to investigate and credential a set of candidate genes previously reported as differentially methylated in endocrine-responsive cancers [19] in order to identify potential prognostic biomarkers in breast cancer. This is one of the first studies to quantify methylation of a large number of candidate genes in a common set of breast cancer specimens by means of a single highly accurate and validated DNA methylation assay.

To ensure accuracy and reproducibility, we used PSQ, an approach particularly suited to situations where the available specimen set for molecular studies is highly limited in either quality or amount. As-
says were specifically designed for future use in FFPE material by limiting the amplicon size but covering a reasonable number of CG sites. This will, in future, allow us to validate our findings in larger sets of samples from clinical trials and develop the biomarkers for clinical use. In addition, our preliminary data show that methylation levels in frozen and matched FFPE specimens are equivalent [19]. To fine-tune the PSQ method, predefined quantitative controls were included within each experiment to allow the adjustment of all data and thus improve the accuracy of comparisons in levels of methylation between the investigated genes. Internal controls to check completeness of bisulfite conversion were also included in the assays. This is an important advantage of the PSQ method, increasing the measurement accuracy compared to other commonly used methods [19].

We used a set of prospectively collected well-annotated Korean breast cancer samples from patients treated at Dong-A University hospital. There are certain important differences between Korean and Western breast cancers, both in risk factors as well as presentation. Apart from dietary [34,35] and ethnic differences, Korean women with breast cancer present at younger mean age of 48 years, approximately 15 years younger than the median age of white women with breast cancer in the United States [36]. Expectedly, ER positivity rates are lower [37], whereas HER2 positivity is much higher in Korean breast cancers [38,39], as seen in our data (ER positive 52%, HER2 positive 39%). Fifty percent of our patients had node positive breast cancer and the same proportion had grade 3 disease. This is consistent with a higher proportion (49% were 50 years or younger) of young patients [37]. Correlations among various histopathological variables were consistent with general breast cancer populations. Although breast conservation rates were low, patients received standard adjuvant treatments. Use of multiple standard chemotherapy regimens and different numbers of cycles (6 or 8), however, did not permit us to use chemotherapy regimen as one of the variables in survival and other analyses as it would have resulted in multiple small subgroups.

We explored the relationship between genes and TTR in two ways, genes treated as continuous variables and as categorized variables (optimum cut-off determination) for the reasons described in methods. Seven genes, TWIST1, SFN, SLIT2, APC, NKX2-5, HLA-A, and SERPINB5 were univariately associated with TTR when analysed as categorical variables: TWIST1 and SLIT2 were also associated with TTR when analysed as continuous variables. Optimum cut-off determination can result in over-fitting and false-discovery, we therefore tested robustness of our discovery by testing cut-offs derived by the quintile-based method through 10000 permutations and 5 of 7 genes showed p-values less than 0.1 and two were between 0.10 and 0.16 (SERPINB5 and SLIT2). We believe that these 7 genes and the cut-offs derived are sufficiently robust for the biomarker discovery-credentialing phase of our study. Of these 7 genes, only SERPINB5 correlated with ER, PgR and grade, SLIT2 correlated with ER (Supplementary Table 2). Correlation among these 7 genes was none to weak (Supplementary Fig. 2), suggesting that the genes can provide independent prognostic information.

Univariate analysis of clinic-pathological variables revealed nodal status as strongly prognostic and histological grade exhibiting a prognostic trend. ER, PgR, HER2, which are very strong predictive but weak prognostic factors, were not prognostic, perhaps due to moderate sample size and high HER2 positivity or due to biological differences between Korean and Western breast cancers.

A step-wise approach was used to identify the best-fit model for primary multivariate analysis, using TWIST1 and SLIT2 as continuous variables, in addition to nodal status and grade. A model with nodal status, grade and TWIST1 was the best model ($p < 0.0001$ vs. null) and TWIST1 may have better prognostic ability than grade although neither reached significance when combined with nodes alone.

Additional exploratory analyses were performed to see if a risk-score based on this 3 variable model or alternatively a gene-only model can classify patients into different risk categories. The gene-only model was developed by a step-wise approach from a larger number of genes (7 significant genes in analysis using genes as categorized variables). TWIST1 and SFN produced the best model ($p = 0.009$ for model vs. null) that could identify a small group of cases with very high risk of recurrence. Such models may be useful when risk-prediction based on nodal status etc is not available, for example, in tailoring neo-adjuvant therapies based on individual patient risk. Stratification of patients into 3 groups based on risk-score showed excellent separation of patients with different risks of recurrence, with none of the low-risk group patients experiencing a recurrence within 5 years and the highest risk patients having less than 50% 5-year survival.

All genes except NKX2-5 have a proven important role in carcinogenesis and tumour progression. For ex-
ample, TWIST1 acts as an oncogene and is a key driver of epithelial-mesenchymal transition (EMT) [40,41] and is directly involved in breast cancer stem cell phenotype [42]. Higher methylation of TWIST1 was associated with better prognosis in our study as expected. SFN acts as a tumour suppressor gene to produce protein 14-3-3 sigma, which acts as a regulator of mitotic translation; deficiency of this protein may cause cells to progress on the path to aneuploidy and tumorigenesis [43]. SLIT2, a tumour suppressor gene, suppresses breast cancer cell growth [44].

Ideally, the question of prognostic potential should be addressed in a systemic therapy naive population. Unfortunately, getting such contemporary sample sets is becoming increasingly difficult to impossible since most patients now receive systemic therapy and those who do not are not representative of a general pool of breast cancer patients; therefore most major biomarker studies now have to resort to sample sets from systematically treated patients. Our study has some other limitations as well. The study was small and not blinded and there were no comparisons to other well established DNA methylation methods such as bisulfite sequencing. However, inclusion of quantitative controls in every experimental run and inclusion of non-CpG C controls wherever possible ensured reliability of our assays. Since, this was an exploratory study of several markers, there were no formal a-priori sample size calculations and we used all samples available from the pathology archives. With high HER2 positivity and a younger population, our patient set is representative of a Korean breast cancer population, but probably not of a western breast cancer population. Although breast conservation rates were lower patient management was generally as per international standards, Dong-A University hospital being a leading cancer centre in Korea. Information on tumour size was not uniform and use of several chemotherapy regimens did not allow us to investigate these variables and their impact on survival, this may have resulted in sub-optimal multivariate analyses. The cut-offs for genes were derived by univariate analysis of the same specimens, thus there were no separate training and validation sets. We chose not to divide our patient set into training and validation sets in view of modest sample size, however, we confirmed robustness of the cut-offs through permutations. Also 2 of 7 genes were significantly associated with TTR when analysed as continuous variables an approach that is less prone to overfitting bias. It should, however, be emphasized that, additional extensive validation of our findings is important in order to establish the clinical utility of these biomarkers and any models based on them.

We identified a set of 7 genes univariately associated with TTR of which TWIST1, SFN and SLIT2 either complemented node status and grade in providing additional prognostic information or stratified patients into different risk groups when evaluated as a gene-only model (Figs 2 and 3). Since these 7 genes did not correlate with conventional clinico-pathological variables, they are likely to provide independent prognostic information. If these data are confirmed in larger independent studies it may be reasonable to explore an approach where a gene-only model helps in tailoring neo-adjuvant therapy and risk-score helps in tailoring adjuvant therapy, with a possibility that low-risk group patients may forgo chemotherapy and avoid associated side-effects.

In summary, PSQ is a useful and accurate method for studying methylation of genes. We identified seven differentially methylated genes in breast cancer that were informative of breast cancer recurrence and deserve further validation as prognostic markers in larger sets of specimens.

Competing interests

ATL is an advisor to Qiagen Inc. and owns Qiagen stock. JMC has given lectures sponsored by Qiagen on an occasional basis. No other relevant competing interests exist.

Acknowledgments

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Sponsors had no role in study design, execution, data analyses or the content of the paper.

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Supplementary material

Supplementary Table 1
Number of samples analysed and methylation values for 20 genes

<table>
<thead>
<tr>
<th>Gene</th>
<th>N</th>
<th>Q1</th>
<th>Median</th>
<th>Q3</th>
<th>MAD</th>
<th>Min</th>
<th>Max</th>
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<tr>
<td>APC</td>
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<td>1.62</td>
<td>5.64</td>
<td>14.05</td>
<td>6.998</td>
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<td>CDH13</td>
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<tr>
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<td>3.9</td>
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<td>61.7</td>
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<tr>
<td>SFN</td>
<td>105</td>
<td>54.853</td>
<td>70.2</td>
<td>82.8</td>
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<td>SERPINB5</td>
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<td>73.1</td>
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<td>95.88</td>
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<td>TWIST1</td>
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<td>13.84</td>
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</table>

Q1: First quartile, Q3: Third quartile, MAD: Median Absolute Deviation.

Supplementary Table 2
Correlation of various clinico-pathological variables with genes

<table>
<thead>
<tr>
<th>Gene</th>
<th>Age</th>
<th>ER</th>
<th>PgR</th>
<th>Grade</th>
<th>HER2</th>
<th>Nodal Status</th>
</tr>
</thead>
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<tr>
<td></td>
<td>Rho</td>
<td>P-value</td>
<td>Rho</td>
<td>P-value</td>
<td>Rho</td>
<td>P-value</td>
</tr>
<tr>
<td>APC</td>
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<td>0.122</td>
<td>0.005</td>
<td>0.957</td>
<td>−0.124</td>
<td>0.187</td>
</tr>
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<td>CDH13</td>
<td>0.014</td>
<td>0.887</td>
<td>0.275</td>
<td>0.003</td>
<td>0.2</td>
<td>0.034</td>
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<tr>
<td>DAPK1</td>
<td>0.076</td>
<td>0.415</td>
<td>−0.061</td>
<td>0.514</td>
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<td>0.757</td>
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<td>0.634</td>
<td>0.397</td>
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<td>EGFR5</td>
<td>−0.12</td>
<td>0.208</td>
<td>0.343</td>
<td>0</td>
<td>0.301</td>
<td>0.001</td>
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<tr>
<td>GSTP1</td>
<td>−0.134</td>
<td>0.148</td>
<td>0.155</td>
<td>0.093</td>
<td>0.07</td>
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<td>HIN1</td>
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<td>0.709</td>
<td>0.449</td>
<td>0</td>
<td>0.375</td>
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<tr>
<td>HLA-A</td>
<td>−0.037</td>
<td>0.72</td>
<td>−0.163</td>
<td>0.111</td>
<td>−0.157</td>
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<tr>
<td>MAL</td>
<td>0.023</td>
<td>0.809</td>
<td>0.002</td>
<td>0.987</td>
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<tr>
<td>MDR1</td>
<td>0.049</td>
<td>0.604</td>
<td>0.056</td>
<td>0.557</td>
<td>0.058</td>
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<td>NKX2-5</td>
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<td>PDLIM4</td>
<td>−0.042</td>
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<td>PTGS2</td>
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<td>0.298</td>
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<td>RARB</td>
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<tr>
<td>SFN</td>
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<td>−0.19</td>
<td>0.052</td>
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<td>0.071</td>
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<tr>
<td>SERPINB5</td>
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<td>−0.376</td>
<td>0</td>
<td>−0.265</td>
<td>0.005</td>
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<tr>
<td>SLIT2</td>
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<td>0.254</td>
<td>0.005</td>
<td>0.169</td>
<td>0.066</td>
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<tr>
<td>TWIST1</td>
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<td>0.622</td>
<td>0.039</td>
<td>0.688</td>
<td>0.033</td>
<td>0.727</td>
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</table>

All significant correlations have been shown in bold and significant negative correlations in italics.
P-values have not been corrected for multiple comparisons.
Supplementary Fig. 1. Methylation distribution of 20 genes, patients separated in two groups, one with recurrence events (E) and other without recurrence events (NE).

Supplementary Fig. 2. Heatmap of correlations among 20 genes. Negative correlations are indicated by “-” sign in this B&W figure. (Colours are visible in the online version of the article; http://dx.doi.org/10.3233/CBM-2012-0266)